SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1 | Soluble and plated anti-CLEC9A Abs induce CLEC9A-ζ signalling. B3Z cells stably expressing CLEC9A ζ were cultured overnight in the presence of anti-CLEC9A (1F6, 397 or 7H11) or isotype-matched control antibodies in soluble form (soluble) or previously adsorbed onto plastic (plated). Lac Z activity was measured at the end of the culture.

Supplementary Figure 2 | Generation and characterization of *clec9a*$^{gfp/gfp}$ mice. a, Targeting strategy used for the generation of *clec9a*$^{gfp/gfp}$ mice. Correct targeting leads to the insertion of a farnesylated form of EGFP in frame with the two first aminoacids of CLEC9A. Half of the exon 1 and the entire exon2 are eliminated and transcription following GFP is terminated by a polyadenylation signal. **b**, GFP expression in the spleen of *clec9a*$^{gfp/gfp}$ mice is restricted to CD8α^{+} DC and correlates with lack of expression of CLEC9A. Splenocytes from *clec9a*$^{gfp/gfp}$ and *clec9a*$^{+/+}$ mice were stained for CLEC9A, CD8α and CD11c. The relative expression of CD11c and GFP by total splenocytes is depicted in the left two panels. The dot plot on the right shows CD8α vs CLEC9A expression by CD11c^{+}GFP^{+} splenocytes from *clec9a*$^{gfp/gfp}$ mice. **c**, Lack of expression of CLEC9A by CD8α^{+} splenic DC from *clec9a*$^{gfp/gfp}$ mice. Splenocytes from *clec9a*$^{gfp/gfp}$ and *clec9a*$^{+/+}$ mice were stained for CLEC9A, CD8α and CD11c. The relative expression of CLEC9A and GFP by CD11c^{+}CD8α^{+} cells of each genotype is shown. **d**, Spleen composition is unaltered by the absence of CLEC9A. Total splenocytes and frequency of T and B lymphocytes, NK and NKT cells, CD4^{+} and CD8^{+} T cells and CD4^{+}, CD8α^{+} and CD4^{-} CD8α^{-} DCs were compared between *clec9a*$^{+/+}$ (CLEC9A^{+}) and *clec9a*$^{gfp/gfp}$ (CLEC9A^{-}) mice. **e**, Thymocyte differentiation is not affected by CLEC9A deficiency. Absolute number of thymocytes and frequency of CD4^{+}CD8^{+} double positive (DP), CD4^{+}CD8^{-} single positive (CD4 SP), CD4^{-}CD8^{+} single positive (CD8 SP) and CD4^{-}CD8^{-} double negative (DN) populations is depicted. **f**, Functional response of DC to innate stimulis is not affected by CLEC9A deficiency. Purified splenic DCs from *clec9a*$^{+/+}$ (CLEC9A^{+}) and *clec9a*$^{gfp/gfp}$

(CLEC9A$^-$) mice were stimulated overnight with curdlan (100 μg/ml; Cdlan), zymosan (100 μg/ml; Zym), CpG-containing DNA oligonucleotide (0.5 μg/ml; CpG) or LPS (1 μg/ml). Supernatants were analyzed for the presence of IL-12 /23 p40, TNF and IL-6 by ELISA. Data in (b, e-f) are the arithmetic mean ± SEM of three mice per group from one representative experiment out of three. c and d: one representative mouse per group is shown.

Supplementary Figure 3 | Phenotypic and functional characterization of Flt3L BMDC generated from *clec9a$^{gfp/gfp}$* mice. Flt3L BMDC were differentiated from bone marrow progenitors isolated from *clec9a$^{gfp/gfp}$* mice or control wild type littermates. **a**, GFP and CLEC9A expression by Flt3L BMDC after gating on CD8α$^+$-like DC (CD24^{hi} CD11b^{lo} B220^{neg}). One representative mouse per group is shown. **b**, Functional response of Flt3L BMDC to innate stimuli is not affected by CLEC9A deficiency. Flt3L BMDC of the indicated genotypes were cultured overnight with curdlan (100 μg/ml; Cdlan), zymosan (100 μg/ml; Zym), CpG-containing DNA oligonucleotide (0.5 μg/ml; CpG) or LPS (1 μg/ml). Culture supernatants were analysed for the presence of IL-12 /23 p40, TNF and IL-6 by ELISA. One representative experiment is shown. Data represent the arithmetic mean ± SEM of three independent Flt3L BMDC cultures from different mice.

Supplementary Figure 4 | Uptake of dead cell-derived material by CD8α-like DC is not affected by loss of CLEC9A. Flt3L BMDC, generated from wild type (CLEC9A$^+$) or *clec9a$^{gfp/gfp}$* (CLEC9A$^-$) littermates, were allowed to interact with PKH26-labelled UVC-treated cells for the indicated periods of time (min) at 4ºC or 37ºC. The association of PKH26-labelled cell material with the CD11c$^+$CD24^{high} CD8α-like subpopulation was then analyzed by flow cytometry. Representative pseudo-color contour plots are depicted.

Supplementary Figure 5 | CLEC9A recruits and signals via Syk kinase

a, Binding of Syk to phosphorylated peptides corresponding to the cytoplasmic domains of CLEC9A or of Dectin-1. Biotinylated peptides bearing the critical tyrosine residue in unphosphorylated or phosphorylated form or mutated to a phenylalanine were incubated with recombinant Syk kinase before precipitation with streptavidin. Precipitates were subjected to SDS-PAGE followed by Western blotting with anti-Syk antibodies. rSyk and (-) mark lanes loaded with recombinant Syk or left bank as a positive and negative control, respectively. **b**, Syk activation in response to CLEC9A triggering in LK cells stably transfected with mCLEC9A. Cells were cultured for 0 or 20 min on plates coated with the indicated antibodies before lysis and SDS-PAGE and Western blotting with anti-phospho-Syk or anti-Syk antibodies. **c**, CLEC9A signals in a Tyr7- and Syk-dependent fashion. B3Z cells stably expressing the wild type or Y7F mutated form of mCLEC9A and co-expressing or not Syk kinase were cultured in plates coated with anti-mCLEC9A. NFAT activity was measured using a colorimetric assay for the Lac Z reporter.

Supplementary Figure 6 | Signalling through CLEC9A requires Syk and a tyrosine at position 7. B3Z cells stably expressing CLEC9A (CLEC9A WT), Syk and CLEC9A (Syk/CLEC9A WT) or Syk and CLEC9A Y7F (Syk/CLEC9A Y7F) were cultured overnight in the presence of anti-CLEC9A (1F6, 397 or 7H11) or isotype-matched control antibodies in soluble form (soluble) or previously adsorbed onto the plate (plated). Lac Z activity was measured at the end of the culture.

Supplementary Figure 7 | Necrotic cells expose ligands for CLEC9A that provoke signalling through Syk kinase. a, Different necrosis-inducing treatments generate dead cells that trigger CLEC9A/Syk signalling. BWZ cells stably co-expressing Syk and wild type CLEC9A were cultured overnight in medium alone (Ctrl) or together with bm1 T MEFs that had been untreated (-) or treated 16h before with UVC (UV) or mitoxantrone (Mtx), MEFs cultured overnight without

serum (serum deprivation; SD) or MEFs subjected to freeze / thawing (FT) just before the assay. LacZ activity (left y axis) is depicted, together with the frequency of TO-PRO-3$^+$ and CLEC9A CTLD$^+$ MEFs at the beginning of the co-culture (right y axis). **b,** Necrotic cell-induced signalling via CLEC9A/Syk can be blocked by soluble anti-CLEC9A mAbs. B3Z cells stably co-expressing Syk and wild type CLEC9A (Syk/CLEC9A wt) or a mutated version (Syk/CLEC9A Y7F) were cultured overnight in the absence (CTRL) or presence of LK cells either untreated (LK) or UVC-irradiated 24h before (240 mJ/cm^2, LK UV). Where indicated, monovalent Fab fragments of anti-mCLEC9A (1F6) or bivalent intact antibodies, including isotype-matched controls (rat IgG1, rat IgG2a) and anti-mCLEC9A (1F6, 397, 7H11), were added to the culture. Lac Z activity was measured at the end of the culture.

