Immunity, Volume *44*

Supplemental Information

The Vaccine Adjuvant Chitosan Promotes Cellular Immunity via DNA Sensor cGAS-STING-Dependent Induction of Type I Interferons

Elizabeth. C. Carroll, Lei Jin, Andres Mori, Natalia Muñoz-Wolf, Ewa Oleszycka, Hannah B.T. Moran, Samira Mansouri, Craig P. McEntee, Eimear Lambe, Else Marie Agger, Peter Andersen, Colm Cunningham, Paul Hertzog, Katherine A. Fitzgerald, Andrew G. Bowie, and Ed C. Lavelle

Figure S1

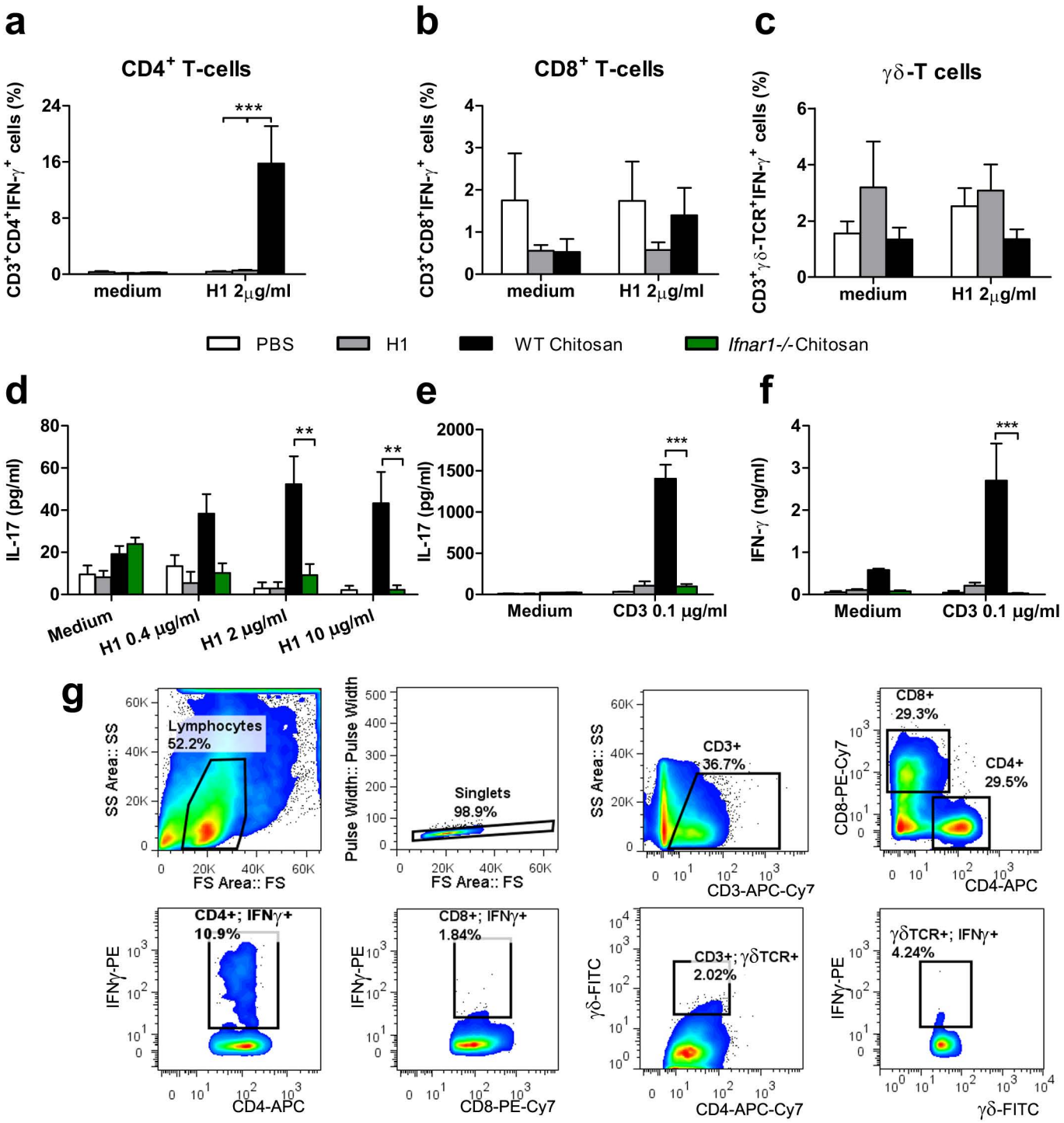


Figure S1, related to Figure 1. CD4+ T cells are the main producers of H1-specific IFN-γ after vaccination and chitosan promotes H1-specific IL-17A secretion in an IFNAR1-dependent manner *in vivo*. Expression of H1-specific intracellular IFN-γ by (a) $CD3^+$ $CD4^+$, (b) $CD3^+CD8^+$ cells and (c) $CD3^+\gamma\delta^+$ PECs isolated from WT mice immunized with H1 alone (grey) or in combination with chitosan (black) or PBS as a control (white). (d) IL-17A production in mLNs isolated from WT and *Ifnar1-/-* (green) mice