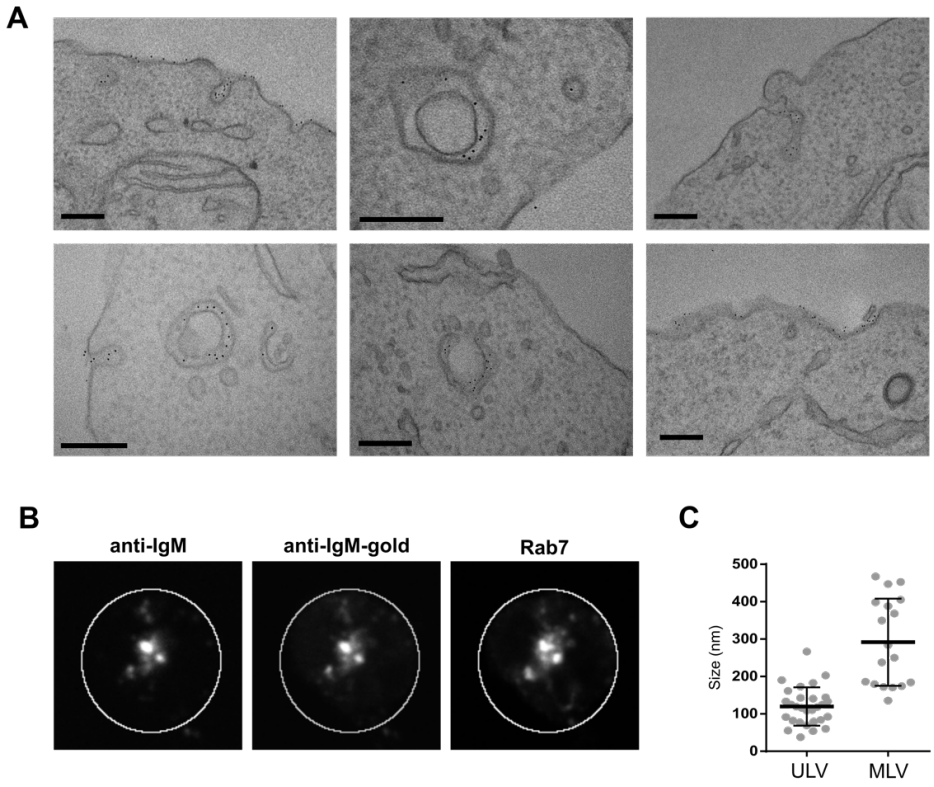


Figure S1. A A20 D1.3 cells were activated with αIgM conjugated with 6nm colloidal gold particles mixed with AF647-αIgM, for 15 min and imaged using Transmission Electron Microscopy. Scale bars 200 nm. **B** The possible effect of colloidal gold-conjugation to the localization of α-IgM was controlled by immunofluorescence analysis of sample duplicates (from A). The cells activated for 75 min were fixed and permeabilised, and gold-conjugated αIgM was stained using an isotype-specific secondary antibody (middle panel) and compared to AF647-αIgM (left). The cells were also immunostained with anti-Rab7 antibody (right). SDCM image shows single section of a representative cell (cell plasma membrane marked with a white circle), where a strong colocalisation of fluorescently labelled α-IgM and gold-conjugated α-IgM was detected together with Rab7, similarly to the samples without colloidal gold (Fig. S1B; see Fig. 1A and Fig. 2B). **C** EM micrographs (as in panel A) were subjected to vesicle size analysis. Vesicles were classified as unilamellar (ULV) or multilamellar (MLV) and their diameter was measured in ImageJ.