Supplementary Figure 8. Syk is necessary for efficient cross-presentation of dead cell-associated antigens. a, Syk-deficient CD8α$^+$-like DC are not impaired in their ability to capture dead cell material. Purified wild type or Syk-deficient CD8α$^+$-like Flt3L BMDC were incubated at 37ºC for 120 min with PKH26-labelled and UVC- treated H-2bm1 splenocytes at a 1 to 5 ratio. Uptake of dead cell material was determined using an ImageStream® multispectral imaging flow cytometer. Results are mean ± SEM of two independent experiments. **b**, Impaired crosspresentation of dead cell-associated antigen by Syk-deficient DC. Purified wild type or Syk-deficient CD8α+-like Flt3L BMDC were cultured with CFSE-labelled OVA-specific OT-I cells and UVC- treated H-2bm1 splenocytes loaded with the indicated amounts ofOVA. After four days, OT-I proliferation was analyzed by flow cytometry (left panel) and IFN-γ levels in the supernatant were determined by ELISA (right panel). Results are mean ± SEM of four independent biological replicates (cells from different mice) and are representative of four independent experiments. p values were determined using one way ANOVA for comparation between different treatments. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, post-hoc Bonferroni test. b. Left panel. One representative experiment is shown out of

four performed.

Supplementary Figure 9. Blockade of CLEC9A reduces crosspriming to dead cell-associated antigen *in vivo*. C57BL/6 mice either untreated or injected with isotype-matched control (rat IgG1) or anti-CLEC9A antibody were immunised i.v. with $7.5x10^5$ UVC-treated H-2^{bm1} transformed MEFs expressing OVA (UVC bm1T OVA) or not (UVC bm1T). Top: six days later, the frequency of OVA-specific splenic $CD8^+$ T cells was determined by staining with H-$2K^b$/ OVA tetramers. Bottom: IFN-γ production by splenic $CD8^+$ T cells was measured by staining after *ex vivo* restimulation for 5 h with OVA peptide. Data from one representative mouse per group is shown.

Supplementary Figure 10. CLEC9A is associated with necrotic cell material that does not enter lysosomal compartments. a, CLEC9A but not DEC-205 is routed to non-lysosomal compartments after internalisation. 20 µg of Alexa488-coupled anti-CLEC9A or anti-DEC205 antibody were injected i.v. into C57BL/6 mice. 1h later, spleen DC were isolated, stained with Lysotracker and plated on fibronectin-coated coverslips. Confocal sections were collected and the intracellular distribution of CLEC9A and DEC205 was analysed. **b**, CLEC9A colocalises with necrotic cell material away from lysosomes. $CD8\alpha^+$ splenic DC from C57BL/6 mice were purified by cell sorting and co-incubated for 2h with Alexa633SE-labelled necrotic bm1T cells at a 1:2 ratio. The intracellular distribution of necrotic cells material associated, or not, with CLEC9A was then analysed by confocal microscopy. Arrows show co-localization of $CLEC9A^+$ vesicles with necrotic material, whereas arrowheads point at $Lysotracker^+$ vesicles containing necrotic material. **a** and **b**. Quantification of the results shows the mean ± SEM of three experiments ($n > 100$ DCs/experiment). All the differences between white and black bars are statistically significant ($p < 0.001$, Student's t test).

FULL METHODS

Mice

All mice were bred at Cancer Research UK in specific pathogen-free conditions. H-2^{bm1} mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. OT-I x RAG-1$^{-/-}$ were a gift from D. Kioussis, NIMR, Mill Hill, London. Radiation chimeras using fetal liver from *syk*$^{-/-}$ mice[1] (kind gift from E. Schweighoffer and V. Tybulewicz, NIMR, Mill Hill, London) were generated as described[2]. All animal experiments were performed in accordance with national and institutional guidelines for animal care and were approved by the Institutional Animal Ethics Committee Review Board, Cancer Research UK, London.

Generation of clec9a$^{gfp/gfp}$ mice.

Red/ET recombineering (Gene Bridges, Heidelberg, Germany) was used to capture the *clec9a* region from BAC clone RP-23 248-K14 (C57BL/6 BAC clone from Invitrogen) into a conventional gene-targeting replacement vector, pFloxRI+tk. Primers included 20 nucleotides pairing with the vector and 70 nucleotides pairing with the desired regions of *clec9a*: Fw 3arm 24330 pFlox 5' ATAATATCAT ATTTCTATAA TATCATTGTA ATGACAAAAC CACTGAACTA GTGCCTGTAA AGGCAGGAGG GGTACCGAGC TCGAATTCTA CCG 3'; Rv pFlox 5arm 5'TGCTATATTA CAGATTTTCA AGTGGGGTAG CCTGGAGTAA CAAGATGGCA GGGCATAATC ACTAGTGCGG CCGCCACCGC GGTGGAGCTC CAGCTTT 3'

Once the desired genomic region was captured into the Amp-resistant vector, a cassette including farnesylated EGFP, and the PGK-gb2 promoter followed by Kan/Neo was used and the recombineering homologous recombination step was repeated followed by selection for Kan. The primers used for amplification of the EGFP-F Kan-Neo resistant cassette were: Rv NeoKan 3arm 5' TGCTTTTGTA CTTACACTTG ATGCCCAAGA AAATGGACGT TGCTAACAAG CCCATACAGA CCACACCTCG AGATAACTTC GTATAATGTA T3' and Fw 5arm EGFP-F 5' TTTGTGCCAG GCTCCTATGT AGACTGCTTC ACCACTCCAA GCGCCTTCAG

CATGCATGTC GACATGGTGA GCAAGGGCGA GGAG 3'. Upon correct targetting, EGFP is inserted immediately downstream and in frame with the first two aminoacids from CLEC9A and disrupts exons 1 and 2, knocking out a region of 1.35 Kb. Transcription is terminated by a strong poly A signal from EGFP. The targeting vector was linearized using Not I prior to transfection into S6B6 hybrid 129S6/C57BL/6 F1 derived embryonic stem (ES) cells by electroporation. Recombinant clones were isolated after culture in G418 and gancyclovir and were screened by PCR using two independent primer pairs with the forward primer in the Neo region and the reverse primer external to the short arm, as indicated (Fig. 3a). The primer pairs used were: Scr Fw1 5' GATCTGTGTG TTGGTTTTTG TGTGC 3'; Scr Rv1, 5' TAGCATGGCA CTTCTCCATT ACCTT 3' Amplicon Fw1Rv1: 2138 bp. Scr Fw2, 5' GCGAATTCGG TACCAATAAA AGAGC 3'; Scr Rv2, 5' CAGAAGCTTC CTGGTTTTGG TTTTT 3' Amplicon Fw2Rv2: 2352. Correctly targeted, karyotypically euploid ES clones were micro-injected into 3.5 day post coitum C57BL/6 blastocysts and resulting offspring with coat-color chimerism were bred with C57BL/6 females to identify germline transmission. Germline transmitting chimeras were subsequently bred with C57BL/6 females. Heterozygous animals were interbred to generate homozygous deficient animal and matched littermate controls. The expression of NK1.1 is linked to CLEC9A deficiency in the *clec9a*$^{gfp/gfp}$ mice (data not shown), indicating that the homologous recombination step targeted the chromosome of C57BL/6 origin in the F1 S6B6 ES cells.

Cells

RPMI 1640 (Invitrogen) supplemented with glutamine, penicillin, streptomycin, 2-mercaptoethanol (all from Invitrogen) and 10% heat-inactivated foetal calf serum (Bioclear) was used for cell culture. RPMI 1640 (Invitrogen) supplemented with glutamine, penicillin, streptomycin, 2-mercaptoethanol (all from Invitrogen) and 10% heat-inactivated foetal calf serum (Bioclear) was used for cell culture. BWZ and B3Z cells containing a reporter plasmid for NFAT coupled to LacZ activity have been previously described[3] and were a generous gift from N. Shastri (UC Berkeley).

LK35.2 cells (LK cells; reference [4]) were from the American Type Culture Collection (HB-98). Mouse embryonic fibroblasts (MEFs) were derived from H-2^{bm1} mice and immortalized with SV40 T large antigen (bm1 T MEFs) using standard protocols. RBL rat leukemia and HEK293 human embryonic kidney cells were from Cancer Research UK. Flt3L BMDC were generated from bone marrow cultured in the presence of 50-100 ng/ml of Flt3L (R&D, Minneapolis, MN) for 10 days or cultured with supernatant from mouse Flt3L-secreting CHO cells.

