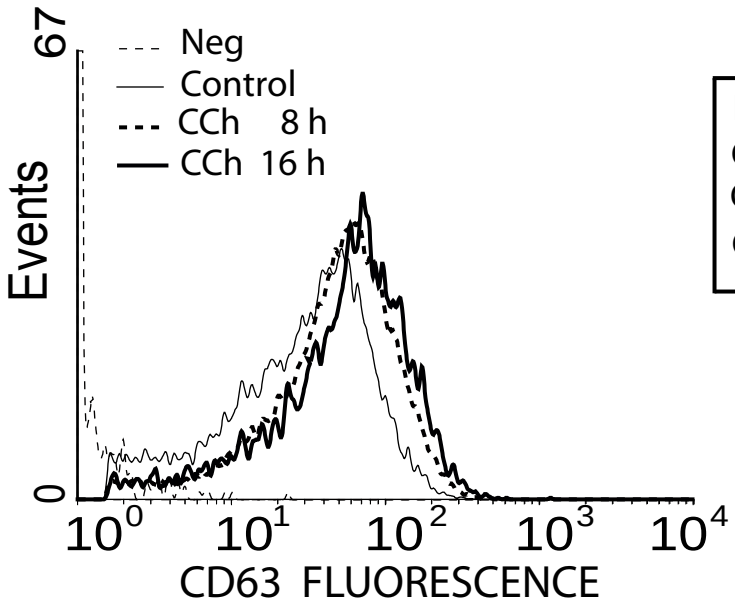
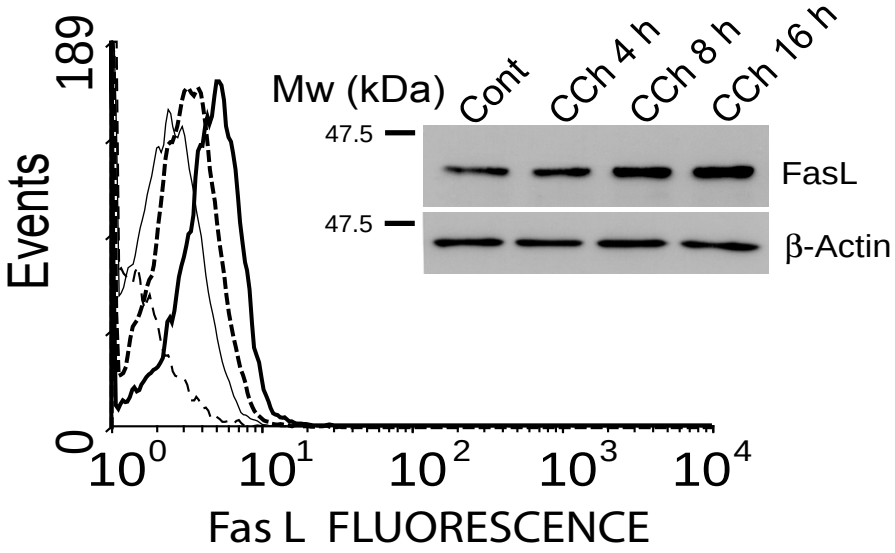
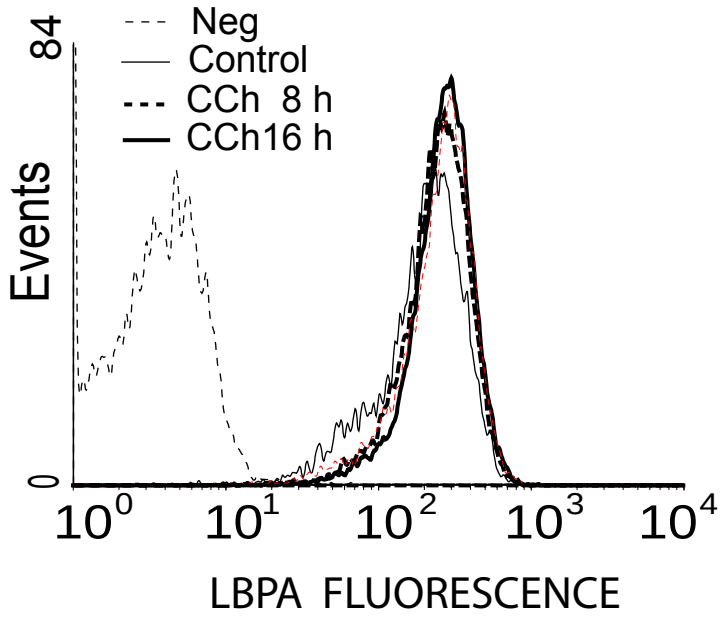