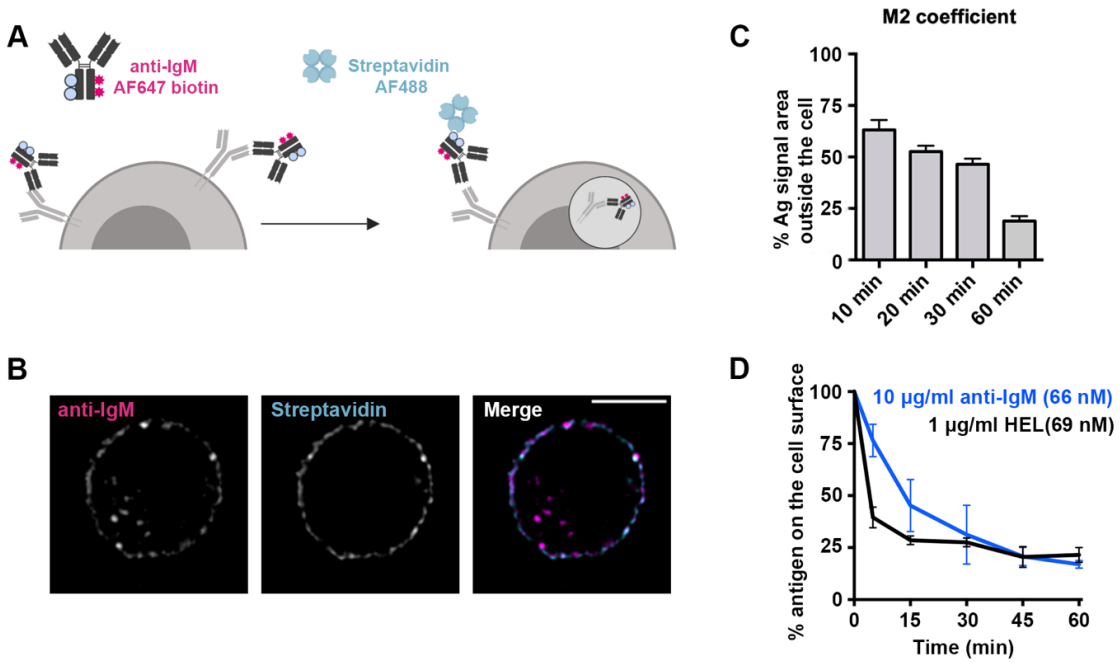


Figure S2. A Schematic representation of the staining to distinguish between internalised AF647-αIgM (magenta) and surface-resident αIgM (probed with AF488-streptavidin; cyan). **B** SDCM imaging of A20 D1.3 cells activated with biotin-AF647-αIgM for 10 min. AF647-αIgM used for activation is shown in magenta, and surface-resident αIgM (AF488-streptavidin) in cyan. SDCM images were deconvolved with Huygens software. Single confocal plane from a representative cell is presented. Scale bar 5 µm. **C** Quantification of the data in B, including additional timepoints. 3D images from cells activated for 10, 20, 30 and 60 minutes were analysed for Manders´ overlap coefficients (M2) using ImageJ. Data shown as mean ±SEM of one experiment (n>20 cells per timepoint). **D** A20 D1.3 cells labelled with biotinylated-αIgM or biotinylated-HEL were incubated at 37 ºC at different timepoints and stained on ice with AF488-streptavidin to detect surface-resident αIgM. Intensity was normalised to time 0 (100%). Data from at least three independent experiments is presented as mean ±SD.

