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Supplemental Information

The Phosphoinositide Kinase PIKfyve Promotes Cathepsin-S-Mediated Major Histocompatibility Complex Class II Antigen Presentation

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SUPPLEMENTAL FIGURES

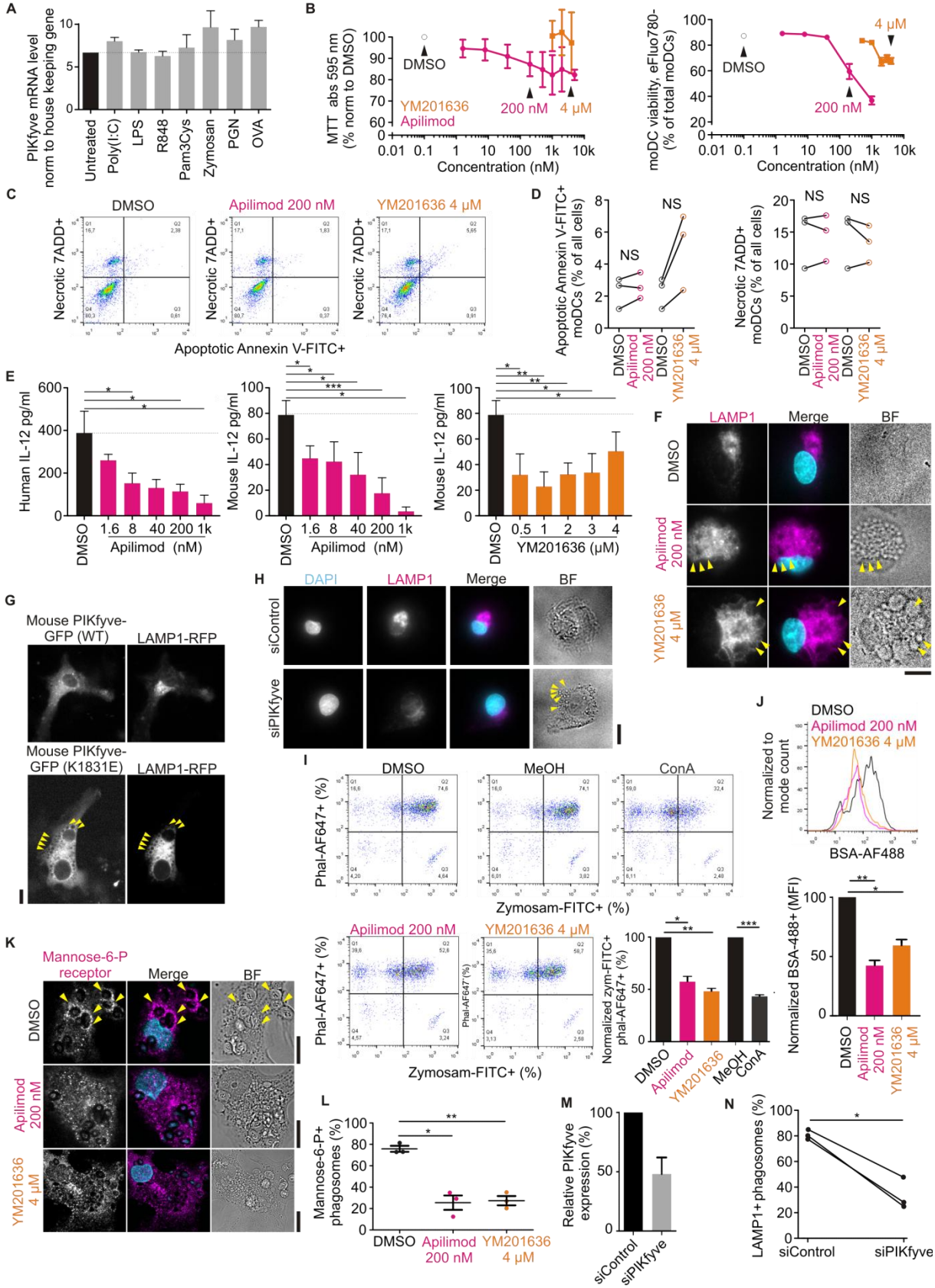


Figure S1. Characterization of PIKfyve inhibitors apilimod and YM201636. Related to Figure 2. (A) Expression of PIKfyve by qPCR in human monocyte-derived dendritic cells after 1 h stimulation with polyionosinic:polycytidylic acid (poly(I:C)) (TLR3 agonist); lipopolysaccharides (LPS) (TLR4 agonist);

resiquimod (R848) (TLR7 agonist); Pam(3)Cys (TLR2 agonist); zymosan (Dectin-1 and TLR2 agonist); pepdidoglycan (PGN) (TLR2 agonist); OVA (CD206 mannose receptor agonist). The expression levels were normalized to hydroxymethyl-bilane synthase (HMBS). (mean ± SEM for multiple donors). (B) Viability of human dendritic cells treated for 3 h with the indicated concentrations of apilimod or YM201636 by MTT assay (left) or fixable viability stain eFluo780 (right) (mean ± SEM for three donors). Black arrowheads: drug concentrations used throughout this study. DMSO: solvent control. (C) Representative flow cytometry dot-blots of dendritic cells stained with annexin-V (early apoptotic cells) and 7ADD (necrotic cells) after 3 h treatment with apilimod (200 nM) or YM201636 (4 µM). (D) Quantification of panel C (individual donors shown). Left panel: quantification of sum of Q2 and Q3 population of annexin-V positive early apoptotic cells. Right panel: quantification of populations in Q1 and Q2 of 7AAD-positive necrotic cells. NS: not significant. (E) IL-12 production measured after 24 h LPS (1 µg/ml) stimulation by human monocyte-derived dendritic cells (left) or murine bone-marrow-derived dendritic cells (middle and right) in the absence or presence of apilimod or YM201636 (mean ± SEM for three donors or mice). (F) Confocal images of monocyte-derived human dendritic cells treated with DMSO, apilimod or YM201636 and immunostained for LAMP1 (magenta in merge). Cyan: DAPI. BF: bright-field. Yellow arrowheads: enlarged vacuoles. (G) Confocal images of representative dendritic cells expressing LAMP1-RFP with mouse wild-type or kinase-dead (K1831E) PIKfyve-GFP. Yellow arrowheads: enlarged vacuoles with the kinase-dead PIKfyve-GFP. (H) Representative images of moDC with siRNA knockdown of PIKfyve (siPIKfyve). siControl: non-targeting siRNA control. Yellow arrowheads: enlarged vacuoles. BF: bright-field. (I) Phagocytosis by dendritic cells measured with flow cytometry and FITC-labeled zymosan particles (zymosan-FITC) and DMSO, apilimod or YM201636 treatment. Cells were labeled with phalloidin-Alexa Fluor 647 (phal-AF647). Bar graphs show quantification of phagocytosing cells (Q2 population in representative scatter plots; mean ± SEM of 3 donors). (J) Same as panel I, but now for endocytosis of BSA-Alexa Fluor 488 (BSA-AF488) for 1 h. Representative histograms shown. (K) Confocal images of zymosan-pulsed dendritic cells immunolabeled for mannose-6-phosphate receptor (magenta in merge) and with 3 h treatment with DMSO (solvent control), apilimod or YM201636. Yellow arrowheads: phagosomes positive for mannose-6-phosphate receptor. (L) Graph: quantification of panel G for 3 donors (~300 phagosomes/condition/donor; mean ± SEM). (M) Quantification of siPIKfyve by qPCR (mean ± SEM of 3 donors). (N) Quantification of LAMP1-positive phagosomes as in main Fig. 2A-D, but now with siPIKfyve. Zymosan was added for 1 h before fixation (~160 phagosomes/condition/donor). Scale bars: 10 µm.