immunized with H1 alone or with chitosan following re-stimulation with H1 or (e) anti-CD3. (f) Production of IFN-γ in response to anti-CD3 stimulation. (g) Gating strategy used for analysis of intracellular cytokine production. Data shown as mean ±SEM; n=5 per group. H1 v H1+chitosan, WT v *Ifnar1-/-*, ** $p<0.01$ *** $p<0.001$.

Figure S2

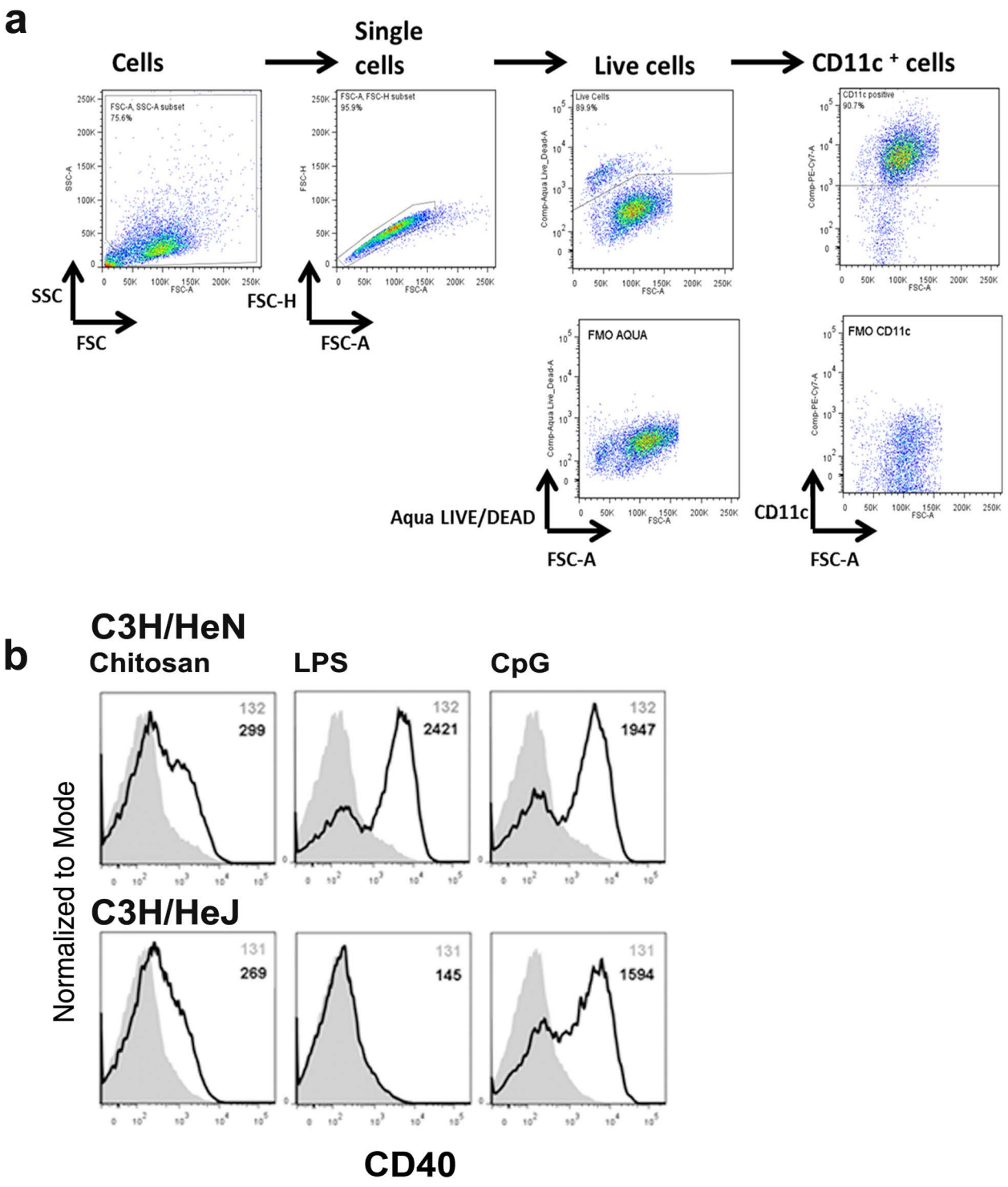


Figure S2, related to Figure 2. Chitosan promotes DC maturation via a TLR4-independent pathway. (a) Gating strategy for analysis of DCs by flow cytometry. Cells were analyzed by FSC-A v SSC-A to exclude debris and doublets eliminated by analyzing cells on FSC-A v FSC-H axis. Live cells were gated as Aqua LIVE/DEAD negative. Expression of maturation markers was analyzed on CD11c positive cells. (b) Representative histograms for CD40 expression in C3H/HeN and C3H/HeJ DCs left untreated (shaded histogram) or stimulated with chitosan, LPS and CpG (black lines). Numbers represent mean fluorescence intensities according to the color code.