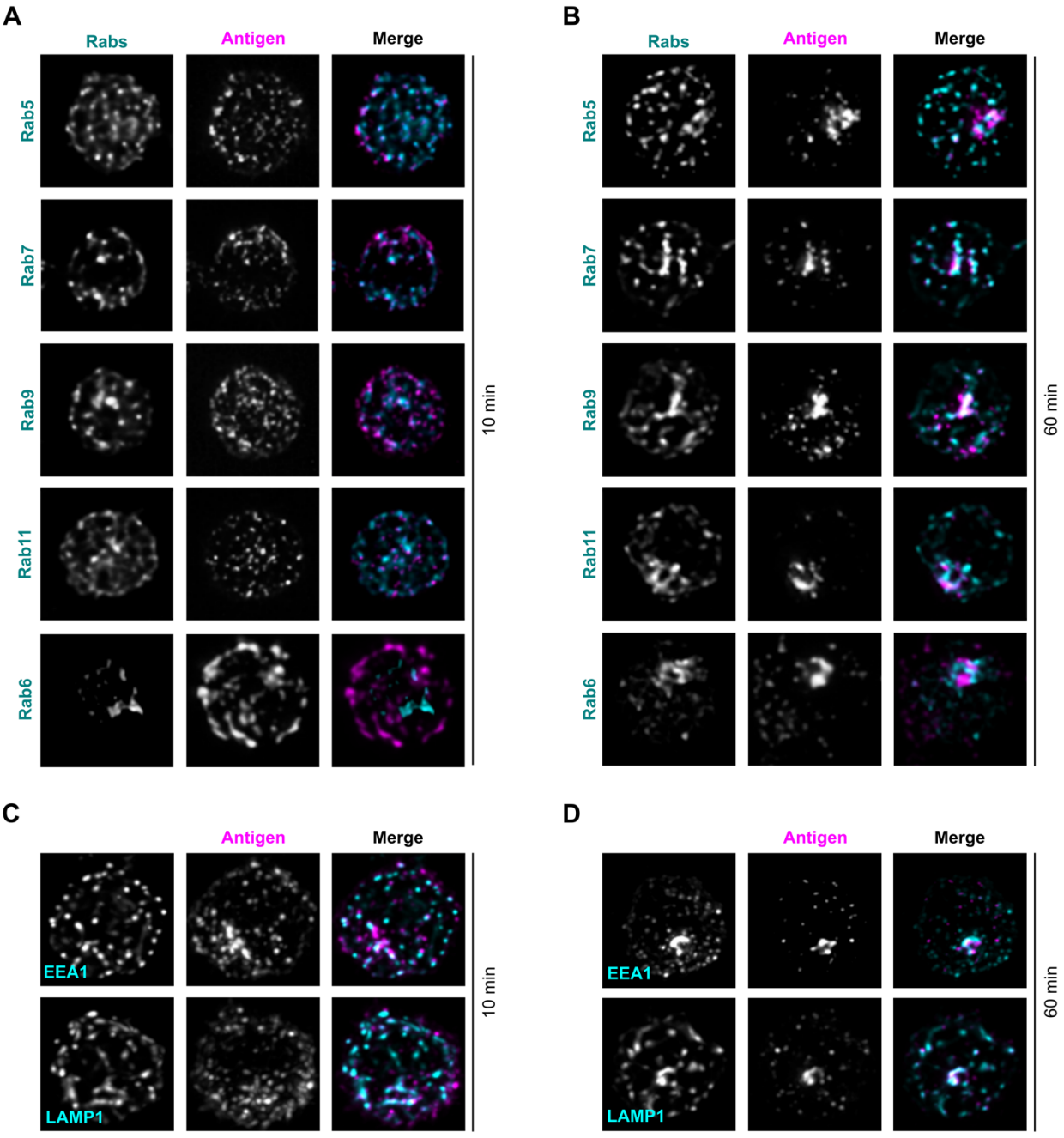


Figure S3. Colocalization of antigen with different Rab-proteins. A-B SDCM imaging of A20 D1.3 cells activated with AF647-αIgM (antigen, magenta) for 10 min **(A)** or 60 min **(B)** and immunostained for different Rab-proteins: Rab5, Rab7, Rab9, Rab11 and Rab6 (cyan). Images were deconvolved with Huygens software. Z-projections of the 3D images from representative cells are shown. **C-D** SDCM imaging of A20 D1.3 cells activated with AF647-αIgM (antigen, magenta) for 10 min **(C)** or 60 min **(D)** and immunostained for EEA1 or LAMP1 (cyan). Images were deconvolved with Huygens software. Z-projections of the 3D images from representative cells are shown.