Reagents

FITC-, PE- or APC-conjugated anti-CD11c (clone HL3), FITC- or PE-coupled anti-CD24 (clone M1/69), PE-conjugated anti-CD172a (SIRPα, clone P84), FITC-, PE- or APC-labelled anti-CD11b (clone M1/70), FITC-, PE-, PECy5- or APC-conjugated anti-B220 (clone RA3-6B2), FITC-labelled anti-Ly6C (clone AL-21), FITC-, PE-, PerCP- or APC-conjugated anti-CD4 (clone RM4-5) and FITC-, PE-, PerCP- or APC-conjugated anti-CD8 (clone 53.6.7) antibodies (all from BD Pharmingen) were used for flow cytometry experiments and/or cell sorting or enrichment. Capture anti-IFN-γ (R4-6A2, BD), anti-IL-2 (JES6-1A12, BD), anti-IL-12p40/p70 (C15.6, BD), anti-IL-6 (MP5-20F3, BD), anti-mouse Flt3L (R&D) and anti-TNF-α (R&D) and detection biotin anti-IFN-γ (XMG1.2, BD), biotin anti-IL-2 (JES6-5H4, BD), biotin anti-IL12/23 p40 (C17.8, BD), biotin anti-IL-6 (MP5-32C11, BD), biotin anti-mouse Flt3L (R&D) and biotin anti-IL-6 (R&D) antibodies were used for ELISA. Purified anti-FcγRIII/II (2.4G2), used to block unspecific Ab binding, was produced by Cancer Research UK antibody production service. ELISAs were developed using extravidin®-alkaline phosphatase and pNPP alkaline phosphatase substrate from Sigma. H-$2K^b$ / OVA iTAg™ MHC tetramers from Beckman Coulter were used for the detection of OVA-specific $CD8^+$ T cells.

Flow Cytometry

Samples were stained in ice-cold PBS supplemented with 2mM EDTA, 1% FCS and 0.2% sodium azide, with the appropriate antibody cocktails. TO-PRO-3 viability dye was added for all cell death

measurements. Flow cytometry data were acquired on a dual-laser FACS Calibur flow cytometer (Becton Dickinson, San Jose CA) and at least 10,000 live events based on a scatter gate were collected for statistical purposes. Flow cytometry data were analyzed using the FlowJo software version 8.7.3 (Treestar, Oregon). Intracellular staining for IFN-γ was performed using Fix&Perm® (ADG, Kaumberg, Austria) following manufacturer's instructions followed by APC-labelled anti-IFN-γ (XMG1.2, BD).

Biochemistry

For peptide pull-downs, biotin-conjugated peptides were dissolved in 40% DMSO before dilution in lysis buffer (50 mM HEPES [pH 7.4], 150 mM sodium chloride, 100 mM sodium fluoride, 10 mM tetrasodium pyrophosphate, 1 mM sodium orthovanadate [pH 10.0], 1 mM EDTA [pH 8.0], 1.5 mM magnesium chloride, 10% glycerol, 1% Triton X-100, 1 mM PMSF, and "Complete" protease inhibitor cocktail tablets [Roche]). Recombinant human Syk (Upstate) diluted in lysis buffer was incubated with the indicated biotinylated peptides corresponding to the CLEC9A and Dectin-1 intracellular tail (Cancer Research UK Peptide Synthesis Laboratory) and streptavidin-sepharose beads (Sigma Biosciences AB, Uppsala, Sweden). After affinity purification, sepharose beads were washed once in lysis buffer and boiled in SDS gel-loading buffer containing 10% β-mercaptoethanol. Proteins were separated by SDS-PAGE, transferred onto Immobilon PVDF membranes (Millipore Corporation, Bedford, MA), and probed with rabbit anti-Syk (a combination of 2131 serum raised against a synthetic peptide corresponding to amino acids 318–330 of murine Syk – kind gift from V. Tybulewicz, NIMR, Mill Hill – and anti-Syk from Cell Signaling Technology, Inc., raised against a synthetic peptide corresponding to the C terminus of human Syk) followed by chemiluminescent detection.

For biochemical detection of phospho-Syk, LK cells stably transduced to express CLEC9A were plated in 6-well plates coated with anti-CLEC9A or isotype control for the indicated times. Cell extracts were prepared in lysis buffer, insoluble material was discarded and a fixed amount of lysate

was separated by SDS-PAGE and transferred and probed with anti-Syk or with anti-phospho-Syk (Cell Signaling) as described above.

NFAT reporter assay in BWZ and B3Z cells

CLEC9A or a mutant Y7F version was introduced with or without Syk into B3Z cells by retroviral transduction. Cells were plated in 96 well plates coated with isotype control or anti-CLEC9A mAb and after overnight culture, were washed in PBS and LacZ activity measured by lysis in CPRG (Roche)-containing buffer. Four hours later O.D. 595 was measured using O.D. 655 as a reference. Alternatively, for detection of CLEC9A ligands, BWZ cells were transduced with a retrovirus encoding a chimera of the extracellular domain of mouse or human CLEC9A fused to the transmembrane region from NKRP1B and the intracellular tail of CD3ζ followed by an IRES sequence and the GFP gene[5]. Ligand binding to the CLEC9A-ζ chimera results in the activation of the NFAT reporter and induction of β-gal activity[5]. To assay basal activation in BWZ cells expressing mouse and human CLEC9A-ζ, 4, 2, 1, or $0.5x10^6$ cells/ml were cultured in 3 ml in a 6 well plate and allowed to (over)grow for two days. Frequency of dead cells was determined using TO-PRO-3 iodide (Invitrogen) and a fixed number of live cells was plated in fresh medium at $2x10^5$/well in 96-well flat bottom plates. After overnight culture, LacZ activity was measured as above.

To induce exposure of CLEC9A ligand, cells were UVC irradiated (240 mJ/cm^2) and cultured for 12-24 h to induce secondary necrotic cell death before adding to $2x10^5$ BWZ expressing CLEC9A-ζ in a 96-well flat bottom plate at a 1:1 ratio in the presence or absence of control Fab or anti-mouse CLEC9A (1F6) and anti-human CLEC9A (8F9) Fab[5]. After overnight culture, LacZ activity was measured as above. In some assays, BWZ cells transfected with different combinations of wild type or mutant CLEC9A and Syk were used to determine if dead cells induce Syk-dependent signalling through CLEC9A and to evaluate the ability of anti-CLEC9A antibodies to block this response. UVC dose response was performed with the following doses of UVC (mJ/cm^2): 0, 0.5, 1.5, 5, 15,

50, 240. Frequency of dead cells was determined 24 h later using the viability dye TO-PRO-3 and cells were plated 1:1 with BWZ cells expressing CLEC9A-ζ ($2x10^5$ cells/well) in fresh medium. After overnight culture, LacZ activity was measured as above.

Different cell death-inducing treatments were also evaluated. MEFs were cultured for 24 h with Mitoxantrone (1 μM) or in the absence of serum (serum deprivation). Death was also induced by submitting a pellet of cells to three cycles of freeze-thaw (freezing in liquid N_2 and thawing at 37ºC). After treatment, the frequency of dead cells was determined by incubation with the viability dye TO-PRO-3 and cells were stained with rsCTLD tetramers and analyzed by flow cytometry and/or were plated 1:1 in fresh medium with $2x10^5$ B3Z cells expressing the CLEC9A-ζ chimera or co-expressing the wild-type form of mouse CLEC9A and Syk. After overnight culture, LacZ activity was measured as above.

Recombinant soluble CTLD generation and staining.

The CTLD for mouse CLEC9A and Dectin-1 was cloned in frame in the p3xFlag-CMV-9 expression vector from Sigma with an added BirA monobiotinylation sequence. Primers used for CTLD amplification were CLEC9A CTLD Fw EcoRI, 5'GAATTCGGTA GTGACTGCAG CCCTTGTCCA3'; CLEC9A CTLD Rev XbaI, 5'GTCTAGAATG CAGGATCCAA ATGCCTTCTT C3'; mDectin1 CTLD BamFw, 5'GGATCCCTCC AAGGCATCCC A3'; mDectin1 CTLD BamRv, 5'GGATCCATTT ACAGTTCCTT CTCA3'. CHO cells were transfected and selected with G418 (1 mg/ml). Stable transfectants were cloned twice by limiting dilution. Clones secreting CTLD from Dectin-1 or CLEC9A were detected by a sandwich ELISA using anti-flag M2 (Sigma) as capture and biotin-2A11 anti-Dectin-1 (kind gift from G. Brown, Cape Town, South Africa) or biotin-1F6 anti-CLEC9A for detection. Concentrated supernatants from CHO clones were generated in CELLine bioreactors (Integra Biosciences, Chur, Switzerland) and were purified using anti-flag M2 agarose (Sigma). Monobiotinylation was performed using standard procedures and tetramers were generated using PE-Streptavidin (Sigma). PE-tetramers of

CLEC9A or Dectin-1 CTLD were used as staining reagents for 30 min at 4 ºC in normal FACS buffer to stain zymosan particles, cells induced to die by several methods or cells fixed in 2% formaldehyde and subsequently permeabilized (0.5% Triton-X-100). Samples were counter-stained with TO-PRO-3 before flow cytometric analysis.