	MFI (A.U.)		
	CD63	FasL	LBPA
Neg	(-)	(-)	(-)
Control	35.01	1.03	150.02
CCh 8 h	65.06	3.02	180.07
CCh16 h	75.03	6.01	250.10





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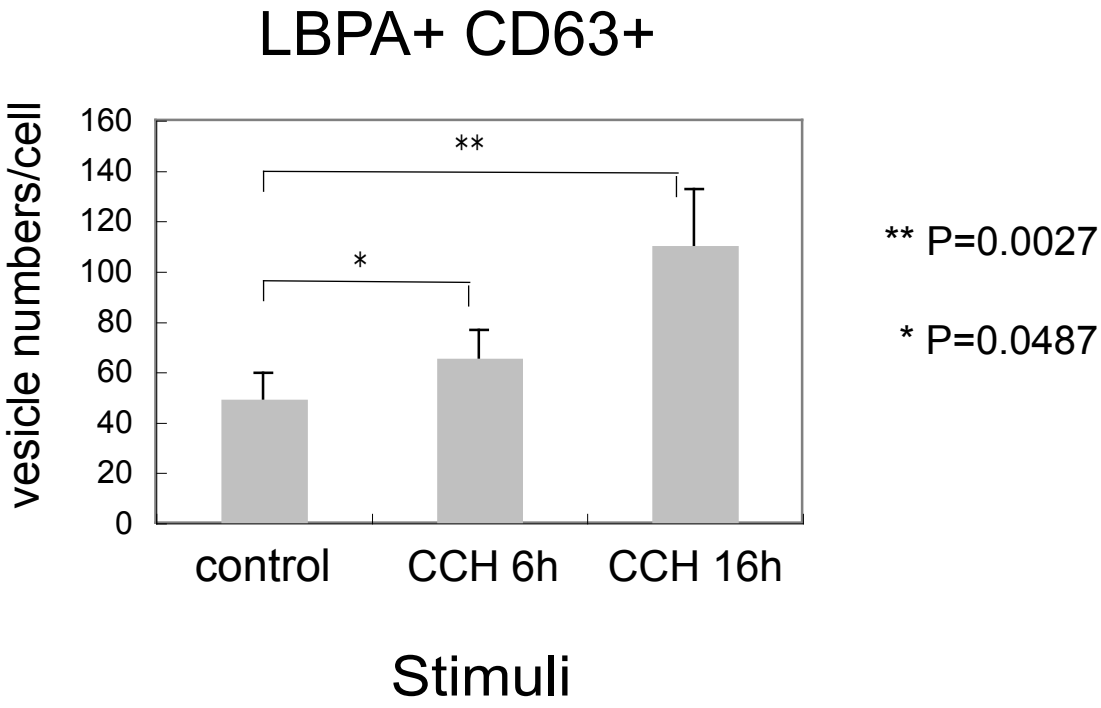
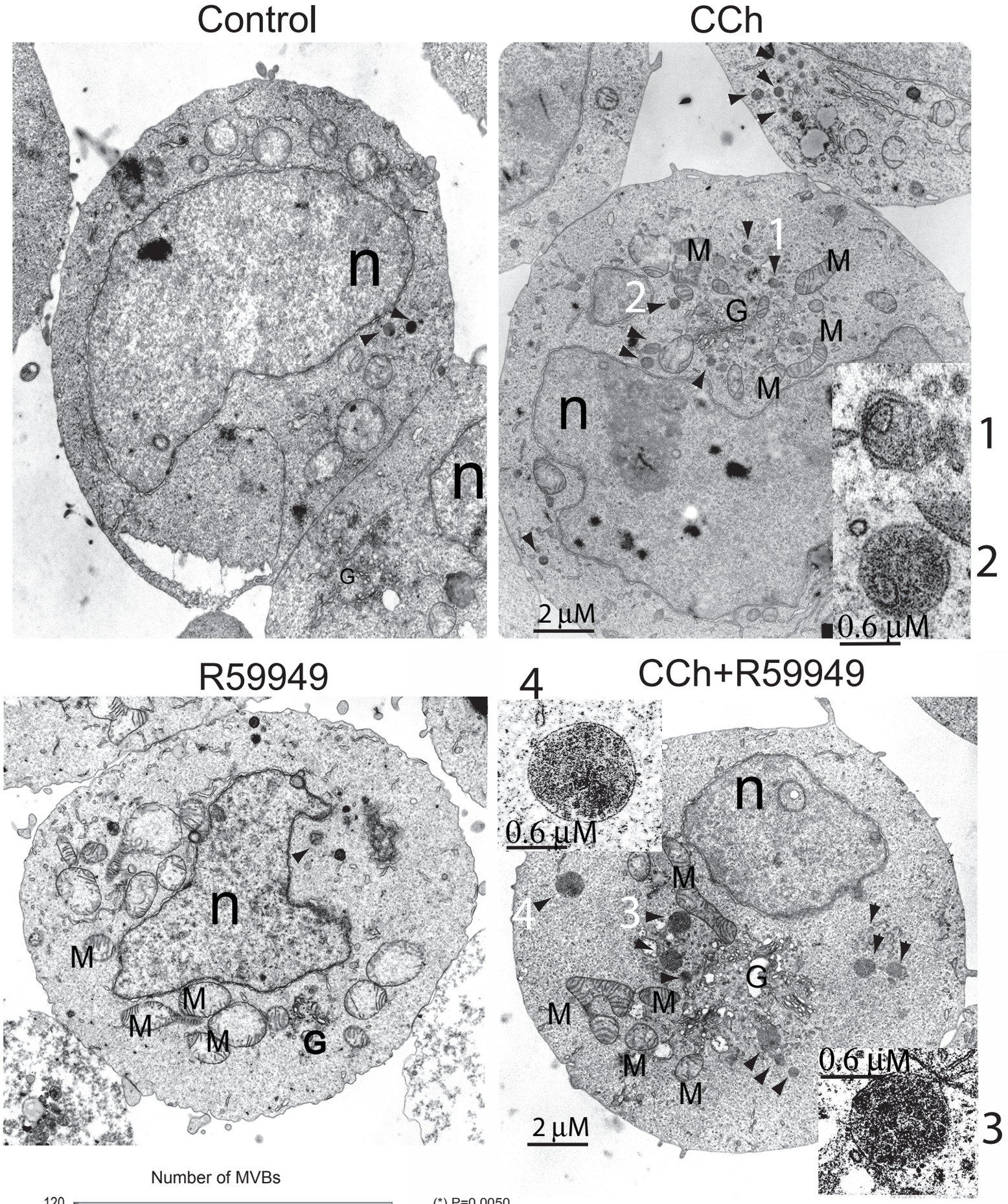
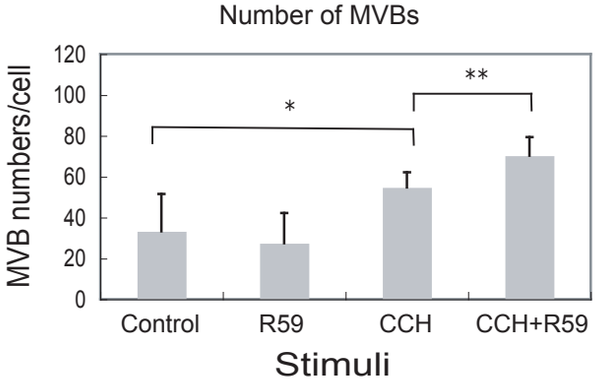