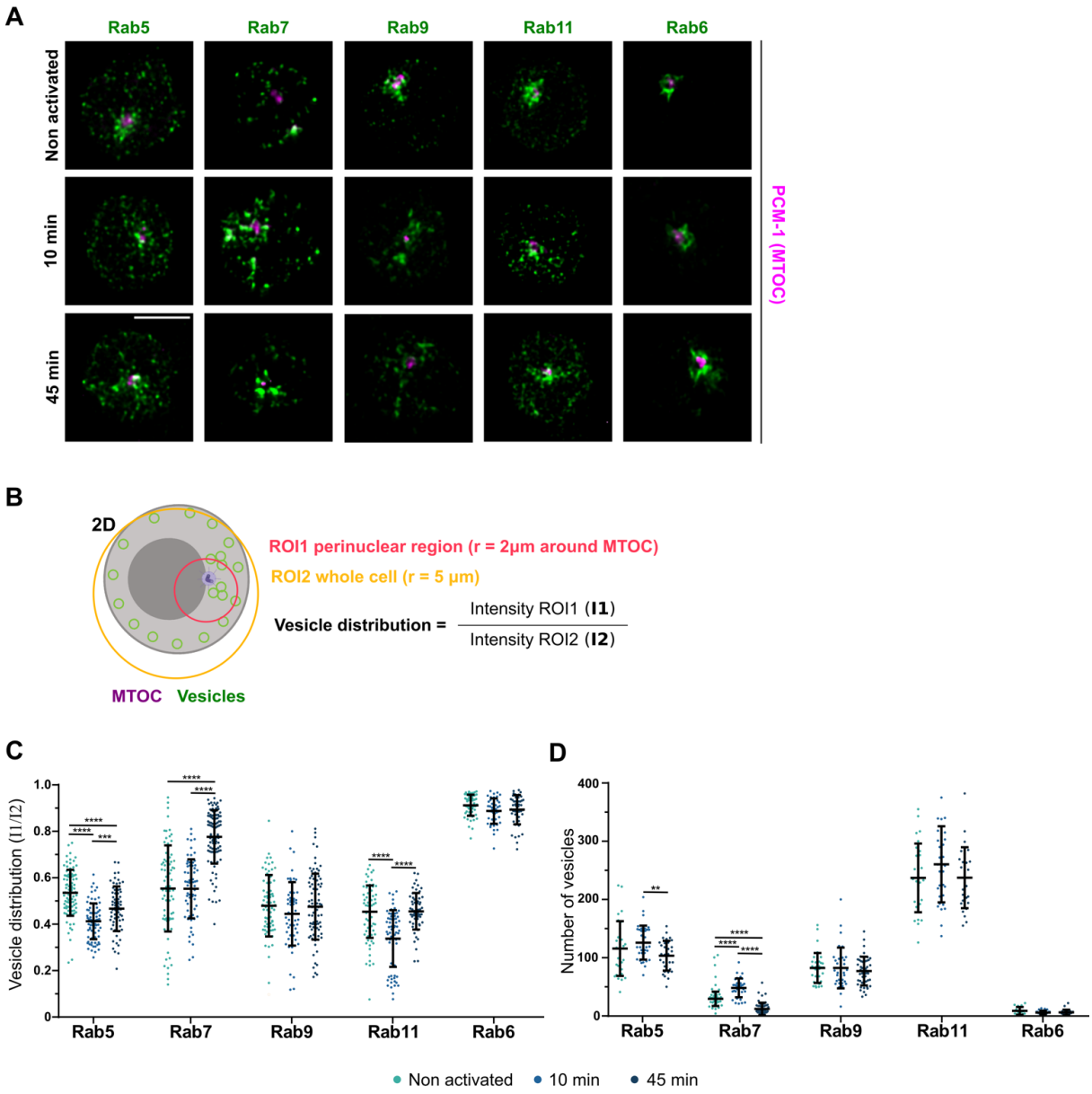


Figure S4. Effect of B cell activation on the distribution of Rab compartments. **A** SDCM imaging of A20 D1.3 cells non-activated or activated with αIgM for 10 min or 45 min and immunostained for different Rab-proteins (Rab5, Rab7, Rab9, Rab11 and Rab6) in green, and an MTOC marker PCM—1 in magenta. Images were deconvolved with Huygens software. Z-projections of the 3D images from representative cells are shown. **B** Schematic representation showing the analyses performed on the images in **A** to generate quantification in **C**. In ImageJ, two different regions of interested (ROI) were selected: ROI1, a circle with radius of 2 µm around the MTOC; and ROI2, a circle around the whole cell. Distribution of the vesicles was quantified as intensity of ROI1/intensity ROI2. **C** Results of the analysis performed as described in B. Data from two independent experiments (mean + SD, 50-100 cells). **D** Results of the quantification of the same data (A) for number of vesicles using the MATLAB script described in Fig.1. Data from one experiment (mean + SD of at least 30 cells).