To analyze the nature of the ligand, 10^6 cells fixed and permeabilized as above were incubated in 50 μL final volume with N-Glycosidase F (PNGaseF, 10U, Roche) at 37º C o/n, O-Glycosidase (0.2 U, Sigma) at 37º C o/n, Benzonase (50 U, Merck) at 37º C o/n, Proteinase K (10μg, Qiagen) for 10 min at 37º C, Trypsin (100μg, Gibco) for 10 min at 37º C, or Pronase E (10 μg, Sigma) for 10 min at 37º C. Alternatively, fixed and permeabilized cells were treated with Gly 0.1M pH 3.5 for 10 min at 37º C to assess acid pH sensitivity or incubated for 5 min at 80º C for heat denaturation. Samples were then stained with PE-tetramers as above.

In another set of experiments, monobiotinylated CTLD were used in combination with streptavidin-Alexa Fluor 488 to stain MEFs previously fixed and permeabilized as indicated above and counterstained with TO-PRO 3. Dead MEFs were then allowed to adhere to poly-L-lysine–coated coverslips at room temperature and mounted using Fluoromount (SouthernBiotech). A confocal series of differential interference contrast and fluorescence images was obtained using a laser scanning confocal microscope (Axioplan 2; Zeiss) with a 63 Plan-Apochromat NA 1.4 oil objective. Image analysis was performed with LSM 510 software (Zeiss).

In vitro and in vivo uptake of dead cells material.

Flt3L BMDC were incubated at 4°C or 37 °C for 0, 30 or 120 min at different ratios with red blood cell-depleted H-2^{bm1} splenocytes previously labelled with PKH26 (Sigma), treated with UVC (240 mJ/cm^2) and allowed to undergo secondary necrosis for 16h. Uptake of dead cell material by $CD11c^+B220^-CD24^{high}$ CD8α-like Flt3L BMDC was then assessed by flow cytometry. The phagocytic index was calculated by subtracting the frequency of positive events at 4°C (binding) from the frequency at 37°C (binding + uptake).

Alternatively, B220^{-}CD11c^{high}CD24^{high} CD8α-like DCs[6] were purified by cell sorting on a high speed MoFlo cell sorter (Beckman Coulter, Fort Collins CO) and co-incubated with UVC-treated and PKH26-labelled splenocytes at 4°C or 37°C for 90 min. Samples were then fixed in 2% paraformaldehyde and acquired on a multispectral imaging flow cytometer (ImageStream®, Amnis Corp., Seattle WA). True uptake of PKH26-labelled dead cell material by DC was determined using an internalization feature that looks at the degree to which the PKH26 staining co-localises with DC defined by tight masking on the brightfield image. Confirmation of internalization was assessed by examination of the image gallery of DCs^{+}PKH26^{+} cells. 10,000 single and focused cells were collected per sample and data was analyzed using IDEAS^{TM} 3.0.245 software (Amnis Corp., Seattle, WA).

For *in vivo* experiments, mice were injected *i.v.* with 20x10^{6} red blood cell-depleted, UVC-treated, and PKH26-labelled H-2^{bm1} splenocytes. Uptake of dead cell material by CD8α^{+} and CD8α^{-} CD11c^{+} splenocytes was assessed by flow cytometry 2h after injection, as reported[7].

Confocal microscopy

CD8α^{+}-like DC from Flt3L-BMDC cultures and UV-treated bm1 T OVA MEFs were brought into contact by brief centrifugation and were cultured on poly-L-lysine-coated coverslips for 20 min at 37°C. Cells were then washed in PBS containing 1mM $MgCl_2$ and 1mM $CaCl_2$, fixed in 3,7% paraformaldehyde/PBS for 10 min and permeabilised in 0.2% Triton-X100/PBS for 4 min. After washing with PBS, cells were blocked with 5% goat serum (Sigma)/PBS and anti-FcγRII/III receptor. Phosphorylated Syk was detected with rabbit polyclonal anti-phospho-ZAP70(Tyr319)/Syk(Tyr352) (Cell Signaling Technology), followed by cross-adsorbed Alexa555-labeled goat anti-rabbit IgG (Molecular Probes, Invitrogen). A confocal series of fluorescence images was obtained with a laser scanning confocal microscope Axiovert 100 (Zeiss). Image analysis was performed with LSM 510 software (Zeiss) and signal intensity was measured using ImageJ software (by Wayne Rasband, NIH). Accumulation of phosphorylated Syk at interface

between CD8α^+-like DC and MEF corpse was quantified by measurement of the z-axis projection of the fluorescence intensity distribution along the major axis of the conjugate. Phospho-Syk was considered to be enriched at the contact area when the signal intensity was more than twice that of the background.

To test the uptake of antibody *in vivo*, C57BL6 mice were injected with 20 μg of anti-DEC205 or anti-CLEC9A antibody and spleens were isolated one hour later. CD8α^+spleen DC were purified using selection with magnetic microbeads (Miltenyi), loaded with Lysotracker dye (50 nM, Molecular Probes, Invitrogen) for 2h at 37 ºC, and allowed to adhere to fibronectin (50 μg/ml, Sigma-Aldrich) coated coverslips, before fixation, mounting and analysis by confocal microscopy as above.

To analyse the localisation of necrotic material within DC, bm1T MEFs were labelled with Alexa633-SE (Molecular Probes, Invitrogen) and then irradiated with UV 2400 J/cm^2 and allowed to undergo necrosis overnight. CD8α^+ DC were sorted and allowed to uptake necrotic material and Lysotracker dye for 2h before being adhered to fibronectin as above. Cells were fixed and stained for CLEC9A using anti-CLEC9A 7H11 Alexa 488 coupled antibody. Samples were analyzed as above.

Crosspriming in vitro.

CD11c$^+$B220$^-$CD11b^{int}CD24^{high}B220^{neg} CD8α-like Flt3L BMDC[6] were sorted on a MoFlo® high speed cell sorter (Beckman Coulter, Fort Collins CO). Clumps of cells were excluded based on pulse width and dead cells were gated out using a scatter gate. Alternatively, the CD8α^+-like DC subset was enriched by negative selection using PE-labelled anti-SIRPα and anti-B220 antibodies (BD) and anti-PE microbeads (Miltenyi). The purified population was routinely more than 95% pure for CD8α^+-like cells, with total absence of B220$^+$ plasmacytoid DC. These DC were co-cultured with antigen and CFSE-labelled OVA-specific CD8 OT-I cells enriched by negative selection (purity > 80%) using a cocktail of PE-labelled mAbs and anti-PE microbeads (Miltenyi).

OT-I priming was measured by analyzing CFSE-dilution profiles by flow cytometry and by monitoring IFN-γ levels in supernatants harvested at day 4 of culture.

As a source of antigen, we used bm1 T MEFs transduced to express a truncated non-secreted OVA-GFP fusion protein and sorted for homogeneous expression of GFP (bm1 T OVA MEFs). Before the assay, OVA-expressing bm1 T MEFs were UVC-irradiated (240 mJ/cm^2) and cultured overnight in complete medium to induce secondary necrosis. As control, low endotoxin OVA protein (Calbiochem) was used as a source of soluble antigen or coated on 3 μm polystyrene beads (Sigma).

In some experiments, bone marrow cells from *clec9a*$^{egfp/egfp}$ mice were transduced at day 0, 1 and 2 of culture with Flt3L with retroviruses with no insert or an insert encoding wild type CLEC9A or CLEC9A Y7F, under the control of a PGK promoter. All retroviruses additionally encoded for GFP under the control of a separate PGK promoter. In transduced cells, GFP correlated directly with CLEC9A expression (not shown). On day 10 of culture, GFP$^+$ B220$^-$CD11b^{int}CD24^{high} CD8α-like transduced Flt3L BMDC were sorted on a MoFlo® high speed cell sorter (Beckman Coulter) and used for cross-presentation assays in vitro as described above.