Suppl. Fig. S2 Alonso et al





(*) P=0.0050
(**) P=0.0030

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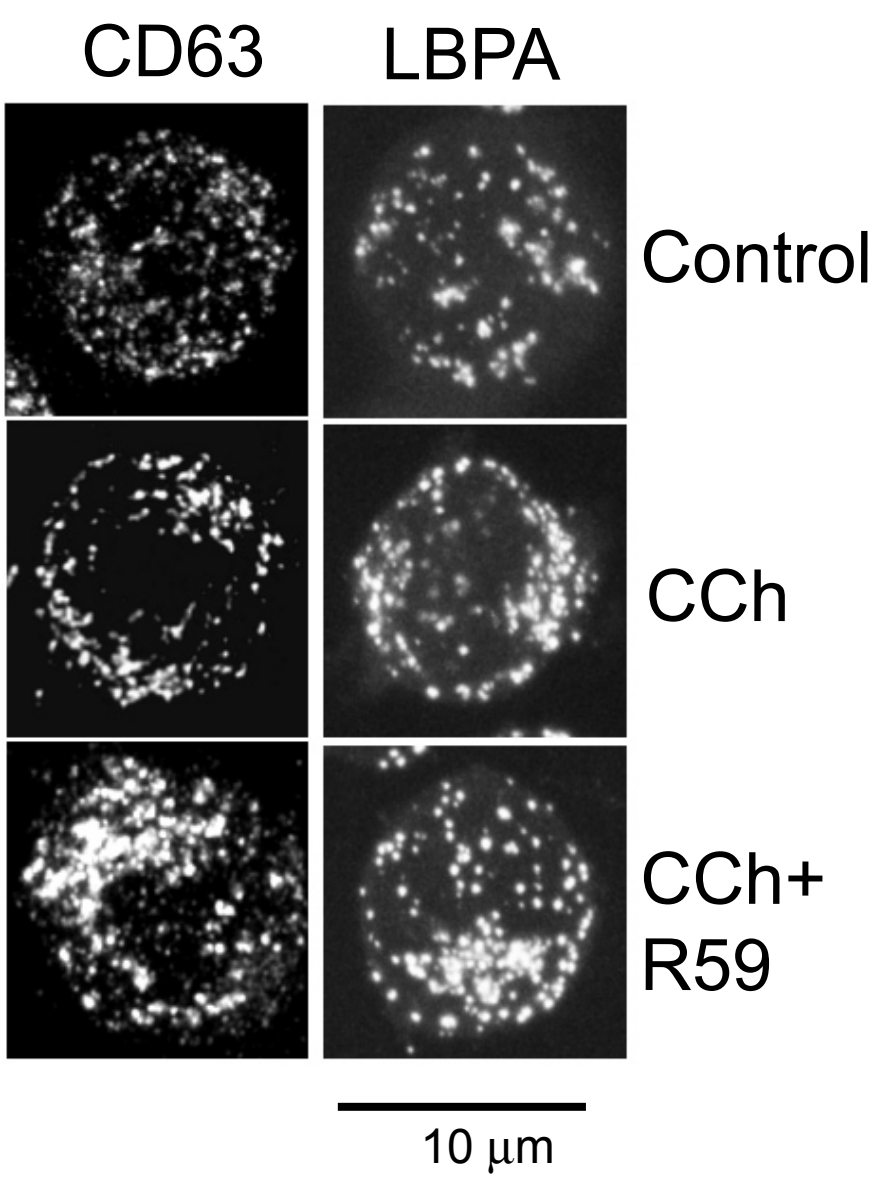