Figure S3

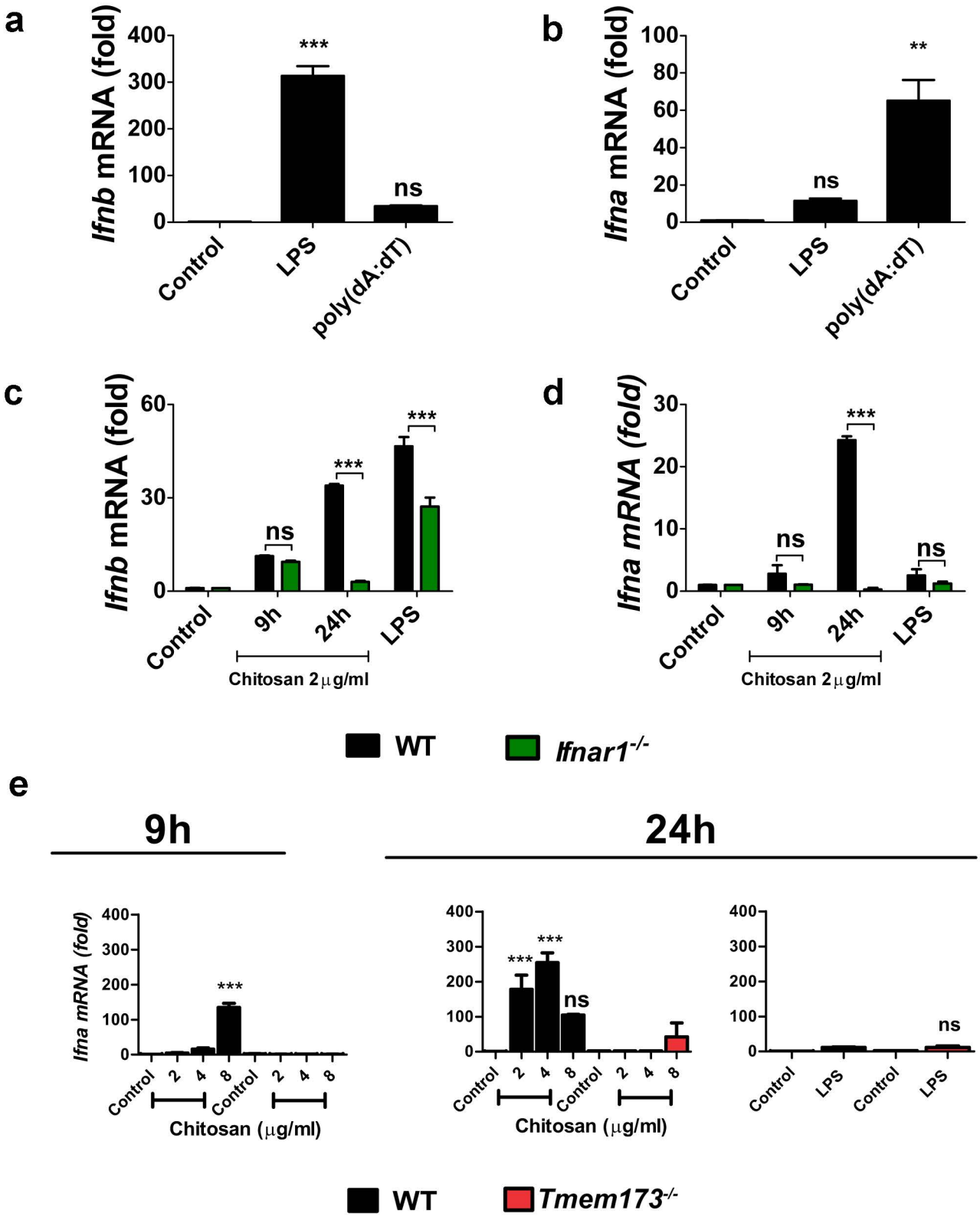


Figure S3, related to Figures 2 and 4. Signaling through IFNAR is required for sustained transcription of *Ifnb* mRNA and induction of *Ifna* in response to chitosan. qPCR analysis of (a) *Ifnb* and (b) *Ifna* mRNA expression in unstimulated DCs and DCs stimulated with LPS or poly(dA:dT). qPCR analysis of (c) *Ifnb* and (d) *Ifna* mRNA expression in WT (black bar) and *Ifnar1*$^{-/-}$ (green bar) DCs left untreated or treated with chitosan (2 μg/ml) for 