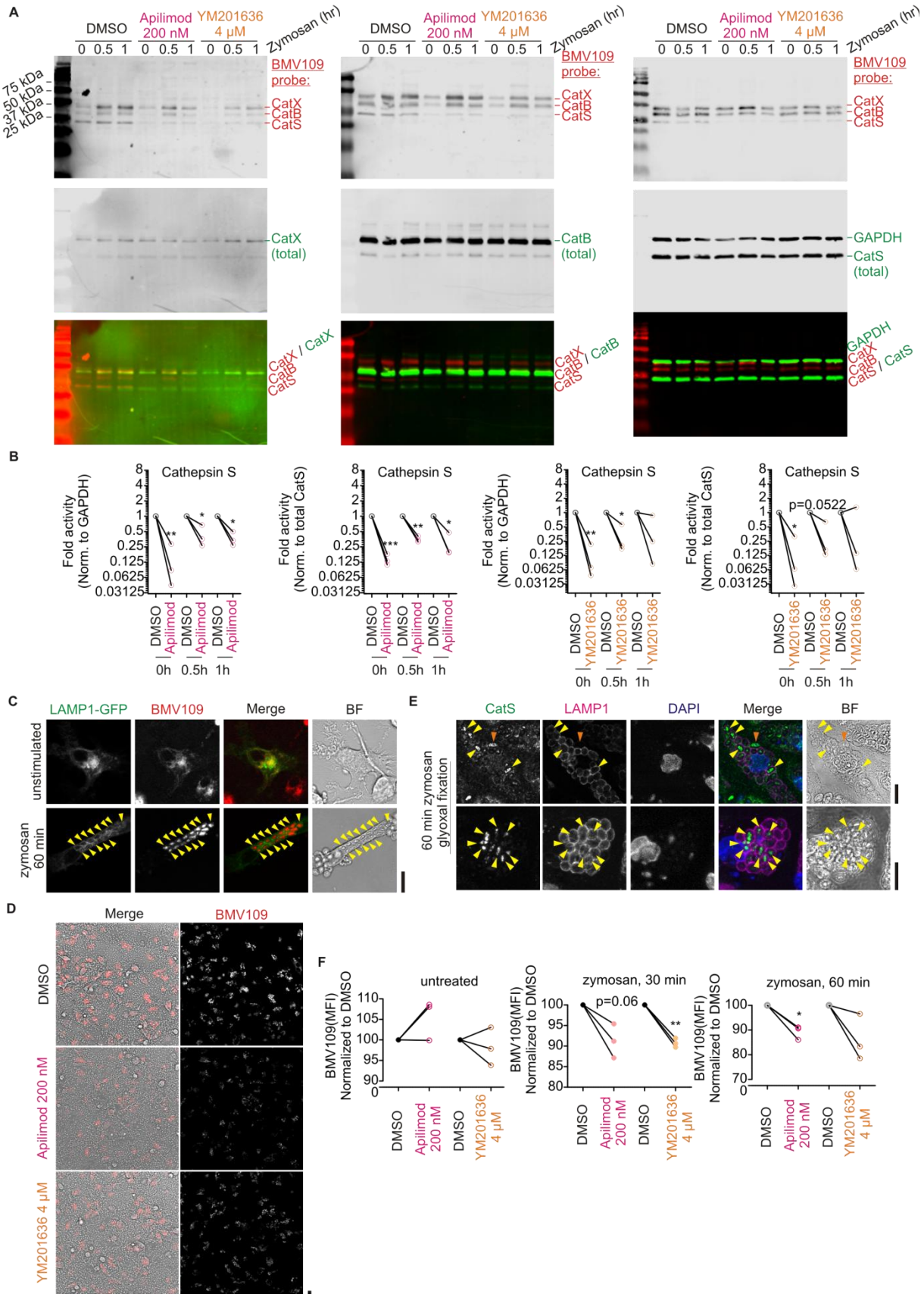


Figure S2. Control experiments for cathepsin activity and trafficking in human dendritic cells. Related to Figure 3. (A) Upper row: SDS-PAGE followed by in-gel fluorescence of human monocyte-derived dendritic cells treated with zymosan and activity-based probe BMV109-Cy5 (red bands in merge). Middle row:

subsequent immunoblotting to PVDF and labeling with antibodies specific for cathepsins (Cat) X, B and S and GAPDH (green bands in merge). Lower row: merged images of BMV109 probe (red) and immunolabeled cathepsins (green) or GAPDH (green, only labeled on the bottom right blot). Every column of images is from a separate donor. Full SDS PAGE/PVDF blots are shown. (B) Quantification of CatS activity measured from panel A. Normalization of each condition is either to GAPDH or total CatS (individual donors shown; normalized to DMSO controls). (C) Confocal live cell imaging of representative dendritic cells expressing GFP-tagged LAMP1 (LAMP1-GFP; green in merge) and pulsed with BMV109 (red in merge) after 1h of zymosan stimulation. Yellow arrowheads: phagosomes positive for LAMP1-GFP and BMV109 signal. BF: bright-field. (D) Representative confocal live cell image of dendritic cells treated as in panel A (1h with zymosan). (E) Confocal image of zymosan-pulsed dendritic cell with immunolabeling for LAMP1 (magenta in merge) and cathepsin S (green). Blue: DAPI. Yellow arrowheads: phagosomes positive for LAMP1 and CatS. Orange arrowhead: phagosome with CatS only. (F) Quantification of panel D (~500 images per condition/donor; mean ± SEM of 3 donors). Scale bars: 10 µm.

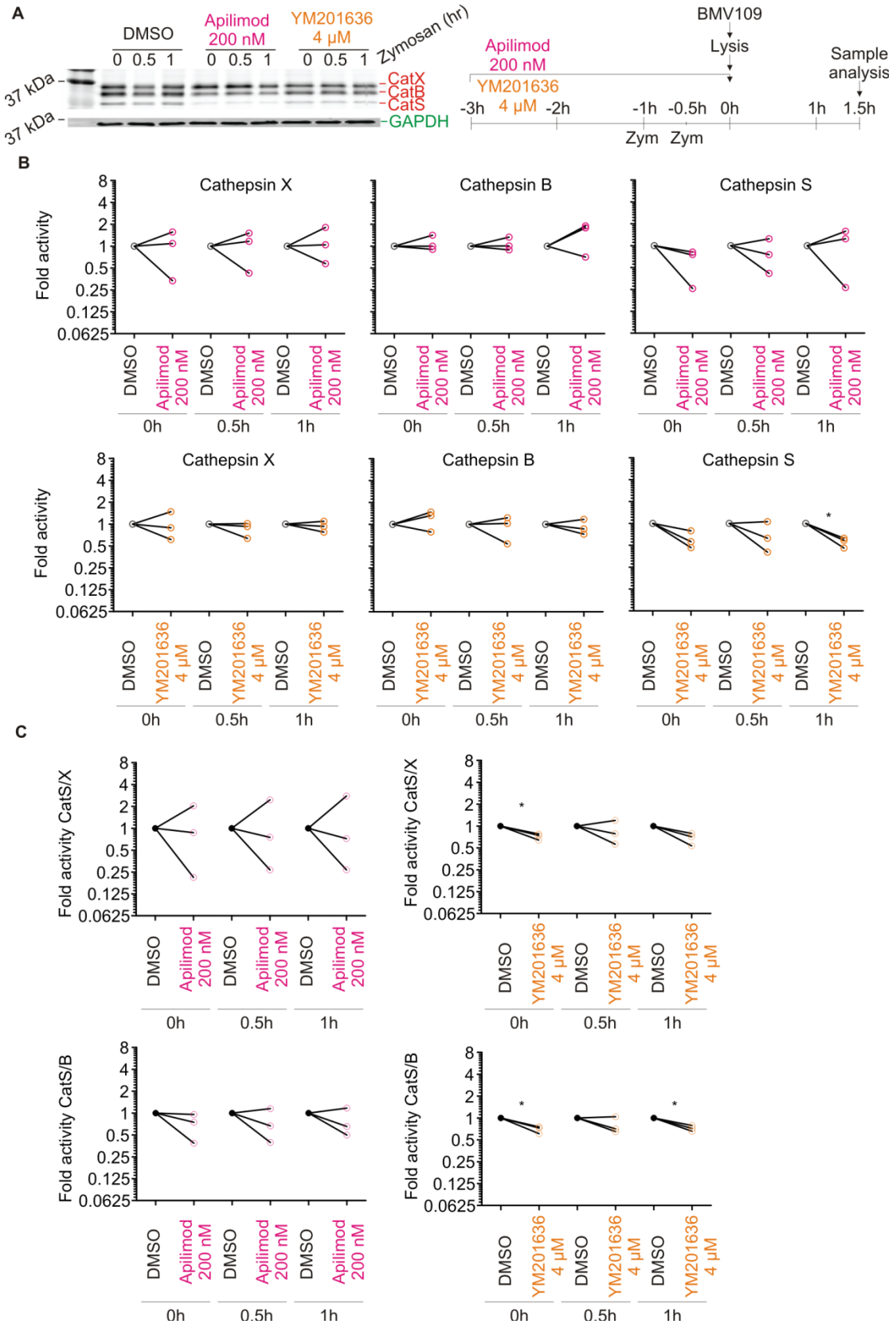


Figure S3. Cathepsin activity in lysate of human monocyte-derived dendritic cells. Related to Figure 3. (A) Zymosan pulsed dendritic cells were lysed and BMV109 probe was added to the cell lysates and incubated on ice for 1.5h. Lysates were resolved with SDS-PAGE and in-gel fluorescence (right-hand scheme). GAPDH: loading control by Western blot. Only part of the SDS PAGE/PVDF blot is shown, the rest of the image carried no information. (B) Quantification of BMV109 signals from panel A (3 donors; individual donors shown;