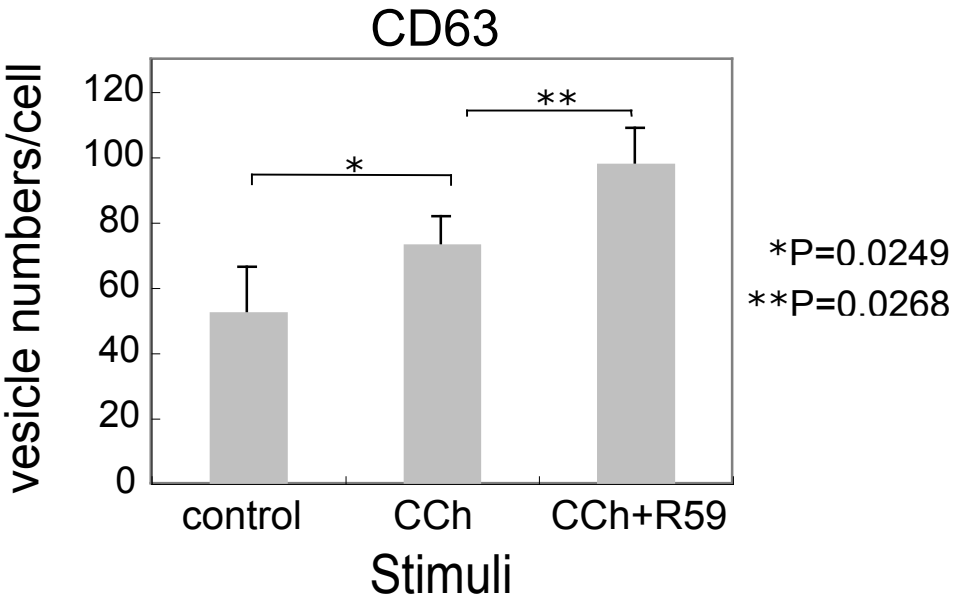

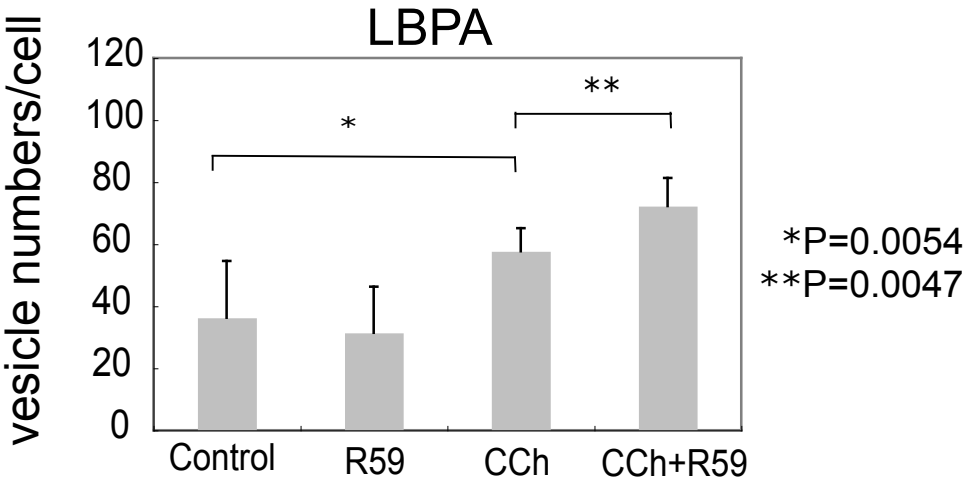