9h or 24h, or as a control LPS for 1h. (e) Induction of *Ifna* mRNA in WT (black) and *Tmem*$^{-/-}$ (red) DCs at 9h and 24h after stimulation with chitosan. Gene induction was calculated using *Actb* mRNA as reference gene; bars represent mean fold changes for treated over control group. Results are expressed as mean ±SEM, representative of two independent experiments. WT v *Ifnar1*$^{-/-}$, ***p<0.001.

Figure S4

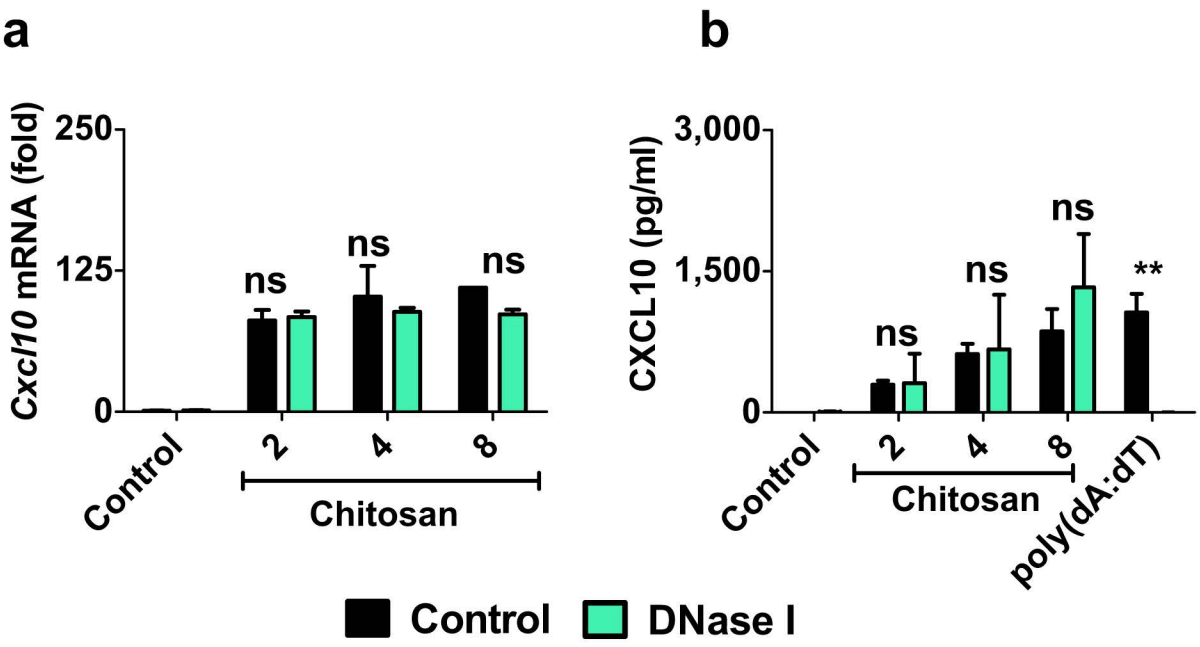
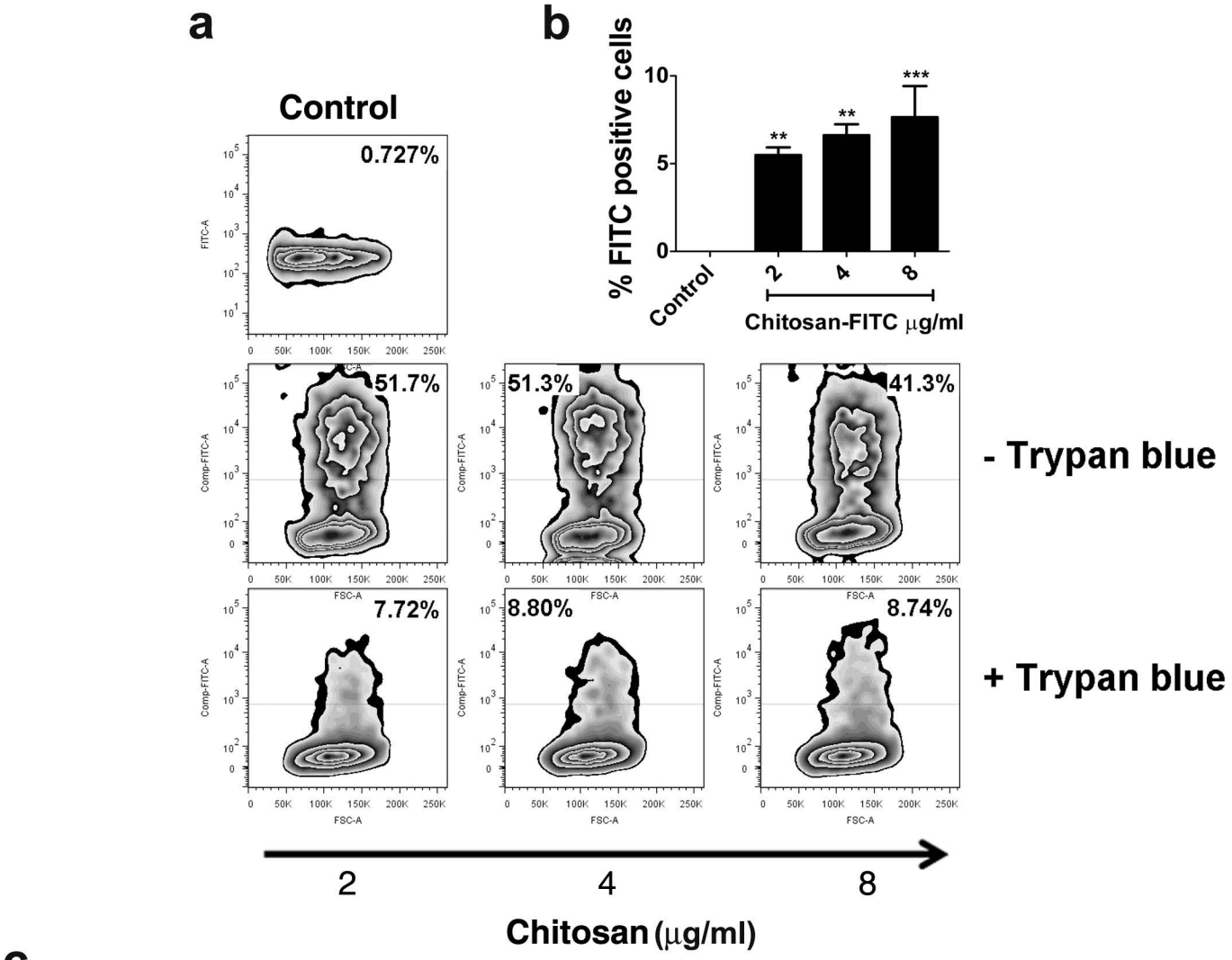