normalized to DMSO controls). (C) Same as panel B, but now BMV109 signal for CatS normalized to CatX and CatB.

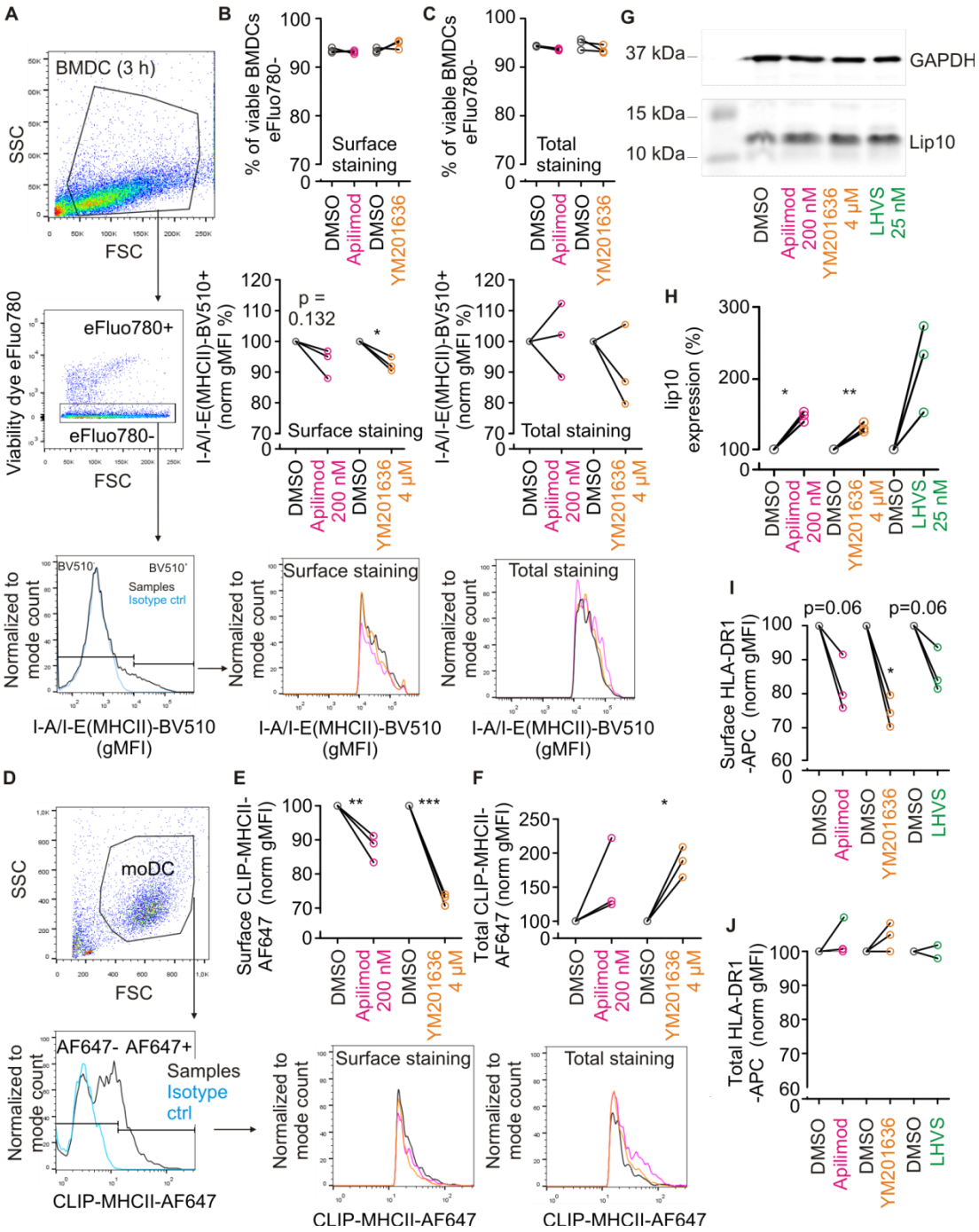


Figure S4. Control experiments for cathepsin activity and trafficking in human and mouse dendritic cells. Related to Figure 4. (A) Gating strategy for flow cytometry experiments with mouse bone-marrow derived dendritic cells (BMDCs) where surface MHC-II (I-A/I-E(MHCII)-BV510) was accessed in non-permeabilized cells negative for the fixable viability dye eFluo780. Representative histograms show the geometric mean fluorescence intensities (gMFI). Cells were treated for 3 h with apilimod or YM201636; SSC: side scatter. FSC: forward scatter. Light blue: isotype control. (B) Quantification of viable eFluo780-negative cells and surface MHC-II signals from panel A normalized to DMSO solvent controls (individual mice shown; normalized to DMSO controls). (C) Same as panels A-B, but now in presence of detergent permeabilization for assessment of total cellular MHC-II. (D) Gating strategy for flow cytometry of human monocyte-derived dendritic cells (moDC) immunolabeled using an antibody recognizing CLIP-bound MHC class II (CLIP-MHCII-AF647) labeled with Alexa Fluor-647 (AF647). (E) Representative histograms and quantification of surface CLIP-bound MHC class II (CLIP-MHCII-AF647) with flow cytometry experiments of moDCs treated for 3h with apilimod

(200 nM) or YM201636 (4 µM) (geometric mean fluorescence intensities (gMFI); individual donors shown; normalized to DMSO control). (F) Same as panel E, but now for total CLIP-bound MHC-II (Total CLIP-MHCII-AF647) with detergent permeabilization. (G) Representative Western blot of lysates from murine BMDCs labeled for CLIP. lip10: 10 kDa precursor fragment of CLIP. LHVS: selective cathepsin S inhibitor. GAPDH: loading control. Only part of the SDS PAGE/PVDF blot is shown, the rest of the image carried no information. (H) Quantification of panel G normalized to GAPDH (BMDCs from different mice shown; normalized to DMSO control). (I-J) Same as D-F, but now using an antibody recognizing MHC-II (HLA-DR1) conjugated with APC and an extra condition with CatS inhibitor LHVS (25 nM) (individual donors shown; normalized to DMSO controls).