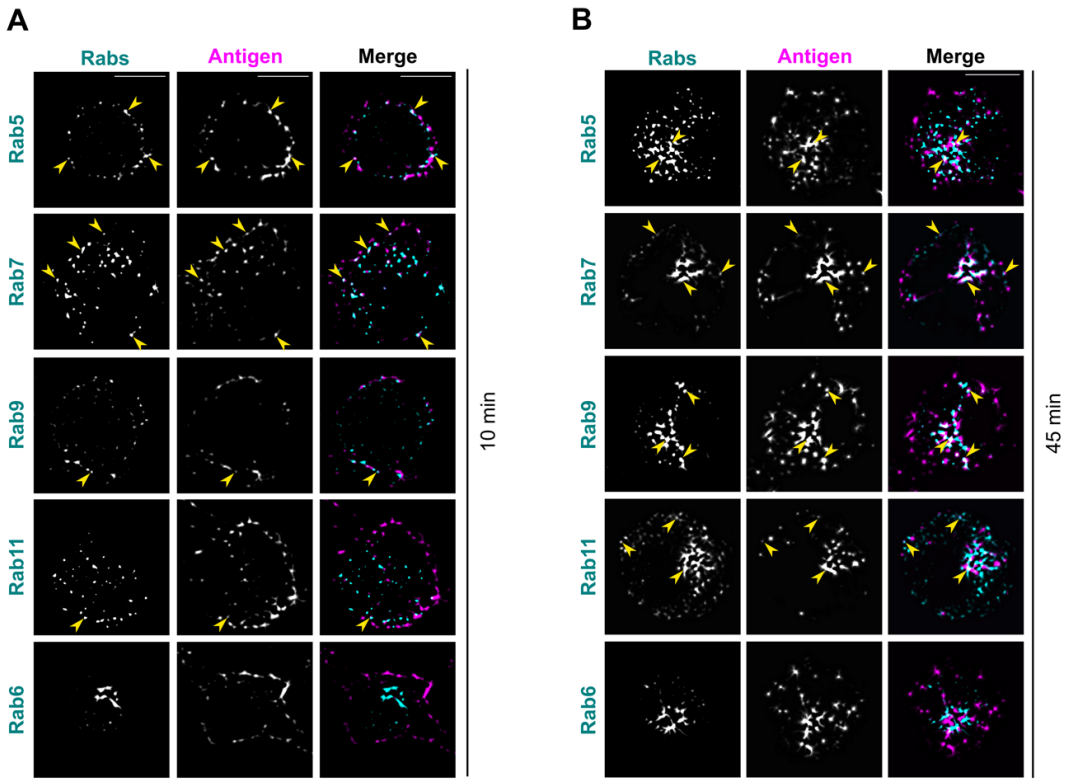


Figure S5. Colocalization of antigen with different Rab-proteins in SRFF. A-B SRFF imaging of A20 D1.3 cells activated with AF647-αIgM (antigen, magenta) for 10 min **(A)** or 60 min **(B)** and immunostained for different Rab-proteins: Rab5, Rab7, Rab9, Rab11 and Rab6 (cyan). Z-projections of the 3D images from representative cells are shown. Scale bar: 5 μm.