Figure S4, related to Figure 2. The ability of chitosan to promote transcription and secretion of CXCL10 by DCs is unaffected by nuclease treatment. Analysis of (a) *Cxcl10* mRNA by qPCR and (b) CXCL10 secretion by ELISA in DCs treated with increasing concentrations of chitosan, LPS or poly(dA:dT) before (black bars) or after treatment with DNase I (turquoise bars). Gene induction was calculated using *Actb* mRNA as reference gene; bars represent mean fold changes for treated over control group. Results are expressed as the mean ±SEM and are representative of two independent experiments. Control v DNase I, **p<0.01.

Figure S5



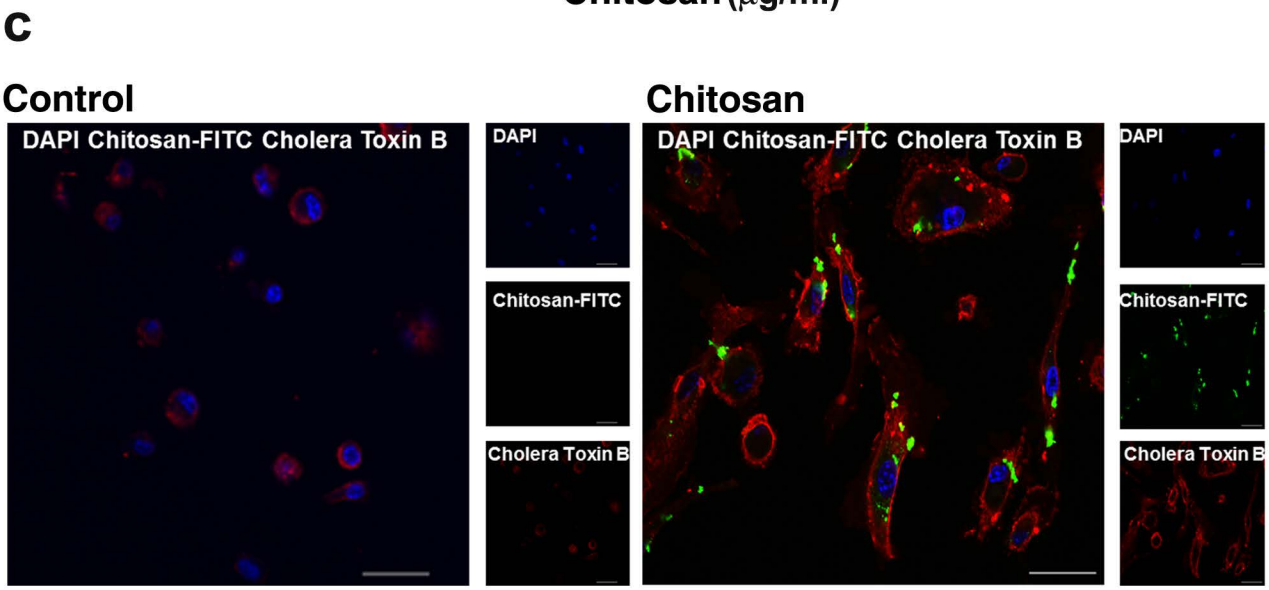


Figure S5, related to Figure 4. Chitosan is efficiently phagocytosed by DCs. (a) Representative plots of the uptake of various concentrations of chitosan-FITC by DCs before and after quenching with trypan blue. (b) Percentage of FITC-positive DCs after incubation with different concentrations of chitosan-FITC for 6h. Results expressed as mean ±SEM of two independent experiments. Medium v chitosan- FITC, **p<0.01, ***p<0.001. (c) DCs were incubated with chitosan-FITC for 6h, after which the cell membranes and nuclei were stained with fluorescent CTB (red) and DAPI (blue) respectively. Cells were visualized by confocal microscopy, scale bar 30 μm. Results are representative of two independent experiments.