Crosspriming in vivo

UVC-treated bm1 T OVA MEFs were washed 3 times in PBS before being injected i.v. (0.75x10^6 cells/mouse) into *clec9a*$^{gfp/gfp}$ or littermate wild type sex-matched control mice or C57BL/6 mice pre-treated 30 min prior immunization with an i.p. injection of PBS or 400μg of isotype-matched control (AFRO-MAC 49) or anti-CLEC9A (1F6) mAb. Injections of antibodies did not affect DC subsets (not shown). Five to six days later, CD8$^+$ T cell responses were measured by quantitating the number of H-2K^b/OVA tetramer positive CD8$^+$ T cells or by OVA peptide restimulation and intracellular staining for IFN-γ. In Figure 4a, data were pooled from sex-matched littermates (in this case, female) from sixteen litters in seven independent experiments. For the litter analysis in Fig. 4b, the mean for CLEC9A$^+$ or CLEC9A$^-$ mice was calculated in each litter. Data from littermates

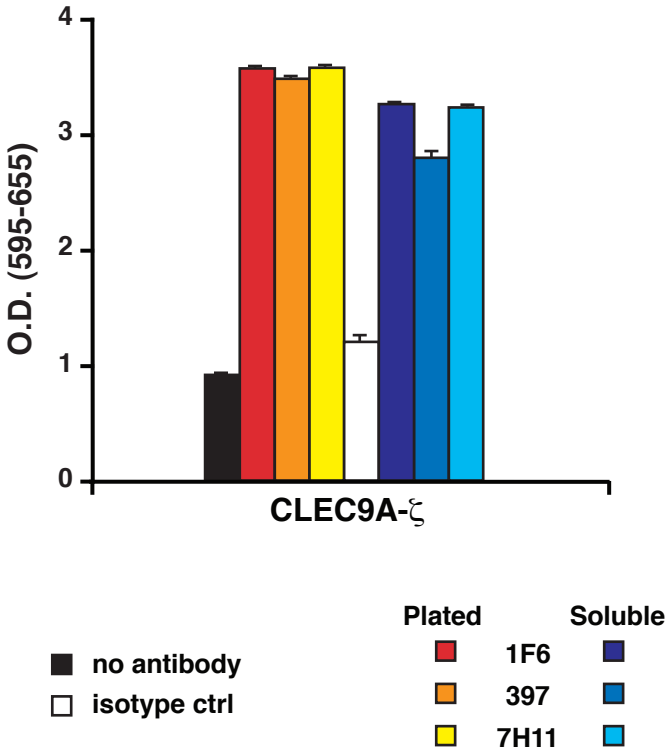
are shown connected with a line.

Statistics

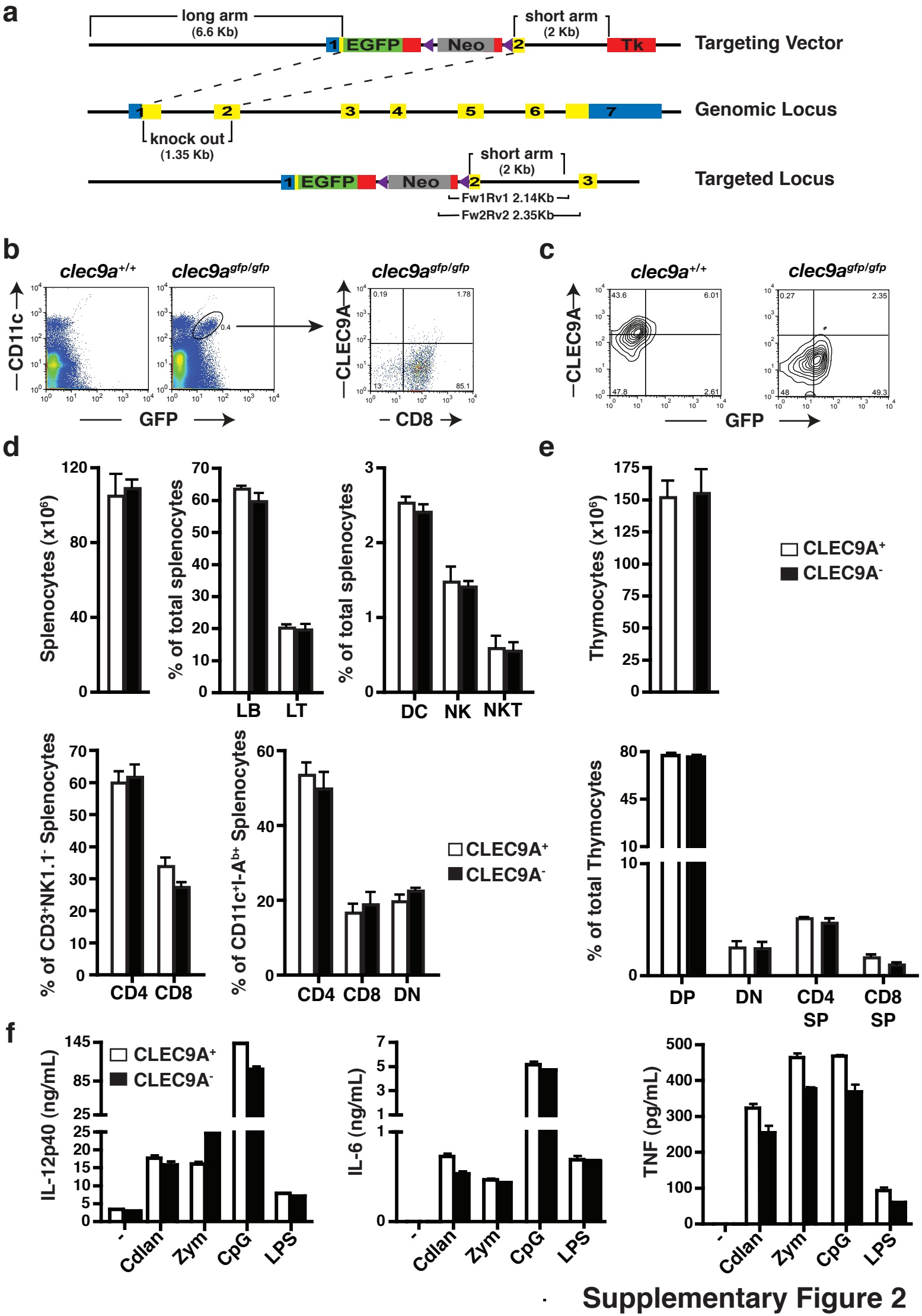
Statistical analysis was performed with a two-tailed Student's t test for differences between groups or U Mann-Whitney when normality of data could not be inferred. Paired Student's t test was used for the analysis of average in each litter (Fig. 4b). Two way ANOVA was used to test for comparations among more than two groups, as in Fig. 3b. $p<0.05$ was considered statistically significant. Quantitative data are expressed as means ± SEM unless otherwise stated.

Supplementary references

1. Turner, M. *et al.* Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature* **378**, 298-302 (1995).
2. Rogers, N.C. *et al.* Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity* **22**, 507-517 (2005).
3. Karttunen, J., Sanderson, S. & Shastri, N. Detection of rare antigen-presenting cells by the lacZ T-cell activation assay suggests an expression cloning strategy for T-cell antigens. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6020-6024 (1992).
4. Kappler, J., White, J., Wegmann, D., Mustain, E. & Marrack, P. Antigen presentation by Ia^+ B cell hybridomas to H-2-restricted T cell hybridomas. *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3604-3607 (1982).
5. Sancho, D. *et al.* Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin. *J Clin Invest* **118**, 2098-2110 (2008).
6. Naik, S. *et al.* Cutting edge: generation of splenic CD8+ and CD8- dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. *J. Immunol.* **174**, 6592-6597 (2005).
7. Iyoda, T. *et al.* The CD8(+) Dendritic Cell Subset Selectively Endocytoses Dying Cells in Culture and In Vivo. *J. Exp. Med.* **195**, 1289-1302. (2002).