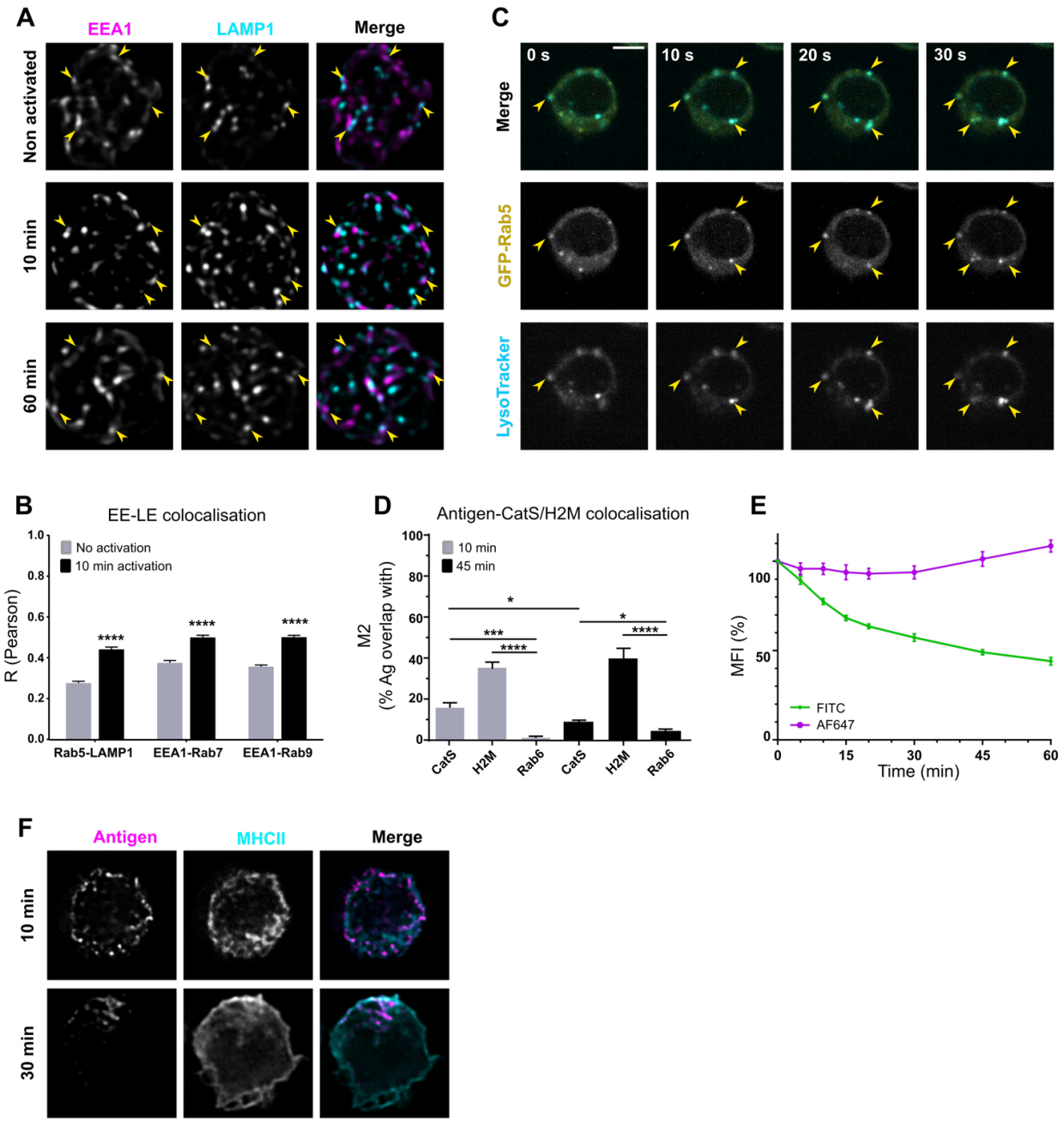
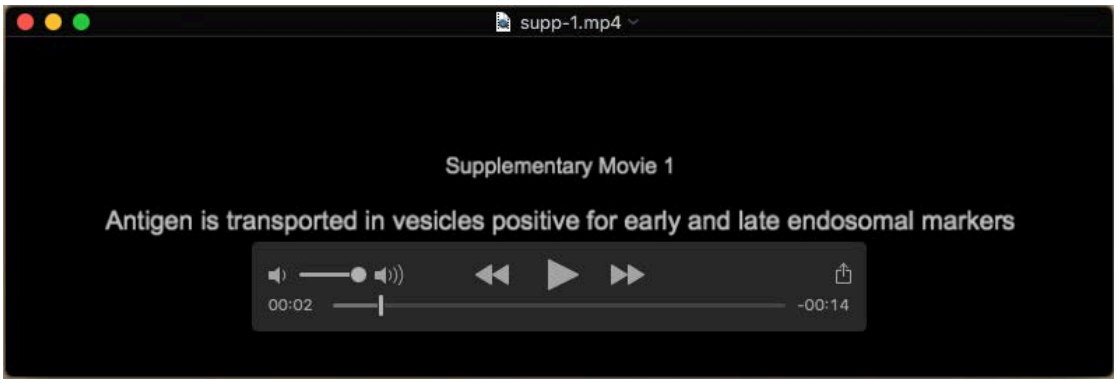
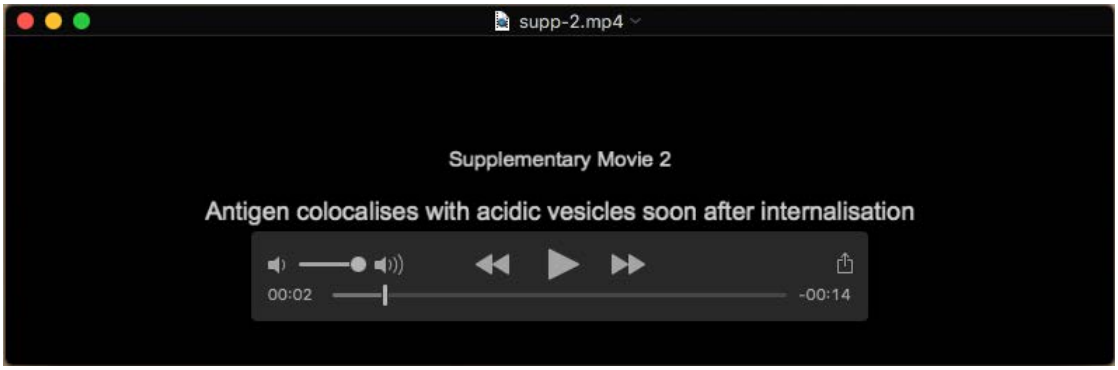


Figure S6. Early and late endosomal markers' colocalization before and after activation. A SDCM imaging of A20 D1.3 cells non-activated or activated with αIgM for 10 min or 60 min and immunostained for EEA1 (magenta) and LAMP1 (cyan). Images were deconvolved with Huygens software. Z-projections of the 3D images from representative cells are shown. **B** Analysis of correlation shown by Pearson´s coefficient of early-late endosomal markers in pairs (Rab5/LAMP1, EEA1/Rab7, EEA1/Rab9) before and after 10 minutes activation. Results from one experiment (n> 40 cells) shown as mean +SEM. **C** Rab5-GFP localises to LysoTracker-positive vesicles already