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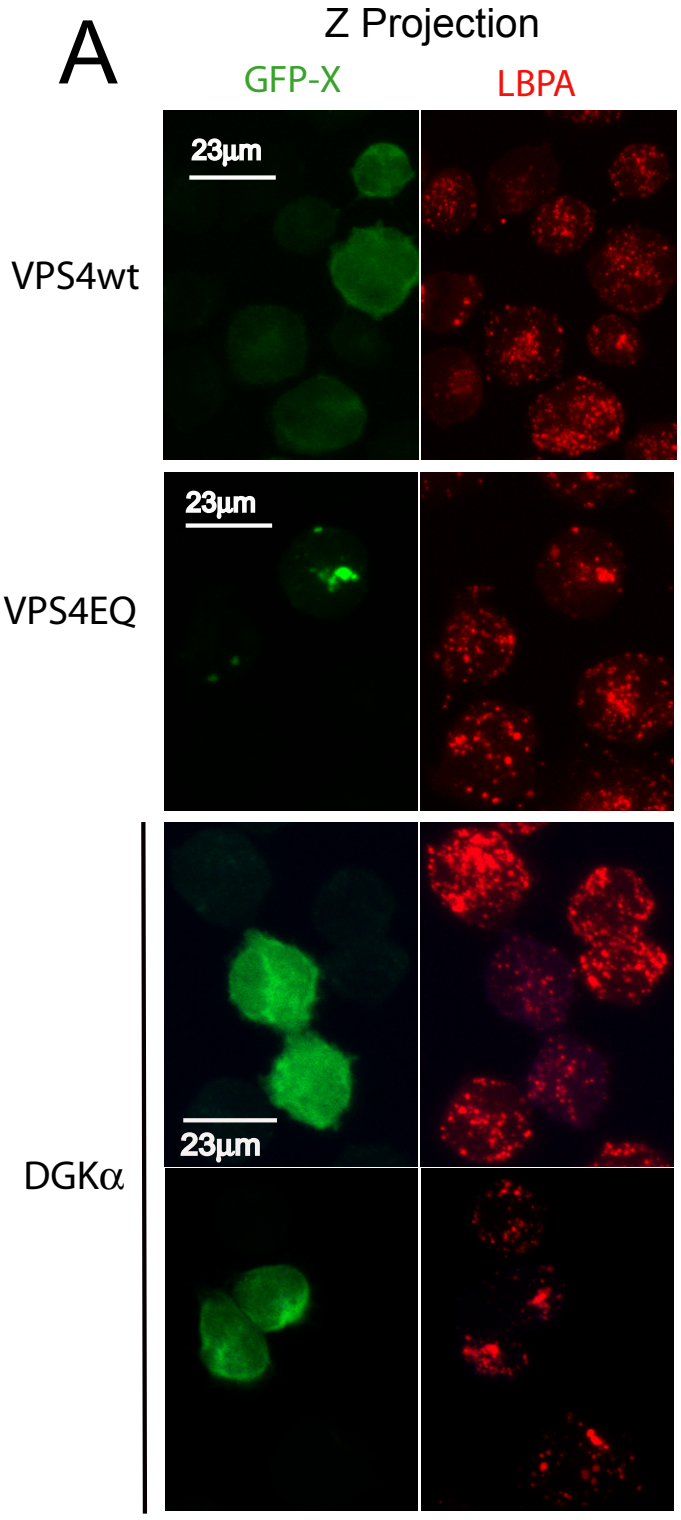
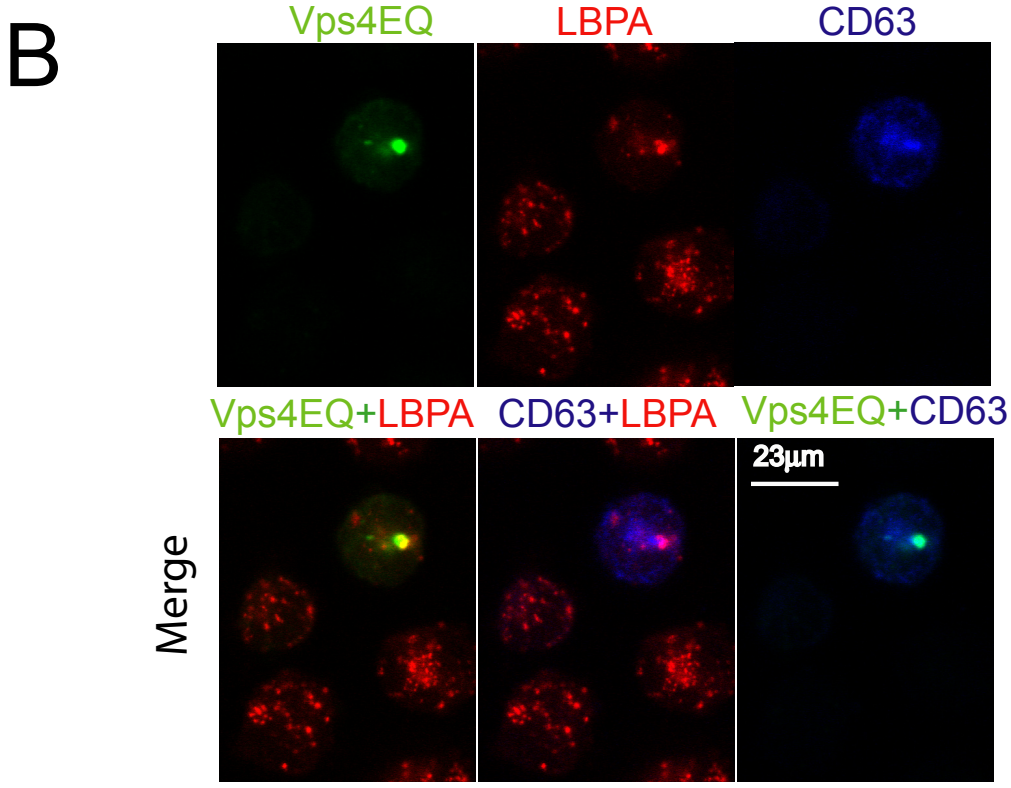