Figure S6

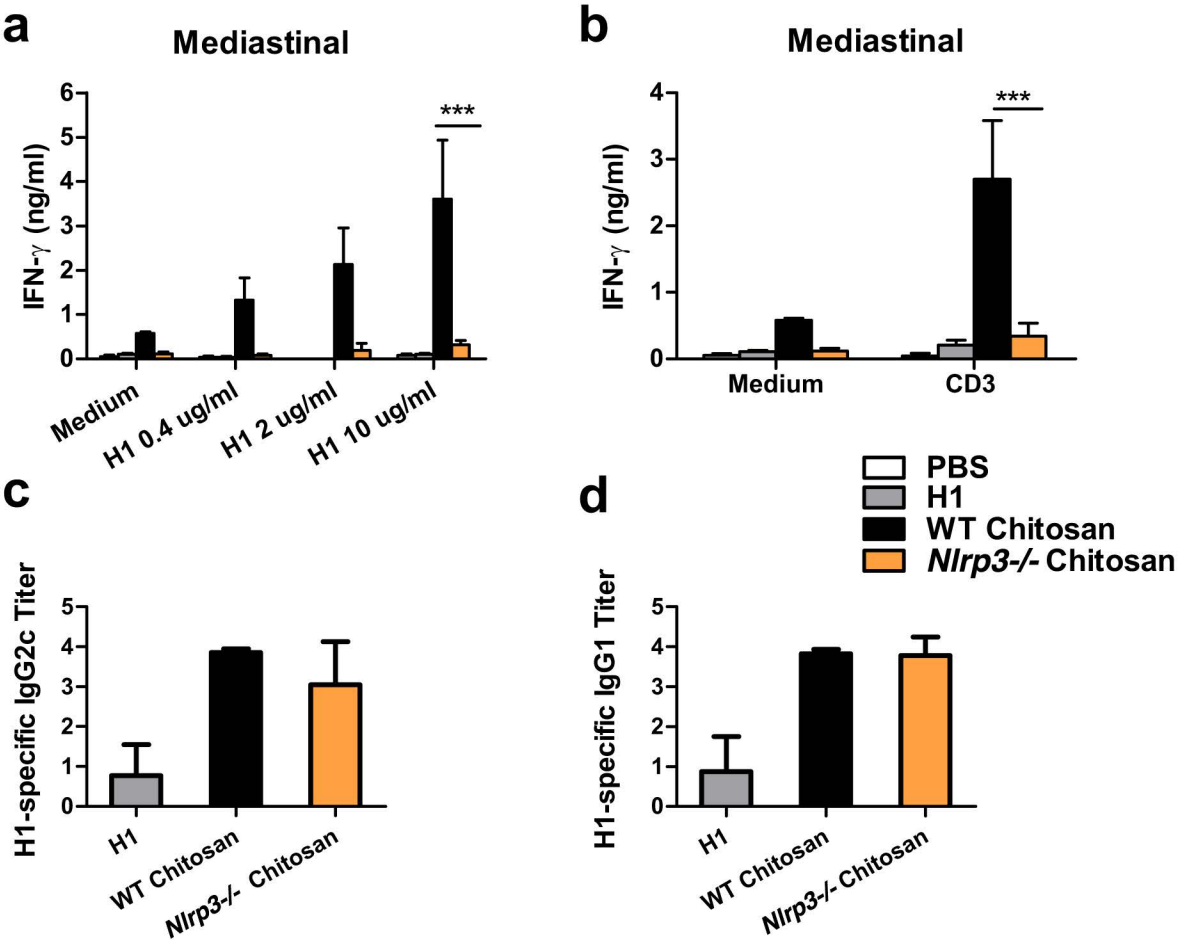


Figure S6, related to Figures 1 and 6. Chitosan promotes NLRP3-dependent Th1 responses. IFN-γ production in mLNs cells from immunized WT (white -PBS, grey -H1, black -H1+chitosan) and *Nlrp3-/-* (orange -H1+chitosan) mice restimulated with (a) H1 or (b) anti-CD3. H1-specific IgG2c (c) and IgG1 (d) measured in serum of WT and *Nlrp3-/-* mice immunized with H1 alone or with chitosan. Data shown as mean ±SEM; n=5 per group. WT v *Nlrp3-/-* ,* $p<0.05$, *** $p<0.001$.