Supplementary Figure 1



Supplementary Figure 2

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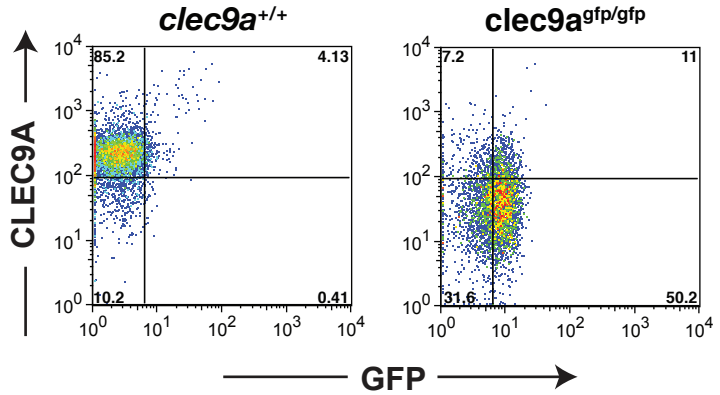

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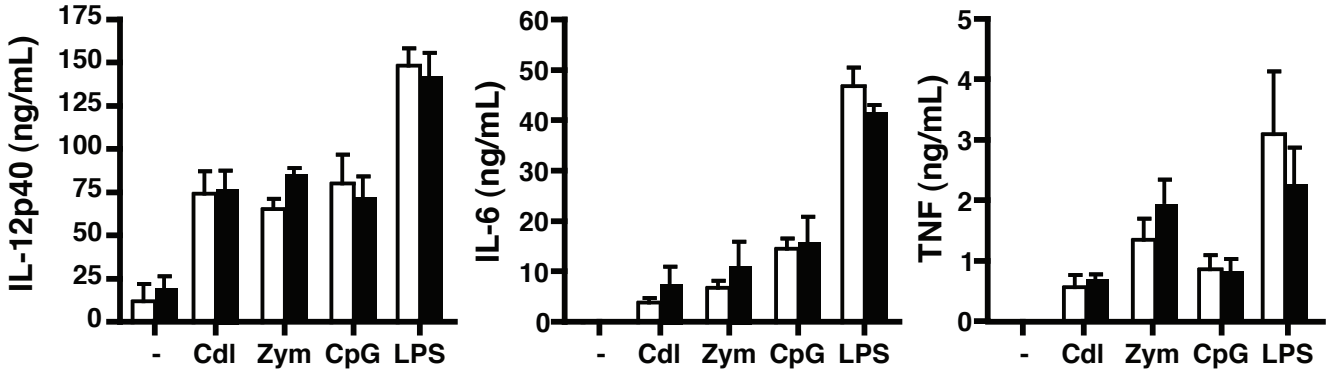
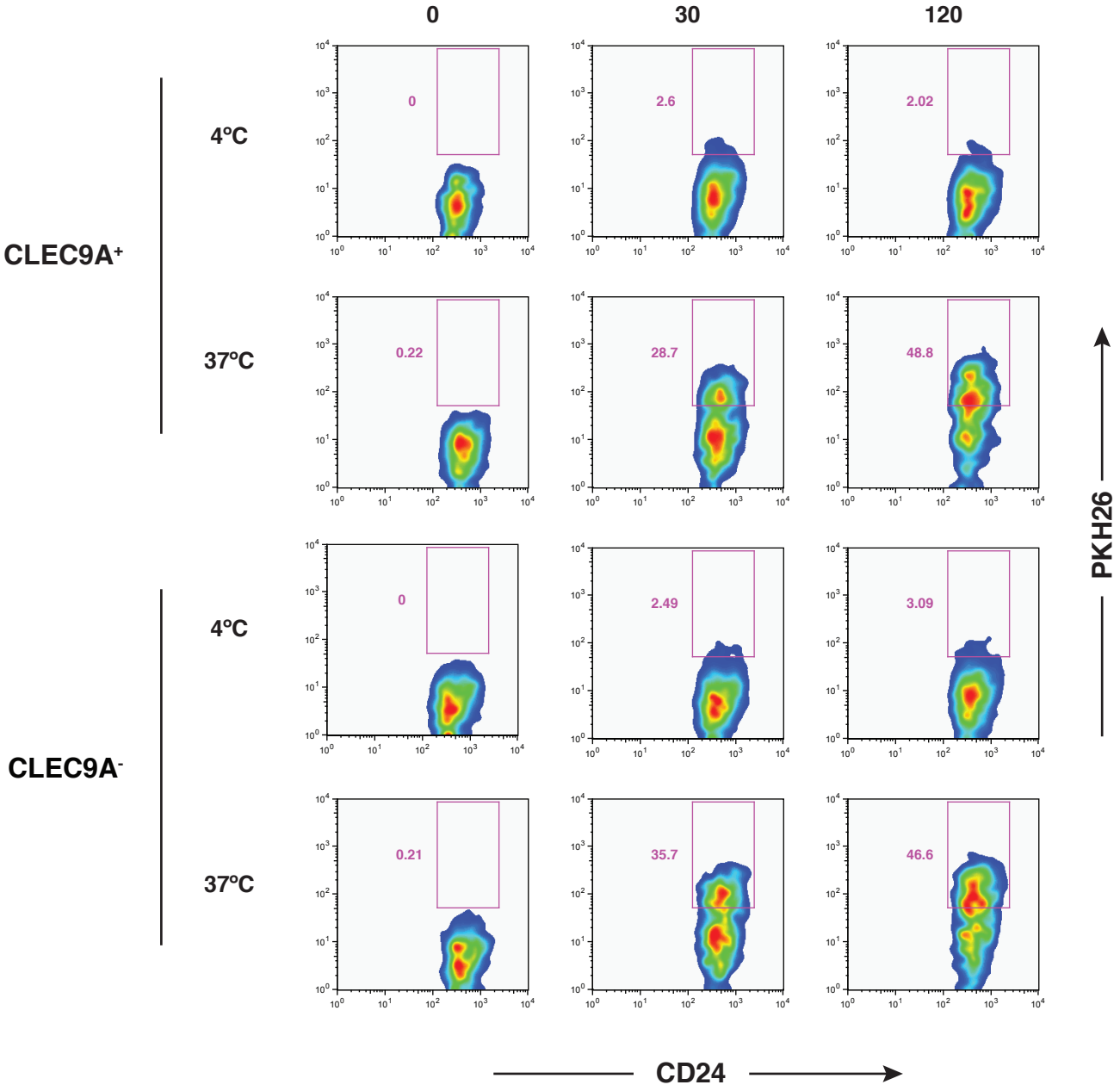


Supplementary Figure 3



Supplementary Figure 4

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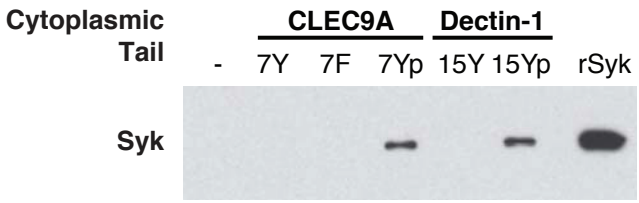

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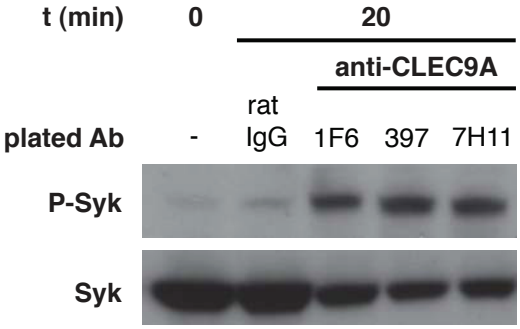