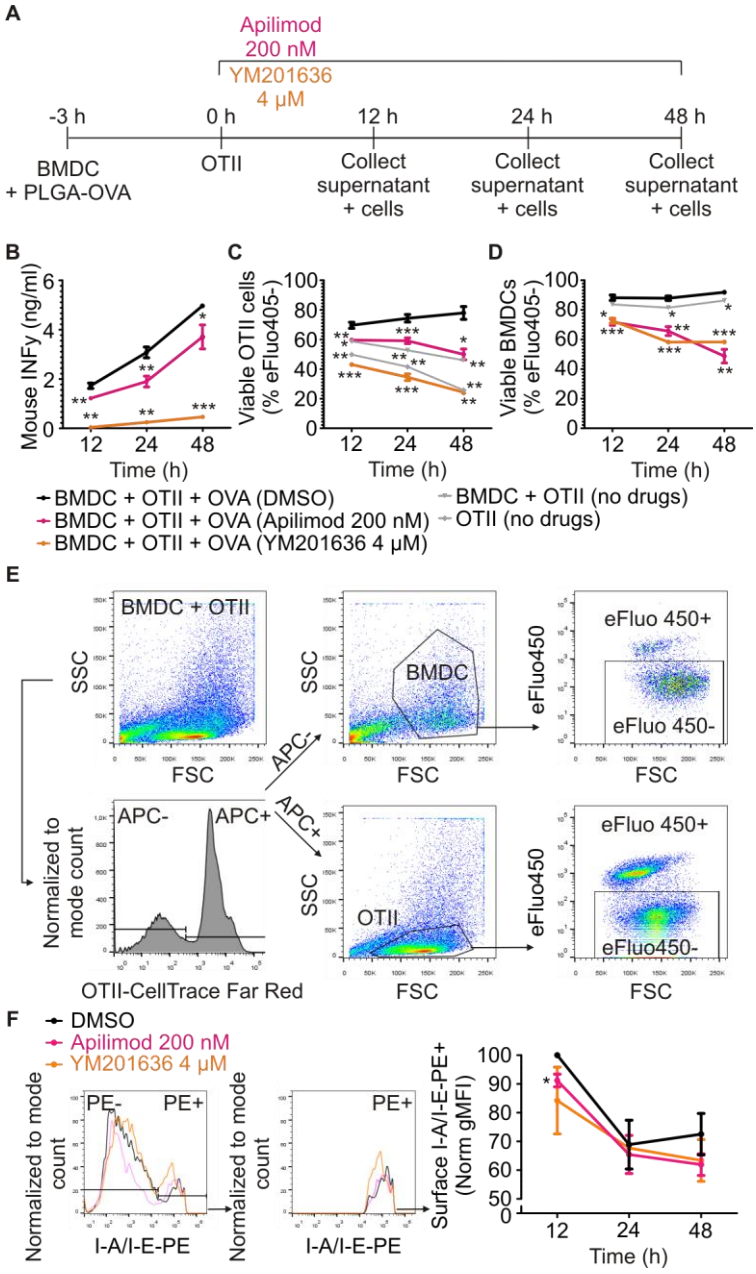


Figure S5. Control experiments for OT-II T cell priming experiments. Related to Figure 4. (A) Time line of murine OT-II T cell activation. Bone-marrow derived dendric cells (BMDC) were pre-incubated with PLGA-OVA particles for 3 hours. Subsequently, $OVA_{323-339}$-recognizing OT-II cells were added and the cells were exposed to apilimod or YM201636 for 12, 24 or 48 hours. (B) INFγ in supernatants by ELISA as indicated in panel A (mean from 3 independent experiments ± SEM). (C-E) Viability of experiment from panels A-B of OT-II cells (C) and BMDCs (D), and gating strategy (E). Viability was assessed with fixable viability dye eFluo405. OT-II cells were identified with OTII-CellTrace Far Red. SSC: side scatter; FSC: forward scatter (mean from 3 independent experiments ± SEM). (F) Surface expression of MHC class II (I-A/I-E-PE) for the experiments from panels A-E. gMFI: geometric mean fluorescence intensity. Representative histogram and quantifications are shown (normalized to DMSO control at 12 h; mean from 3 independent experiments ± SEM).

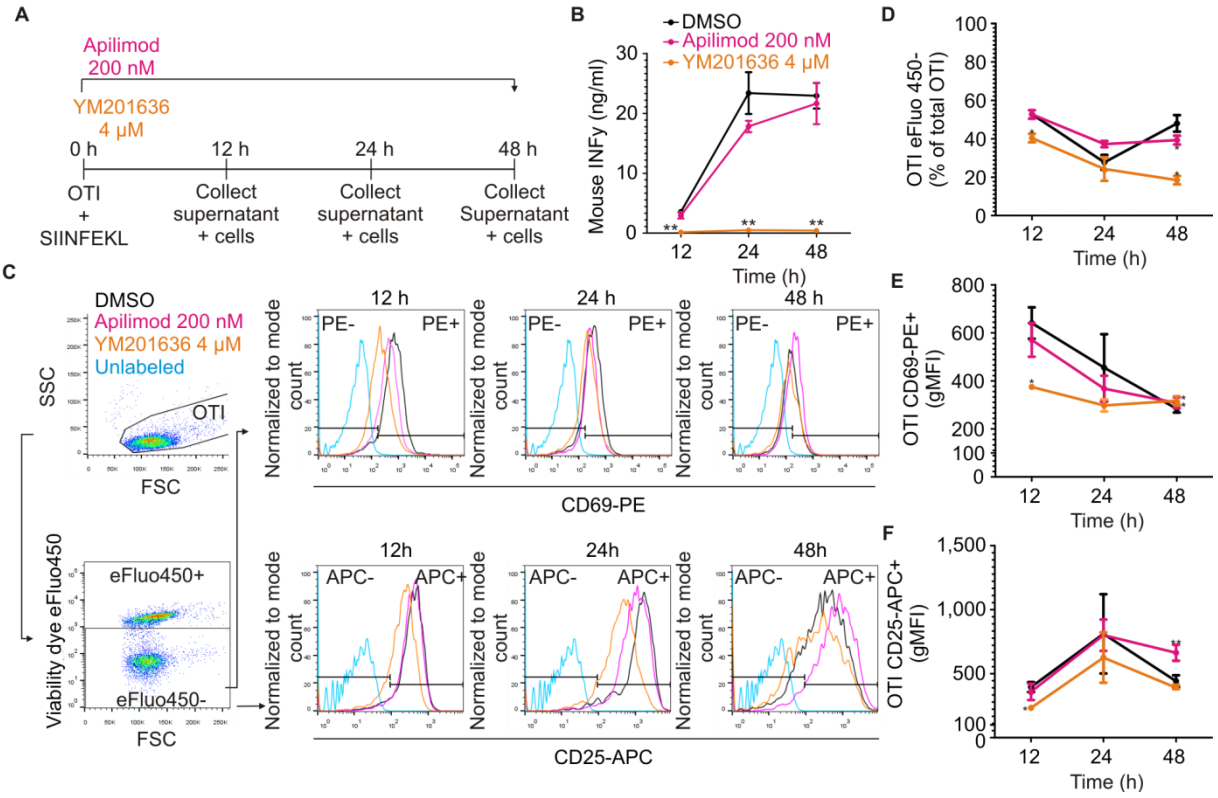


Figure S6. Apilimod and YM201636 impair T cell function. Related to Figure 4. (A) Scheme of experiment. OT-I T cells specifically recognizing ovalbumin residues 257-264 (SIINFEKL) in context of MHC-I were incubated with the epitope and PIKfyve inhibiting drugs apilimod or YM201636. (B) INFγ in the supernatants of the OT-I T cells from panel A. Note that YM201636 (but not or less apilimod) results in reduced production of INFγ. (C-F) Flow cytometry gating strategy and representative histograms (C) for T cell viability (D) and activation (E-F). Viability of OT-I T cells with fixable viability dye eFluo405 (D). Viable OT-I T cells were gated for early activation marker CD69-PE (E) or CD25-APC (F). SSC: side scatter; FSC: forward scatter; gMFI: geometric mean fluorescence intensity (mean from 3 independent experiments ± SEM).