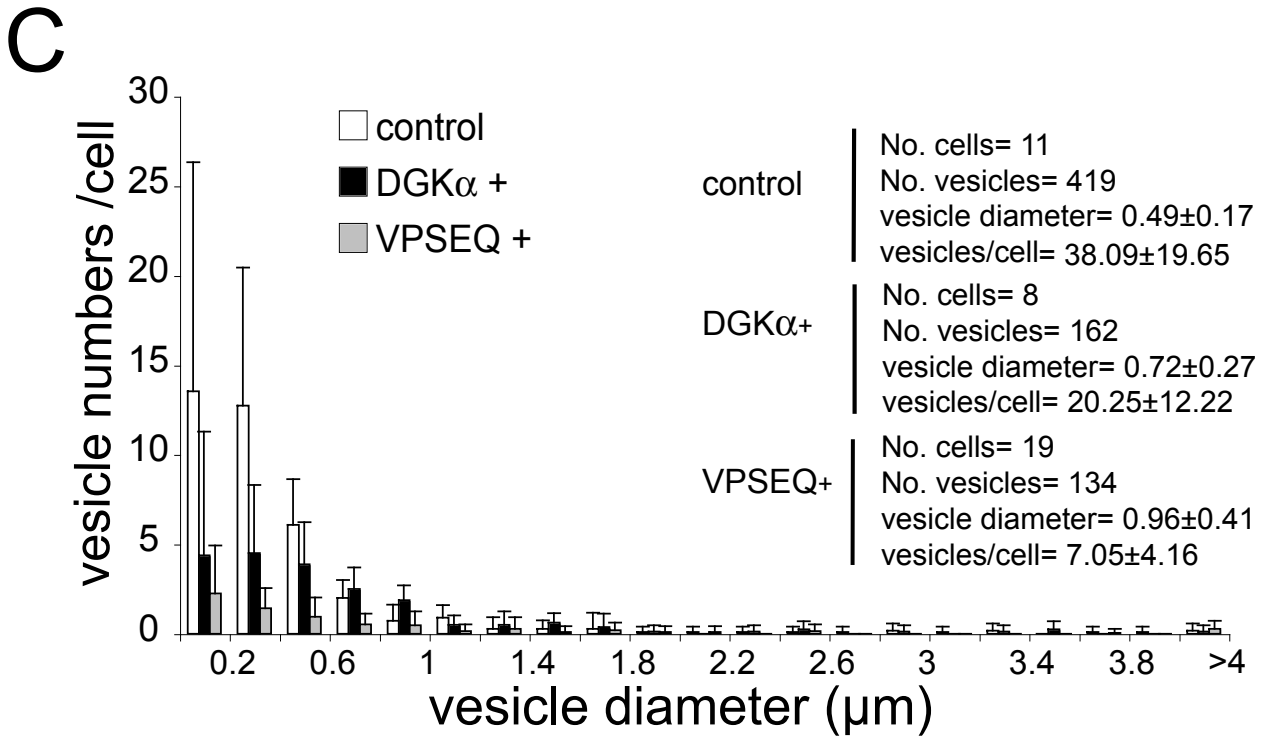
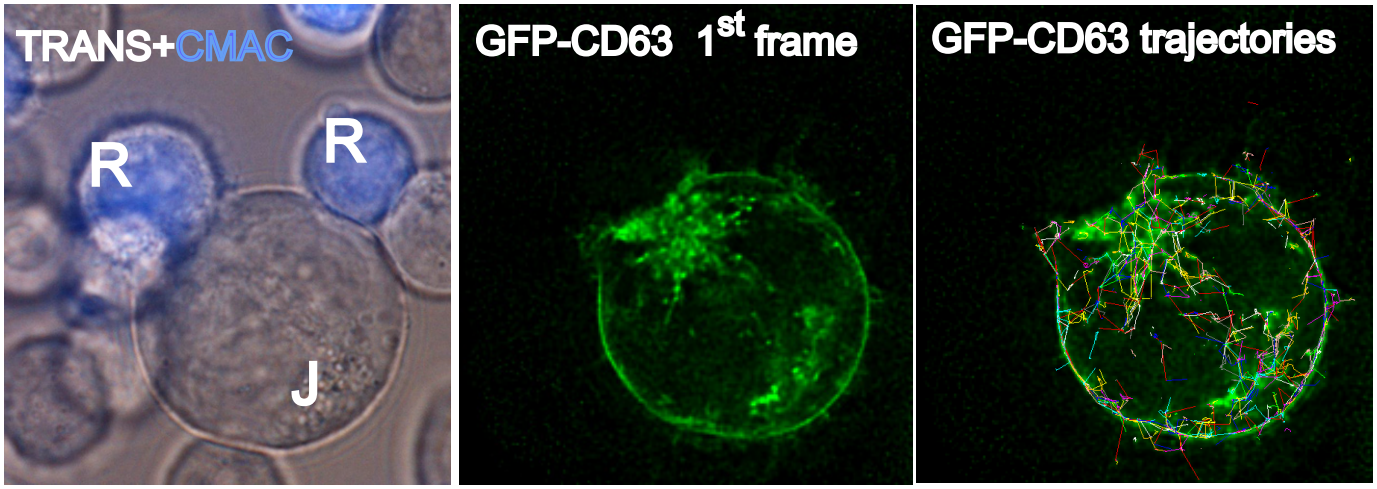