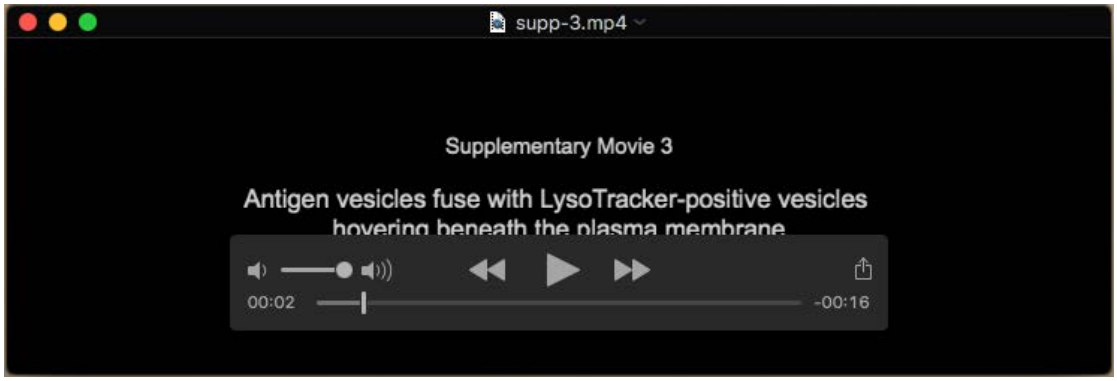
before activation. A20 D1.3 cells were transfected with GFP-Rab5 (yellow) and loaded with LysoTracker (LT; cyan). Live-imaging was performed with SDCM (with sCMOS Orca Flash4 v2 camera) on a single plane. On the upper panel, a merge image of a representative cell is shown as a timelapse for 30 seconds. Split channels for GFP-Rab5 and LysoTracker are shown in the middle and bottom panel respectively. Examples of colocalizing vesicles pointed to with yellow arrow-heads. Scale bar 5 µm. **D** Quantification of the data shown in Fig. 4D and Fig. 5E. Antigen colocalization with CatS and H2M, compared to the negative control Rab6, was measured from SRRF images by analysing Manders´ overlap coefficients using ImageJ. Data from two independent experiments (>30 cells/timepoint). Results are shown as mean ±SEM. **E** Antigen enters low pH compartments after internalisation. Flow cytometric analysis of pH-sensitive FITC- and pH-stable AF647-conjugated anti-IgM for different timepoints. 3 experiments mean + SD. **F** A20 D1.3 cells were activated with AF647-αIgM (antigen in magenta) for 10 or 30 minutes. Samples were then fixed, permeabilised and stained with anti-MHCII (cyan). SDCM images were deconvolved with Huygens software. Single confocal sections from representative cells are shown.



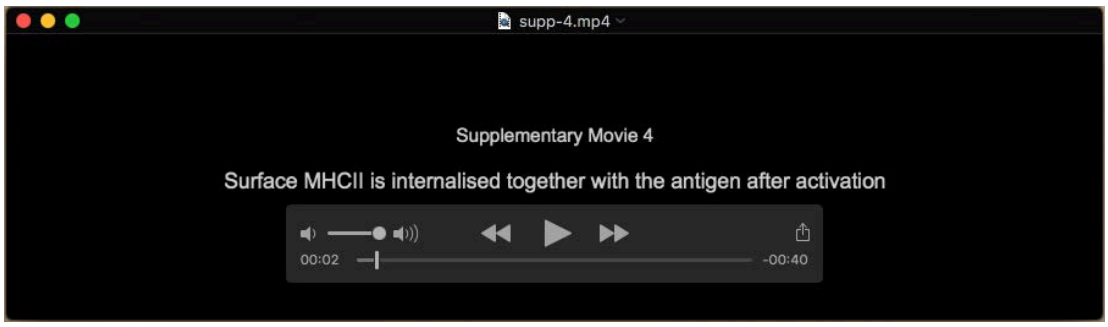
Movie 1. Antigen is transported in vesicles positive for early and late endosomal markers. A20 D1.3 cells were transfected with GFP-Rab5 (right panel), loaded with LysoTracker (left panel) and activated with RRx-αIgM (middle panel). Live-imaging was performed with SDCM (sCMOS Orca Flash4 v2 camera) on a single plane. Triple-positive vesicles are highlighted with a purple circle tracking the spots in the antigen channel. Movie was recorded 10 min after activation and imaged every 2 s.



Movie 2. Antigen colocalises with acidic vesicles soon after internalisation. A20 D1.3 cells were loaded with LysoTracker (middle panel) and activated with AF488-αIgM (left panel). Right panel shows merge image of antigen channel (magenta) and LysoTracker channel (cyan). Live-imaging was performed with SDCM (EVOLVE camera) on a single plane every 500 ms. Double-positive vesicles are highlighted with a purple circle tracking the spots in the antigen channel. Movie was recorded 1 min after cell activation.