Figure S7

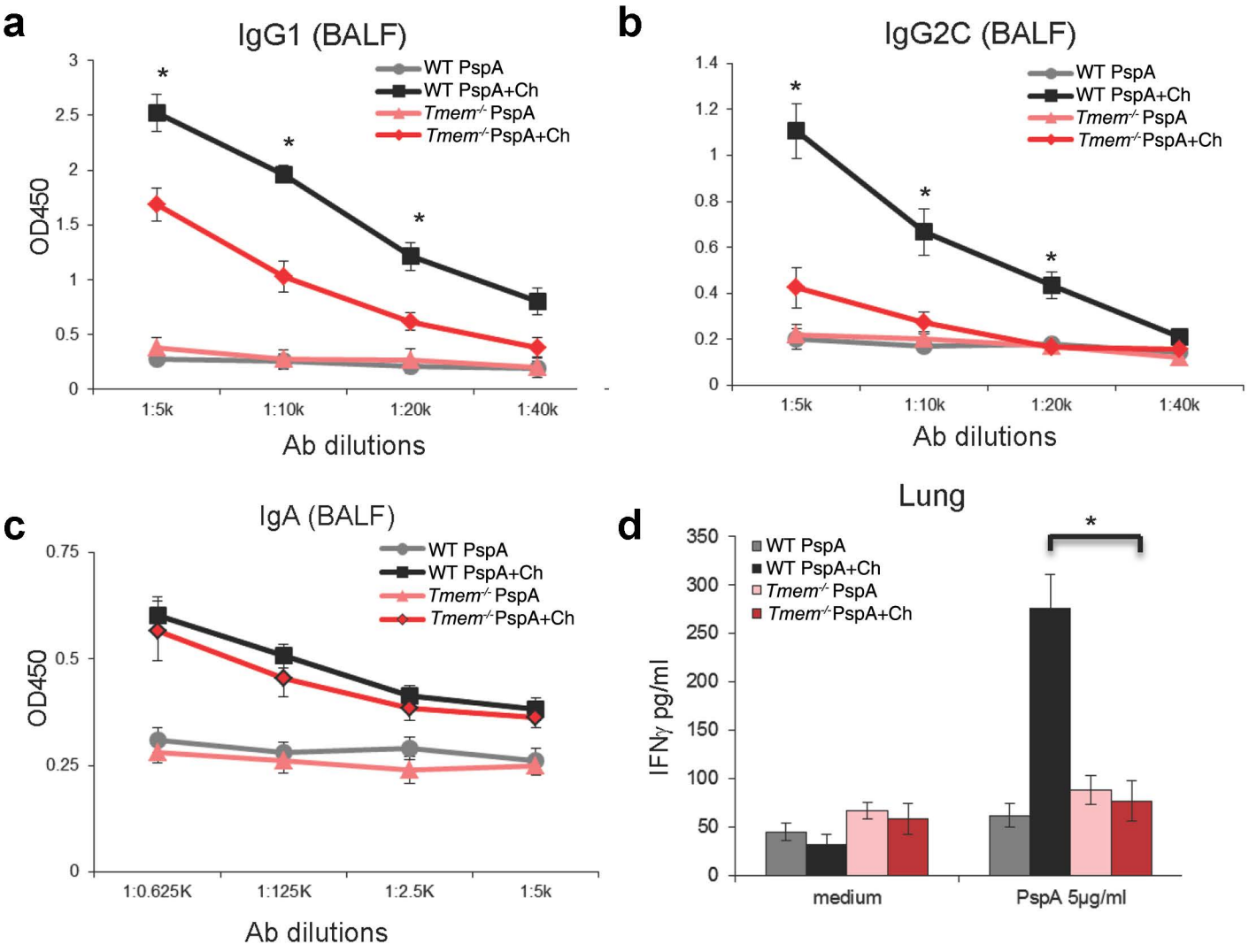


Figure S7, related to Figure 6. STING is essential for mucosal Th1 and IgG2c responses following intranasal immunization with Chitosan and the pneumococcal antigen PspA. Refers to Figure 6. WT and *Tmem*$^{-/-}$ mice were immunized i.n. on days 0 and 14 with either PspA (2 µg) alone (grey -WT or pink -*Tmem*$^{-/-}$) or together with 50 µg chitosan (black -WT, red -*Tmem*$^{-/-}$). Bronchoalveolar lavage fluid (BALF) was collected 14 days after the last immunization. Levels of PspA-specific (a) IgG1, (b) IgG2c and (c) IgA in BALF were quantified by ELISA. (d) IFN-γ secretion by cells isolated from the lung of WT and *Tmem*$^{-/-}$ mice immunized with PspA alone or in combination with chitosan and re-stimulated ex vivo with 5 µg/ml PspA. Results expressed as the mean ±SEM of two independent experiments. WT v *Tmem*$^{-/-}$; *p<0.05.

Supplemental Experimental Procedures.

Preparation of FITC-labeled chitosan and uptake analysis.

FITC-conjugated chitosan was prepared using a protocol adapted from Hunang *et al* (Huang et al., 2002) involving a reaction between the isothiocyanate group of FITC and the primary amino group of chitosan (Onishi and Machida, 1999). 35 ml of dehydrated methanol containing 25 mg of FITC was mixed with 25 ml of a 1% w/v chitosan in 0.1 M acetic acid solution. After 3h of reaction in the dark at room temperature, FITC-labeled chitosan was precipitated with 0.2 M sodium hydroxide, centrifuged for 30 min at 4500 x g and the resultant pellet washed with 70% v/v methanol in water. The wash step was repeated twice and the pellet resuspended in 15 ml of 0.1 M acetic acid solution and stirred overnight. The polymer solution was dialyzed in 2.5 L of distilled water for 3 days in the dark before freeze-drying. Care was taken to avoid endotoxin contamination of the buffers and equipment used in the protocol. For the analysis of uptake of FITC-labeled chitosan, cells were incubated with AQUA LIVE/DEAD fluorescent dye as before. Trypan blue was added to samples immediately before