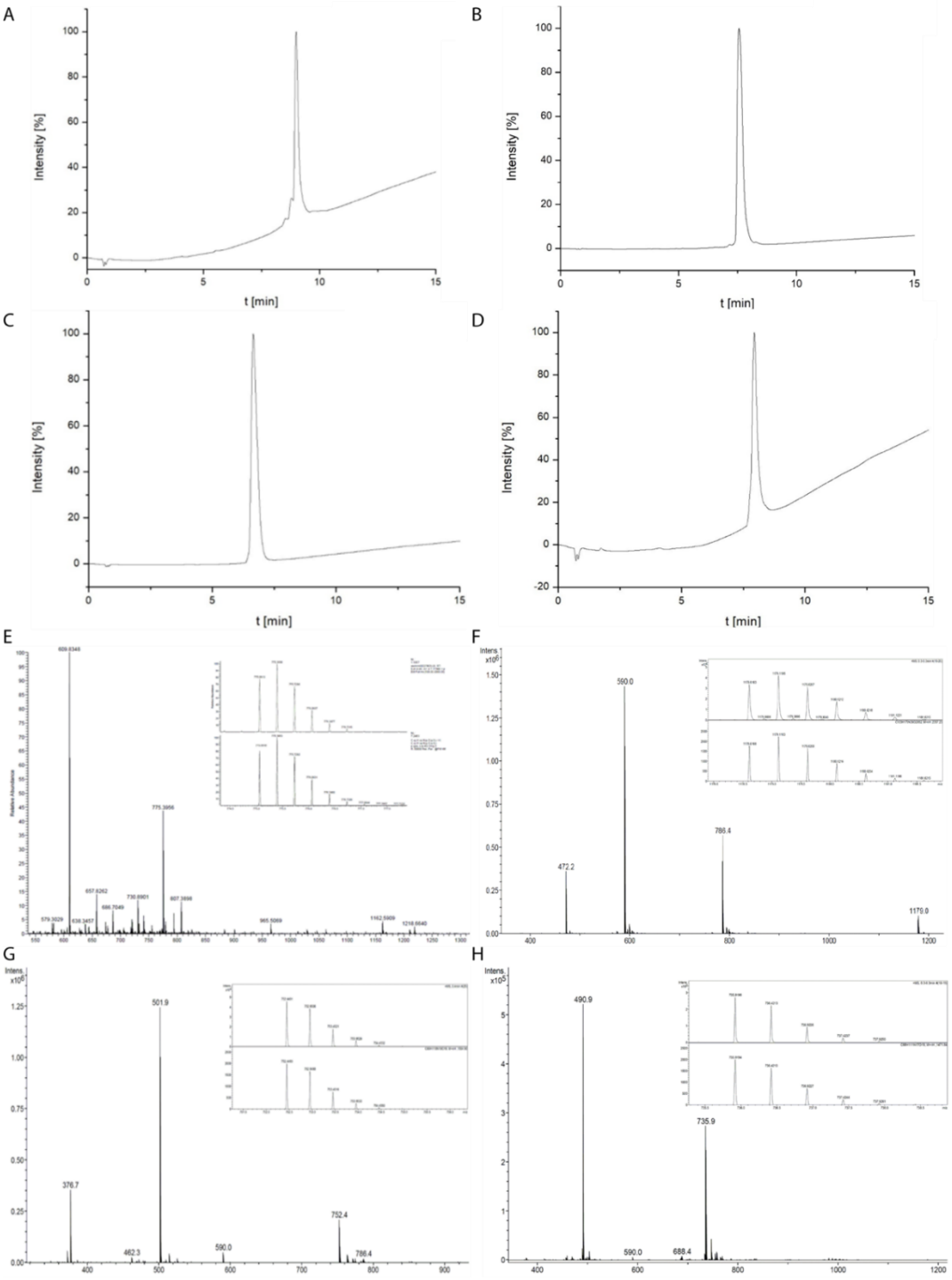


Figure S7. Analytical HPLC profiles and mass spectrometry validation of synthesized peptides. Related to Figure 6. Analytical HPLC chromatograms of: (A) hemagglutinin (HA) (residues 318-338) long clickable, (B) HA(318-338) long non clickable, (C) HA(322-334) short non clickable, and (D) HA(322-334) short clickable. ESI and HR-ESI-MS spectra of (E) HA(318-338) long clickable *m/z* (ESI) = 775.3956 $[M+3H]^{3+}$, 1162.5909 $[M+2H]^{2+}$ and *m/z* (HR-ESI-MS) = calculated: 775.0610 $[M+3H]^{3+}$, found: 775.0613 $[M+3H]^{3+}$, (F) HA(318-338) long non clickable *m/z* (ESI) = 472.2 $[M+5H]^{5+}$, 590.0 $[M+4H]^{4+}$, 786.4 $[M+3H]^{3+}$, 1079.0 $[M+2H]^{2+}$ and *m/z* (HR-ESI-MS) = calculated: 1178.6168 $[M+2H]^{2+}$, found: 1178.6183 $[M+2H]^{2+}$, (G) HA(322-334) short non clickable *m/z* (ESI) = 376.7 $[M+4H]^{4+}$, 501.9 $[M+3H]^{3+}$, 752.4 $[M+2H]^{2+}$ and *m/z* (HR-ESI-MS) = calculated: 752.4483 $[M+2H]^{2+}$, found: 752.4491 $[M+2H]^{2+}$ and (H) HA(322-334) short clickable *m/z* (ESI) = 490.9

$[M+3H]^{3+}$, 735.9 $[M+2H]^{2+}$ and *m/z* (HR-ESI-MS) = calculated: 735.9194 $[M+2H]^{2+}$, found: 735.9198 $[M+2H]^{2+}$.

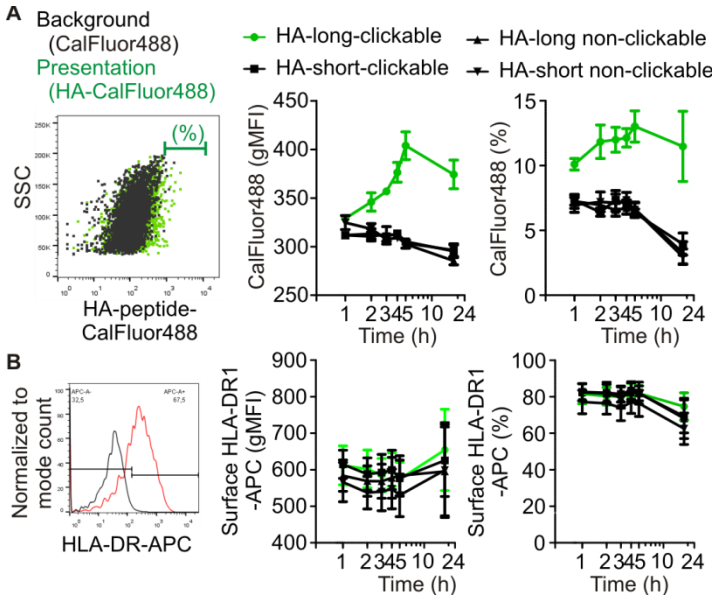
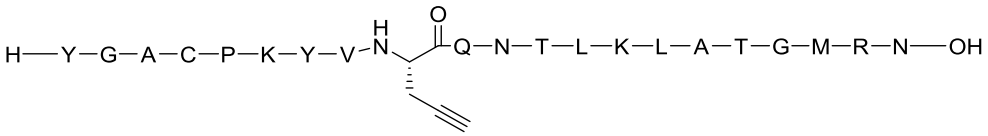
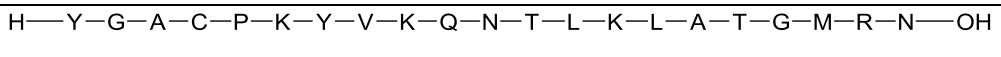
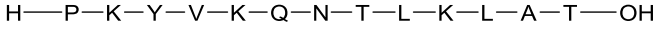
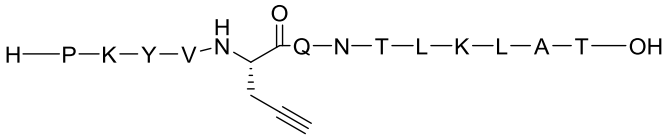


Figure S8. Control experiments for bio-orthogonal labeling of MHC-II presented epitope. Related to Figure 6. (A) Human dendritic cells were incubated with hemagglutinin (HA) peptides carrying an unnatural propargylglycine amino acid amendable to bio-orthoginal labeling with CalFluor488. Left: flow cytometry dot-blots with dendritic cells with (green) and without (background; black) HA peptide. SSC: side scatter. Right: gMFI (geometric mean fluorescence intensity) and population of HA-presenting cells incubated for the indicated times with long (residues 318-338) or short (residues 322-334) HA peptides with or without clickable propargylglycine amino acids. (B) The cells from panel A were tested for surface exposed MHC-II (HLA-DR) by flow cytometry. Representative histograms (left) and quantification by gMFI and population of HLA-DR-positive cells (right) are shown (mean from 5 donors ± SEM).