Movie 3. Antigen vesicles fuse with LysoTracker-positive vesicles hovering beneath the plasma membrane. A20 D1.3 cells were loaded with LysoTracker (middle panel) and activated with AF488-αIgM (left panel). Right panel shows merge image of antigen channel (magenta) and LysoTracker channel (cyan). Live-imaging was performed with SDCM (EVOLVE camera) on a single plane every 500 ms. An antigen vesicle fusing with LysoTracker after pinching from the plasma membrane is highlighted with a purple circle. Movie was recorded 80 s after activation.



Movie 4. Surface MHCII is internalised together with the antigen after activation. A20 D1.3 cells were labelled on ice with AF488-anti-MHCII (left panel) and RRx-αIgM (middle panel). Cells were shifted at 37 ºC to start activation and recorded after 40 s. Right panel shows merge image of antigen channel (magenta) and MHCII channel (cyan). Live-imaging was performed with SDCM (sCMOS Orca Flash4 v2 camera) on a single plane every 4 s. Vesicles positive for MHCII and antigen were tracked with circles (shown in different colours) in the antigen channel.

Table S1. Key resources/reagents table

	Reagents	Source/Brand	Cat. number	Dilution or concentration	Use
Antigens	Anti-IgM-biotin	SouthernBiotech	1021-08	10 µg/ml	Antigen internalisation (FACS) DAB ablation
	6nm Gold rat anti-mouse IgM	Jackson ImmunoResearch (JIR)	115-195-075	1:650	EM
	Rhodamine Red-X-AffiniPure Donkey anti-mouse IgM	JIR	715-295-140	10 µg/ml	IF/Live imaging
	Alexa Fluor 647AffiniPure Donkey anti-mouse IgM	JIR	715-605-140	5-10 µg/ml	IF/Live imaging/FACS
	Alexa Fluor 488 AffiniPure F(ab´)2 Fragment Donkey anti-mouse IgM	JIR	715-546-020	10 µg/ml	Live imaging
	FITC anti-mouse IgM	JIR	715-095-140	5 µg/ml	pH probe (FACS)
	Donkey anti-mouse IgM AlexaFluor647-biotin	In-house	715-605-140 + Thermo 21343	10 µg/ml	IF
	Hen Egg Lysozyme (HEL)	Sigma-Aldrich	#L6876	10 µg/ml	ELISA
	HEL-biotin	In-house	Sigma-Aldrich # L6876 + Thermo 21338	1-10 µg/ml	For DQ-Ova probe/FACS
Antibodies (IF)	Anti-Rab5	CST	3547	1:150	IF
	Anti-Rab6	CST	9625S	1:200	IF
	Anti-Rab7	Santa Cruz	Sc-376362	1:100	IF
	Anti-Rab9	CST	5118	1:150	IF
	Anti-Rab11	CST	5589	1:200	IF
	Anti-EEA1	Santa Cruz	Sc-6415	1:50	IF
	Anti-LAMP1	DSHB	1D4B	1:75	IF
	Anti-CathepsinS	LSbio	B2550	1:50	IF
	Anti-CathepsinS	Santa Cruz	sc-271619	1:50	IF
	Anti-MHCII	Santa Cruz	Sc-59322	1:50	IF
	Anti-MHCII-AF488	In-house	Sc-59322 + Thermo A20000	1:50	IF/Live imaging
	Anti-PCM1-AF647	Santa Cruz	Sc-398365 AF647	1:200	IF
	Donkey-anti-rabbit IgG (H+L) AlexaFluor 488/555/647	Thermo	A21206, A31572, A31573	1:500	IF

	Donkey anti-goat IgG (H+L) AlexaFluor 488/555	Thermo	A11055, A21432	1:500	IF
	Mouse anti-rat IgG Fcy Fragment Specific AlexaFluor 488/RRx/647	JIR	212-545-104 212-295-104 212-605-104	1:500	IF
	Goat-anti-mouse IgG Fcy subclass 1 AlexaFluor 488/ RRx/647	JIR	115-545-205 115-295-205 115-605-205	1:500	IF
ELISA	Anti-mouse IL-2	Nordic Biosite	503804	4 µg/ml	ELISA
	Anti-mouse IL-2, biotin	Nordic Biosite	503702	1 µg/ml	ELISA
	ExtrAvidin-AP (alkaline phosphatase)	Sigma-Aldrich	E2636	1:5000	ELISA
	FAST pNPP substrate tablet	Sigma-Aldrich	N2770-5SET	-	ELISA
Other	LysoTracker Deep Red	Thermo	L12492	125 nM	Live imaging
	DQ-OVA-biotin	In-house	Thermo D12053 +EZ-Link Maleimide-PEG2-biotin (Thermo 21901BID)	1:10	FACS
	Fibronectin	Sigma	F4759-2MG	4 µg/ml	IF