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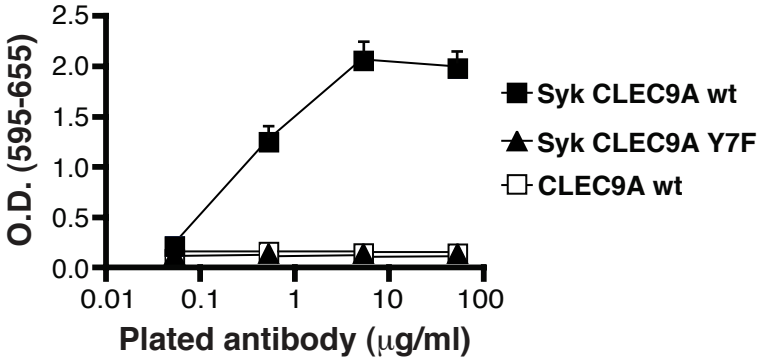
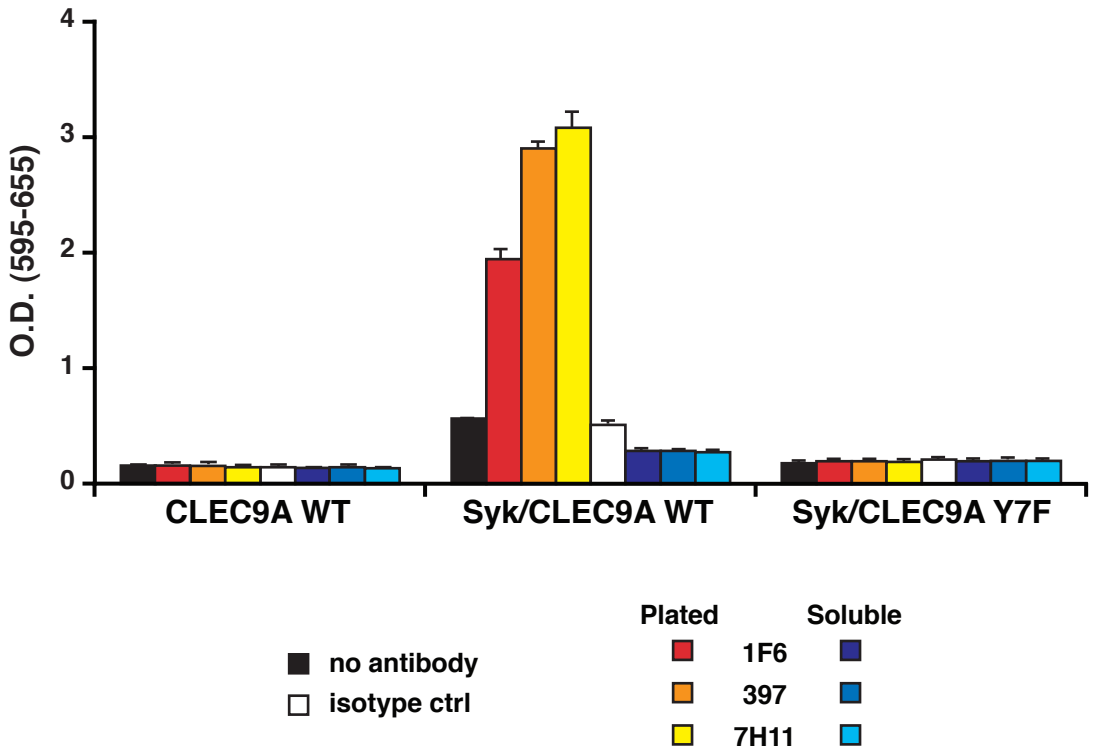


Supplementary Figure 5



Supplementary Figure 6

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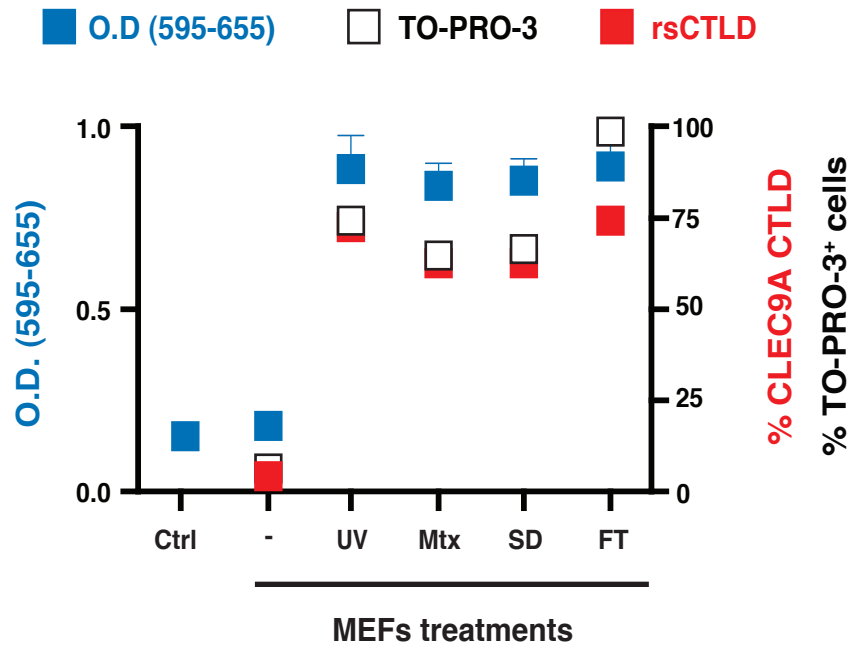