Table S1. Related to Figure 6. Synthesized hemagglutinin (HA) peptides with and without propargylglycine.

HA(318-338) long clickable	$C_{102}H_{163}N_{29}O_{29}S_2$ 2323.7 [g/mol]	
HA(318-338) long non clickable	$C_{103}H_{170}N_{30}O_{29}S_2$ 2356.8 [g/mol]	
HA(322-334) short non clickable	$C_{69}H_{118}N_{18}O_{19}$ 1503.8 [g/mol]	
HA(322-334) short clickable	$C_{68}H_{111}N_{17}O_{19}$ 1469.8 [g/mol]	

TRANSPARENT METHODS

Antibodies and reagents

The following antibodies were used: mouse monoclonal IgG1 anti-EEA1 at 1:100 (BD Biosciences; 610456 and 610457); rabbit polyclonal anti-LAMP-1 (CD107a) at 1:100 (Sigma; L1418); mouse monoclonal IgG1, κ anti-LAMP-1 (CD107a) at 1:100 (Biolegend; 328601, clone H4A3); mouse monoclonal IgG2a anti-M6PR at 1:50 in methanol fixed samples (Abcam; ab2733); rabbit polyclonal anti-GAPDH (14C10) at 1:1,000 (Cell Signaling; 2118); goat monoclonal IgG anti-Cathepsin X at 1:500 (R&D Systems; AF1033-SP); rabbit polyclonal anti-Cathepsin S at 1:250 (Novus Biologicals; NBP2-85807); mouse monoclonal IgG2a anti-Cathepsin B at 1:500 (Calbiochem; IM27L); mouse IgG2a,κ anti-HLA-DR, DP, DQ conjugated to FITC at 1:4 (BD Biosciences; clone Tü39) with isotype control mouse FITC-conjugated IgG2a,κ at 1:10 (BioLegend; 400208; clone MOPC-173); mouse monoclonal IgG1 anti-HLA-DR in complex with CLIP at 1:50 (Santa Cruz; sc-12725; clone CerCLIP.1); mouse IgG1, κ isotype control conjugated to APC at 1:10 (eBioscience; 17-4714-82); rat IgG2 anti-mouse CD74 at 1:500 (Biolegend; 151002); mouse monoclonal IgG1 anti-gp91^{phox} at 1:100 (D162-3; MBL); mouse anti-human-HLA-DR conjugated to APC at 1:100 (BD Biosciences; 559866; clone G46-6, RUO); mouse monoclonal IgG2a, κ anti-human-HLA-DR at 1:100 (Biolegend; 307614; clone L243); rat IgG2b,k anti-mouse I-A/I-E (MHC-II) conjugated to BV510 or PE at 1:200 (BioLegend; clone M5/114.15.2) with isotype control rat IgG2b,κ – BV510 or PE at 1:400 (Biolegend; 400645; clone RTK4530); anti-mouse CD69-PE from hamster at 1:100 (BD Biosciences; 553237; clone H1.2F3,RUO); anti-mouse CD25-APC from rat at 1:400 (eBioscience; 17-0251-82; clone PC61.5); OT-II were labeled with CellTrace Far Red labeling following the manufacturer's guidelines (ThermoFisher; C34564). The following secondary antibodies were used at (1:400): donkey-anti-mouse IgG (H&L) Alexa Fluor 647 (ThermoFisher; A-31571); donkey-anti-rabbit IgG (H&L) Alexa Fluor 568 (ThermoFisher; A-10042); goat-anti-mouse IgG (H&L) Alexa Fluor 488 (ThermoFisher; A-11029); goat-anti-Rabbit IgG (H + L) labeled with IRDye 800CW at 1:5,000 (LI-COR; 926-32211); goat-anti-rat Alexa Fluor 680 at 1:5,000 (ThermoFisher; A-21096).

Cell viability was assessed with the fixable viability dye eFluor450 (ThermoFisher; 65-0863-14) or eFluor780 (ThermoFisher; 65-0865-18) at 1:2,000 in PBS for 30 min on ice prior to 4% PFA fixation. The labeling for Flow cytometry was done as described (Baranov et al., 2016; Wimmers et al., 2016). All surface flow cytometry stainings were in PBA buffer (0.5% BSA and 0.01% NaN3 in PBS) and intracellular/total staining in PBA supplemented with 0.1% saponin as a detergent, all labeling was done for 10-30 min, PBS was used for washing. Annexin V-FITC (BD Biosciences;556419) and 7AAD (eBioscience; 00–6993–50) labeling was performed as described (Baranov et al., 2017) on ice for 10 min in buffer containing 1.5 mM $CaCl_2$. The MTT assay was performed as described (Baranov et al., 2016; Baranov et al., 2017) as follows: 0.66 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma M2128-1G) was in contact with the cells for 2 h before lysis with the following buffer: 90% isopropanol, 0.0125% SDS and 0.04 M HCl. Absorbance was analyzed at 595 nm.

Apilimod (STA-5326) was from Axon Medchem (ID 1369; CAS 541550-19-0) and MT-Diagnostics (USbiol-002800) and was used at 200 nM. YM201636 was from Calbiochem (ID 524611; CAS 371942-69-7) and used at 4 µM. Phenylarsine oxide (PAO) (at 1 µM) was incubated for 1 hour as described (Dingjan et al., 2016). LHVS (at 25 nM) was used as selective inhibitor for cathepsin S (provided by Dr. M. Bogyo, Stanford School of Medicine, CA, USA) (Koblansky et al., 2013). For control conditions, 1% DMSO was used (*i.e.*, the highest vehicle content in all drug dilutions). In the cathepsin activity and confocal microscopy experiments, phagocytosis was induced using un-opsonized zymosan particles at a 1:10 and 1:5 cell-to-particle ratio respectively. For live cell microscopy, IgG-opsonized zymoson was used as described (Baranov et al., 2016). Briefly, 20 mg/ml zymosan suspension with an equal volume of opsonizing solution from ThermoFisher (Z2850) was incubated for 60 min at 37^{o}C and vigorously washed in PBS. BSA conjugated to Alexa fluor 488 was used at 10 µg/ml (ThermoFisher; A13100). Human IL12 was measured by ELISA using 96 flat bottom well plates (Microlon; 655092), coating antibody at 3 µg/ml (Pierce Endogen; M122), IL-12/IL-23 p40 detection antibody at 250 ng/ml (Thermo Scientific; M121B). Mouse IL12p70 (eBioscience; 88-7121-88) and INFγ (Invitrogen; 88-7314-88) were measured by ELISA following manufacturer's kit guidelines. All flow cytometry experiments were done on a FACS-Calibur cytometer or a FACS Verse system (both BD Bioscience).

Animals

OVA-T cell receptor transgenic OT-I mice purchased from Charles River Laboratories (Sherbrooke, PQ) and OT-II mice purchased from Jackson Laboratories (Bar Harbor, ME) were held in a pathogen-free environment in Nijmegen Central Animal Laboratory, Netherlands, in accordance with institutional and European guidelines and approved by the Animal Experimental Committee (Radboud UMC). CD8+ T cells recognizing $OVA_{257-264}$ SIINFEKL peptide or $CD4^+$ T cells recognizing $OVA_{323-339}$ peptide presented on H-$2IA^b$ MHC-II, were isolated from spleen as described (den Brok et al., 2016; Dolen et al., 2016).