sample acquisition to quench external fluorescence, enabling internalized FITC-labeled chitosan fluorescence to be distinguished from surface bound FITC-labeled chitosan. Samples were acquired on BD FACSCanto II or Cyan using FACSDiva (BD) or Summit (Dako, Colorado) software and the data analyzed using FlowJo software (Treestar, Oregon).

Fixed cell imaging

DCs were plated on sterile cover slips in 12-well plates at 0.625 x 10^6 cells/ml. Non-adherent cells were removed the following day. For uptake assays, fluorescent chitosan was incubated with cells for 6h, after which coverslips were washed with PBS and fixed by 2% paraformaldehyde for 40 min. The cover slips were washed again with PBS and blocked with 1% BSA in PBS for 30 min. Coverslips were washed once again with PBS and incubated with cholera toxin subunit B (CTB) conjugated with Alexa Fluor 647 (Life Technologies) for 30 min. Finally the cover slips were washed again in PBS and rinsed with sterile water before mounting using Prolong Gold Antifade Mountant with DAPI (Life Technologies). Cells were viewed using Point Scanning Confocal Microscope (Olympus FV100 LSM Confocal Microscope).

For DNase I transfection efficiency assays, fluorescent Alexa Fluor-488 DNAse I (Fisher) either active or heat inactivated as previously described was encapsulated in DOTAP transfection reagent (Roche) as per manufacturer instructions. DCs were incubated with the fluorescent DNase I-DOTAP for 40 min. Cells were then washed and fixed in 2% paraformaldehyde for 40 min. Coverslips were washed again and mounted using Prolong Gold Antifade Mountant (Life Technologies). Prepared slides were dried at room temperature in the dark overnight and then stored at 4^{o}C. Cells were viewed using a Leica SP8 scanning confocal microscope.

Supplemental references

Huang, M., Ma, Z., Khor, E., and Lim, L.-Y. (2002). Uptake of FITC-chitosan nanoparticles by A549 cells. Pharm. Res. 19, 1488–1494.

Onishi, H., and Machida, Y. (1999). Biodegradation and distribution of water-soluble chitosan in mice. Biomaterials 20, 175–182.