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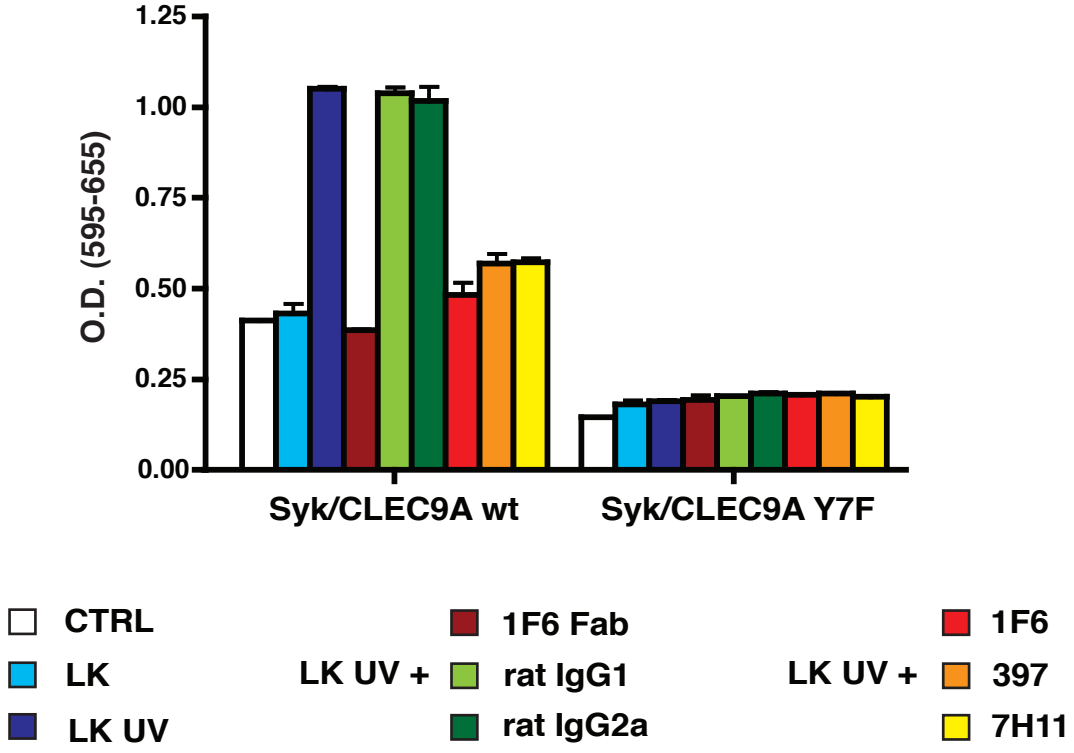
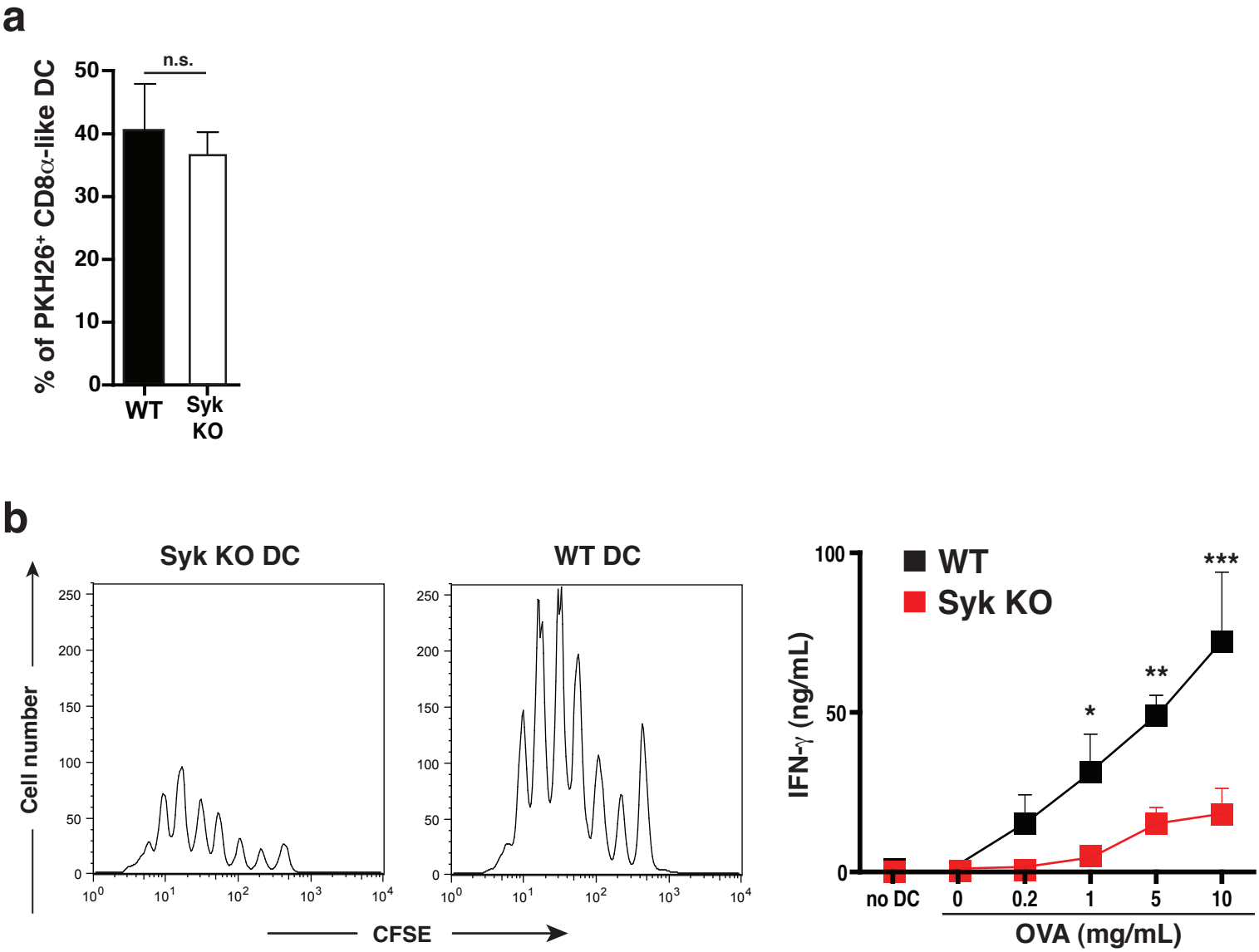
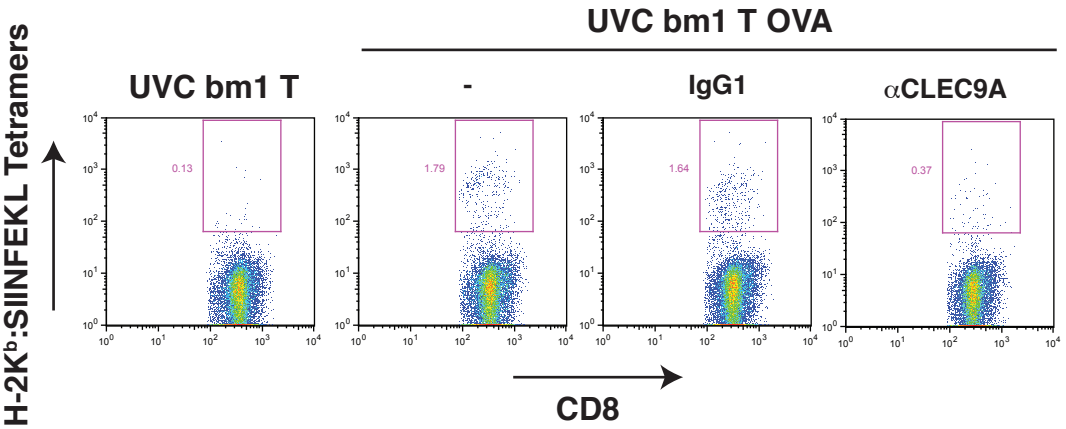
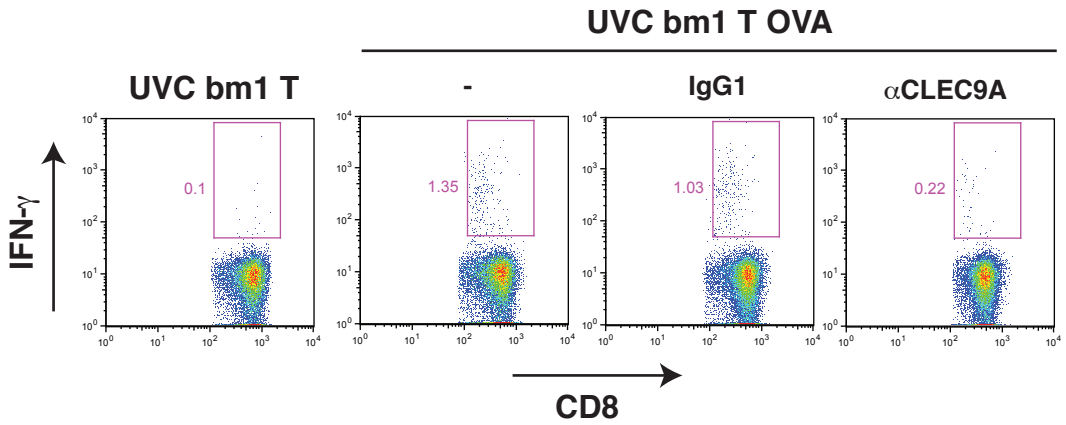


Supplementary Figure 7



Supplementary Figure 8





Supplementary Figure 9

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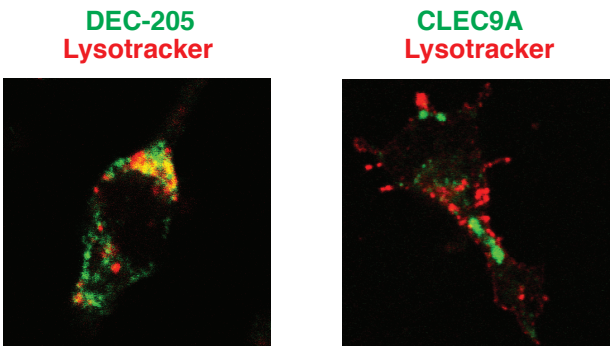

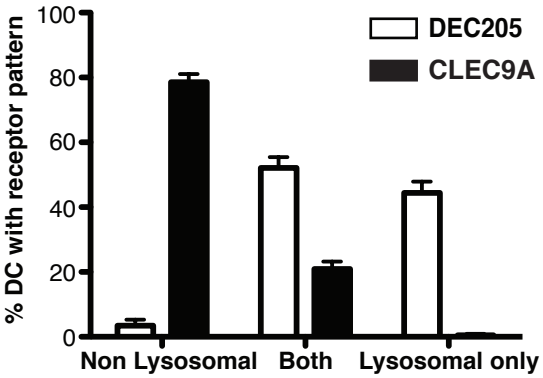

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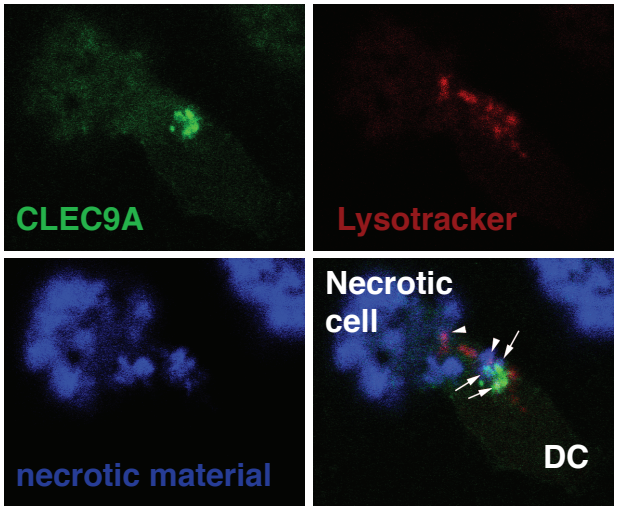

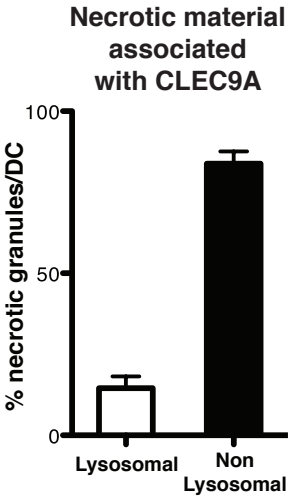

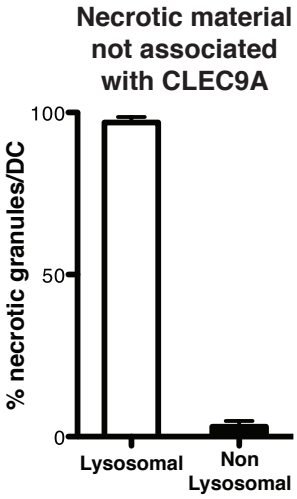

Supplementary Fig. 10