MHC-II-restricted OVA presentation to OT-II

For BMDCs differentiation, bone marrow cells of 7-11 weeks old mice was isolated and cultured in RPMI 1640 medium (Gibco) (10% fetal calf serum (FCS), 1% L-alanyl-L-glutamine, 0.1% 2-Mercaptoethanol and 0.5% antibiotics/anti-mycotics (AA, Gibco)) supplemented with 20 ng/ml GM-CSF (Peprotech). BMDCs were collected between day 6-7 for experiments (Baranov et al., 2017). Poly(lactic-co-glycolic acid) (PLGA) particles containing OVA were prepared as described (Dolen et al., 2016) and 200 µg of particles containing 10 µg OVA were used to stimulate 50,000 GM-CSF-cultured day 6-7 mouse BMDCs in 100 µl per well (96 well plate) starvation serum-free RPMI (2.1 mM ultra-glutamine and 50 µM 2-Mercaptoethanol) for 3 hours before drug addition to ensure comparable OVA-PLGA uptake. Next, free OVA-PLGA particles were washed away and the medium was changed to 200 µl per well complete RPMI (10% FBS, 2.1 mM ultra-glutamine and 50 µM 2-Mercaptoethanol) containing 100,000 OT-II pre-labeled with CellTrace Far Red (DMSO, Apilimod and YM201636 were added in concentrations mentioned above). The supernatants for ELISA and cells for flow cytometry were collected at 12, 24 and 48 hours and the cells were immediately pretreated with viability dye eFluor on ice as mentioned above, then fixed and antibody-labeled for flow cytometry. OT-I cells were isolated from splenocytes as described (Baranov et al., 2017) from a 6 month old OT-I male mouse with a CD8a+ T Cell Isolation Kit (Miltenyi Biotec; 130–104–075) and directly stimulated with $OVA_{257-264}$ peptide (SIINFEKL) at 1 µg/ml.

Receptor stimulation, RNA isolation, cDNA synthesis and qPCR

For stimulation of pathogen recognition receptors the following ligands were used: polyionosinic:polycytidylic acid (Poly:IC)(TLR3 agonist) at 100 µg/ml for 4 hours; lipopolysaccharides (LPS) (TLR4 agonist) at 1 µg/ml for 4 hours; resiquimod (R848) (TLR7 agonist) at 2.5 µg/ml for 4 hours; Pam(3)Cys (TLR2 agonist) at 5 µg/ml for 4 hours; zymosan (Dectin-1 and TLR2 agonist) at $2x10^8$ particles/ml for 1 hour; pepdidoglycan (PGN) (LTR2 agonist) at 10 µg/ml for 1 hour; OVA (CD206 mannose receptor agonist) at 1 µg/ml for 1 h. After stimulation, RNA isolation was performed from 1 million dendritic cells using Quick-RNA MiniPrep kit (ZymoResearch) in accordance with the manufacturer's guidelines. For generating cDNA, master mix 1 (1 µl Random Hexamers (100 µM), 1.2 µl dNTPs (10 mM), RNA (1 µg) and H_2O) was made. This mix was incubated at 65°C for 5 minutes and directly chilled on ice. Mastermix 2 (4 µl 1st Buffer (5x), 2 µl DTT, 1µl RNAsin, 1 µl Reverse Transcriptase or H_2O) was added and the RT-program on a PCR machine was started. The RT-program consisted of the following steps: 10 min 25°C, 50 min 37°C, 15 min 70°C, pause at 4°C. After cDNA synthesis, qPCR was performed using 10 µl Sybr Green, 1.2 µl Primer Mix F + R (10 µM), 4.8 µl MQ and 4 µl cDNA (80 ng) dilution per well. The qPCR-program consisted of the following steps: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds, 60 °C for 1 minute and repeated 39 times. The results were analyzed using the Bio-RAD Prime PCR program. The primers that were used for qPCR of PIKfyve were 5'-TGTCT GTGCT TGATC CAAGT G-3' and 5'-GCCAG GCCAA ATCAT CCTCT AA-3'.

siRNA knockdown assays

Post 72 hour knock-down in human dendritic cells was performed as described (Baranov et al., 2016). As control, siRNA irrelevant ON-TARGET plus non-targeting (NT) (Dharmacon) was used. The following cocktail of siRNAs was used for knockdown of PIKfyve (Invitrogen): 5'-GGAAA GGAAU UAGUC AACUG GCUAA-3', 5'-GGAGA CCUCC GAGCU UGCAC AUAUU-3', 5'-GAGGC CAGGG AGAAC AGCAG CCUUU-3'. Knockdown efficiency was measured with qPCR.

Microscopy and image processing

For the microscopy experiments, 100,000 dendritic cells were plated on 12 mm-diameter glass coverslips in serum free RPMI medium with 1% antibiotics/antimycotics and 2 mM ultra-glutamine and incubated at 37ºC and 5% CO_2. Fixation in most cases was done with 4% PFA solution in PBS. For CatS immunostaining, we performed antigen retrieval according to the manufacturer's protocol (R&D Systems; CTS013, CTS014, CTS015, CTS016). Fixation in 3% v/v glyoxal (Sigma-Aldrich #128465) for CatS and LAMP1 immunolabeling was done for 20 min as described (Richter and Revelo, 2018) and the solution mix was made with following ratios: 19.84 ml ddH_2O, 5.52 ml ethanol (absolute, for analysis), 2.18 ml glyoxal, 0.21 ml acetic acid, 1M NaOH was used to adjust pH to 4 or 5. Immunolabeling and blocking was performed with saponin permeabilization, as described (Baranov et al., 2016), in CLSM buffer (PBS with 20 mM glycine and 3% (w/v) BSA). Primary antibodies were incubated overnight, and secondary for 0,5-1h. In between, washing was done with PBS. Live cell imaging for BMV109 was done for 50,000 cells per well in µCLEAR F-bottom 96 well cell culture microplates (Greiner bio-one; 65500) in phenol red free and antibiotic free RPMI medium. The inhibitor and BMV109 concentrations and pipetting schemes were identical as for the in-gel fluorescence experiments. Samples were imaged with a Leica SP8 confocal laser scanning microscope with a 63x 1.20 NA water immersion objective (Leica HC PL APO 63x/1.20 W CORR CS2) or Leica DMI6000 epi-fluorescence microscope fitted with a 40× 0.85 NA dry objective, a metal halide EL6000 lamp for excitation, a DFC365FX CCD camera, and GFP and DsRed filter sets (all from Leica). Images were analyzed with Fiji (ImageJ).

Solid-phase peptide synthesis

Peptides were synthesized on a pre-loaded *Wang* resin (0.05 mmol, 0.27-0.32 mmol/g; Nova Biochem) on a Liberty Blue *CEM* microwave-assisted peptide synthesizer. The synthesis was conducted via a standard Fmoc/tBu-protocol using the recommended coupling (5 eq. amino acids, 5 eq. DIC, 5 eq. OxymaPure in DMF, 1: 75 °C, 170 W, 15 s, 2: 90 °C, 30 W, 50 s) and deprotection methods (piperidine/DMF, 1:4, *v/v*, 1: 75 °C, 155W, 15 s, 2: 90 °C, 30W, 50 s). The following L-amino acid building blocks were used in the automated synthesis: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Pro-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Val-OH and Fmoc-Pra-OH (GL Biochem; ABCR). For all amino acids, except for arginine, single couplings were performed. Special care was taken for the incorporation of Cys, Pra and Arg residues. For cysteine and propargylglycine, the temperature of the microwave-assisted coupling was reduced and the reaction time elongated (1: 25 °C, 0 W, 120 s, 2: 50 °C, 30 W, 480 s). Arginine was introduced by double coupling ((a) 1: 25 °C, 0 W, 1500 s, 2: 75 °C, 30 W, 120 s, (b) 1: 75 °C, 30 W, 300 s). After synthesis, the resin was filtered off, washed successively with DMF (5 times), methanol (5 times) and DCM (10 times) and dried *in vacuo.* Acidic cleavage from the resin was achieved by treatment with a mixture of trifluoroacetic acid (TFA) / triisopropylsilane / ethanedithiol / water (94:2.5:2.5:1, 5 ml, 2 h). The resin was extracted with additional TFA (5 ml), and the combined extracts were concentrated to 2 ml under a flow of nitrogen. The crude peptide was then precipitated in cold diethylether (10 ml) and isolated by centrifugation and decantation of the supernatant. The precipitate was washed twice with ice-cold diethylether and subsequently dissolved in 5 mL of water and then freeze-dried to give a fine white solid.

Peptide purification

Peptides were purified by preparative reversed-phase HPLC using a Pharmacia Äcta Basic 900 device (pump type P-900, variable wavelength detector UV-900) at flow rates of 10 ml/min, and a Macherey-Nagel Nucleodur 100-5-C18 ec, (250 mm by 21 mm, 5 µm) reversed-phase column. Linear gradients of water and acetonitrile or methanol (solvent A: water, 0.1 % TFA, solvent B: acetonitrile / water 4:1, 0.1 % TFA or methanol, 0.1 % TFA) over 30 min were used for purification.

Peptide characterization

Peptides were characterized by electrospray ionisation (ESI) and high-resolution (HR-MS-ESI) mass spectrometry on a Bruker maXis spectrometer (Billerica, USA). Analytical HPLC measurements were

performed using a Thermo chromatography system (pumps UltiMate 3000, detector UltiMate 3000, autosampler UltiMate 3000 diode array) and an ACE Excel 2 C18 (2 µm, 2.1 x 100 mm) reversed-phase column at a flow rate of 0.4 ml/min. For peptide characterization, a linear gradient of water and methanol run (buffer A: water, 0.1 % TFA, buffer B: methanol, 0.85 % TFA) from 20-90 % buffer B over 15 min was used. Chromatograms were monitored at 220 nm wavelengths. The column was run at a controlled temperature of 50 °C.

Cell culture and transfection

Cultures of human monocyte-derived dendritic cells derived from peripheral blood monocytes (PBMCs) were obtained from buffy coats of healthy donors as described (Baranov et al., 2014; Baranov et al., 2016). Briefly, adherent monocytes isolated from the blood of healthy donors were in culture for 6 days in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS, Greiner Bio-one), 1 mM ultra-glutamine (BioWhittaker), antibiotics (100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 0.25 µg ml^{-1} amphotericin B, Gibco), IL-4 (500 U ml^{-1}) and GM-CSF (800 U ml^{-1}). After that moDCs were detached from the plastic within 1h under cold PBS at $4^{O}C$ before freezing in liquid nitrogen in 10% DMSO and 40% FBS. Mouse GM-CSF differentiated BMDCs were generated as described (Baranov et al., 2017) and outlined above. The construct for full-length human PIKfyve was from Invivogen (cat nr puno1< hPIKFYVEb >) and subcloned in the EcoRI/BamHI sites of pEGFP-C1. pEGFP-HA-PIKfyve and pEGFP-HA-$PIKfyve^{K1831E}$ within pEGFP-C2 were obtained from Assia Shisheva (Ikonomov et al., 2001). LifeAct-RFP was a gift from Michael Sixt (Max Planck Institute of Biochemistry, Martinsried, Germany) (Riedl et al., 2008). The mRFP-tagged LAMP1 is described (Sherer et al., 2003) and LAMP1-mGFP was a gift from Esteban Dell'Angelica (Addgene plasmid # 34831 (Falcon-Perez et al., 2005)). NCF4-PX-EGFP was a gift from Michael Yaffe (Addgene plasmid #19010, (Kanai et al., 2001)). The YFP was replaced by mCherry in HLA-DRA-IRES-HLA-DRB-YFP, which was a gift from Jacques Neefjes (Netherlands Cancer Institute, Amsterdam, Netherlands) (Zwart et al., 2005). Transfection was performed as described (Baranov et al., 2014; Baranov et al., 2016) with a Neon Transfection system (Invitrogen), a ratio of 3 µg of DNA per $1*10^{6}$ cells was used, resuspended in 100 µl buffer R (Invitrogen) and electroporated (2 pulses of 40 ms, 1,000 V). After transfection, the cells were kept in antibiotic-free and serum-free RPMI medium (Invitrogen) for 3h before addition of full medium. Zymosan uptake and imaging experiments were performed 5–12 h post-transfection.

pHrodo zymosan

Dendritic cells (200,000 cells/well) were cultured in RPMI-1640 with 1% glutamine without phenol red in the presence of Apilimod or YM201636 for 3 h, in the final hour with drugs the cells were stimulated with 200 µl pHrodo Red Zymosan Bioparticles Conjugate for Phagocytosis (0.5 mg/ml) (ThermoFisher; P35364). Hoechst (5 µg/ml) was added 15 minutes before imaging. Samples were imaged with a Leica SP8 confocal laser scanning microscope with a 63x 1.20 NA water immersion objective (Leica HC PL APO 63x/1.20 W CORR CS2). Images were independently analyzed with Fiji (ImageJ) with identical treshholding parameters.

Cathepsin activity-based probe

Dendritic cells (300,000 cells/well) were cultured in RPMI-1640 with 1% ultra-glutamine (without Phenol Red) and incubated with different pharmacological treatments. Cells were stimulated using 15 µl unlabeled zymosan per well for 0, 0.5 or 1 h prior to lysis. Cathepsin probe BMV109-Cy5 at 1 µM (Verdoes et al., 2013) was added 0.5 h before lysis or in lysate control experiments probe BMV109-Cy5 was added immediately after lysis and incubated on ice for 1.5h. In lysate control experiments probe BMV109-Cy5 was added immediately after lysis. Cells were centrifuged and washed with ice-cold PBS, lysed in hypotonic lysis buffer (50 mM PIPES pH 7.4, 10 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 4 mM DTT, and 1% NP-40). The cells were cooled on ice for 10 minutes. The lysates were transferred to new tubes and BCA protein determination was performed (Micro BCA™ Protein Assay Kit, Thermo Scientific; 23235). The samples were resolved on 15% SDS-PAGE and in-gel fluorescence of BMV109-Cy5 of active cathepsins was tested with a TRIO+ Typhoon scanner (670BP-500PMT-Rex-ex-Focal plane 3x). The SDS gel was subsequently blotted and immunostained for GAPDH loading control. The membrane was incubated with secondary antibody coupled with IRDye 680 or 800 at a dilution of 1:5,000 for 1

h. The membrane was washed three times with 0.05% Tween-20 for 10 minutes each and scanned with an Odyssey scanner (LI-COR). PAO was added to dendritic cells at 1 µM (Dingjan et al., 2016).

ROS measurements

ROS in apilimod treated dendritic cells was measured with the Amplex Red assay as described (Dingjan et al., 2016). Zymosan particles were co-labeled with ROS-sensor OxyBurst-Green H2DCFDA (ThermoFisher; D-2935) and Alexa-Fluor-633 C5-maleimide (A-20342, ThermoFisher) and live cell imaging was performed as described (Dingjan et al., 2017). For analysis, the OxyBurst fluorescence signal from each zymosan particle was divided by that of Alexa-Fluor-633.

HLA-DR1-restricted presentation of viral influenza HA

Peptides containing influenza A virus haemagglutin (strain A/Aichi/2/1968 H3N2) peptide (HA) residues 322-334 with or without lysine 326 converted into L-propargylglycine were synthesized (as described in ***Solid-phase peptide synthesis*** section). 100,000 dendritic cells were pre-stimulated for 5 h in 100 µl serum-free RPMI with a water solution of HA-peptide at 5 µM final concentration. Apilimod or YM201636 were added 3 h prior to the end of the HA-stimulation. As a control, cells were incubated for 15 min at 37^{o}C with trypsin-containing buffer (NaCl 0.8% (w/v), KCl 0.04% (w/v), D-glucose 0.1% (w/v), $NaHCO_3$ 0.058% (w/v), EDTA 0.02% (w/v), trypsin 0.05% (w/v) (BD Difco 250; 215240)). Cell viability was traced with eFluor780 (ThermoFisher; 65-0865-18) and cells were fixed in 4% PFA in PBS. At all steps before and during viability staining or click-labeling, the cells were washed at least 2 times with either PBS or HBS (N_2-bubbled, NaCl 150 mM, HEPES 20 mM, pH 7.4). Autofluorescence was reduced after 20 min by washing with quenching buffer (100 mM glycine, 100 mM NH_4Cl), surface presentation of HA was labeled for 1 h with CalFluor488 (Shieh et al., 2015) in HBS containing 1 mM $CuSO_4$, 0.5 mM THPTA (Tris(3-hydroxypropyltriazolylmethyl)amine), 5 mM sodium L-ascorbate; 0.01% (w/v) BSA, aminoguanidine 2.2% (w/v). Finally, surface HLA-DR1 was labeled with mouse anti-human-HLA-DR conjugated to APC at 1:100 (BD Biosciences; 559866; clone G46-6, RUO) in PBA buffer (0.5% BSA and 0.01% NaN_3 in PBS).

Statistical Analysis

All data were analyzed using Student's two-tailed paired t tests. A p value < 0.05 was regarded as statistically significant ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

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