Suppl. Fig. S5 Alonso et al



Suppl. Fig. S6 Alonso et al

Videos 1 and 2

J-HM1-2.2 cells expressing CFP-CD63 were either left untreated (Video 1) or stimulated with CCh for 24 h (Video 2). After this period, videos were captured at 8 frames per second and a frame size of 640x480 pixels using a Nikon DS-5M camera. The videos were edited using ImageJ and VirtualDub software. The final frame display rate is 6 frames per second. Blue represents the chimerical CFP-CD63 molecule.

Videos 3 and 4

J-HM1-2.2 cells expressing GFP-CD63 were stimulated with CCh for 3 h (Video 3) or 6 h (Video 4). Videos were captured and processed as previously indicated; green represents GFP-CD63. In video 3, the movement of a GFP-CD63$^+$ vesicle towards the plasma membrane and its disappearance in proximity to the membrane can be observed in the lower part of the cell located in the left side. In video 4, the extinction of a ring-shaped, GFP-CD63-decorated vesicle can be observed in the right side of the cell.

Video 5

Raji B cells labelled with cell tracker blue (CMAC, blue) were pulsed with SEE for 30 min and synapses with Jurkat cells expressing GFP-CD63 were formed as indicated in Material and Methods. 4 hours after synapse formation, videos corresponding to GFP-CD63 were captured (7 frames per second) and a representative example out of 47 synapses recorded is shown. See also Supplementary Fig. S6 to visualize CMAC fluorescence corresponding to this experiment.

Video 6

Same as video 5, but the formation of a double synapse between one Jurkat cell and two Raji cells (CMAC, blue) was recorded from the beginning (10 frames per second). Simultaneous capture of GFP-CD63 and CMAC fluorescence was performed using NIS-AR software. Deconvolution of the GFP-CD63 fluorescence channel was performed as indicated in Material and Methods. A representative example out of 11 synapses recorded is shown.

Supplementary Fig. S1. J-HM1-2.2 cells were stimulated with CCh or not (Control) for the indicated times, stained with anti-CD63, anti-FasL, or anti-LBPA, and the fluorescence (Mean Fluorescence Intensity, MFI) measured by flow cytometry. Negative samples (Neg) correspond to cells stained only with secondary antibody. In the inset from the middle panel, a WB of cell extracts developed with anti-FasL (Q-20 Ab) is shown.

Supplementary Fig. S2. Quantitation of LBPA+ CD63+ vesicles and statistical analysis of the results obtained in similar experiments to that represented in Fig. 1B was performed as described in Material and Methods, in cells stimulated or not (control) with CCH (6 and 16 h). Results are represented as average number ($\pm$SD) of vesicles per cell and summarize the results obtained in 4 independent experiments.

Supplementary Fig. S3. Electron microscopy of MVBs. J-HM1-2.2 cells were stimulated with CCh for 8 hours in the presence or the absence of R59949 and processed for electron microscopy as indicated in Material and Methods. Representative sections of the results obtained with at least 30 different cells are shown. Black arrowheads label vesicles resembling MVBs. Some enlarged areas (insets 1 to 4) showed the presence of electron-dense ILVs inside MVBs. KEY: n; nucleus; M; mitochondria; G; Golgi. Lower panel: the mean number ($\pm$SD) of MVBs per cell is represented. Quantitation was performed by counting vesicles on randomly chosen sections, and summarize the results from 4 different experiments.

Supplementary Fig. S4. Cells pretreated or not with R59949 (10 μM) were stimulated with CCh for 6 h. Left panel: confocal microscopy of cells after CD63 and LBPA labelling (Z-projections). Representative images of the results obtained in 3 independent experiments are shown. Right panels: quantitation of the number of $CD63^+$ and $LBPA^+$ vesicles. Results are represented as average numbers ($\pm$ SD) of vesicles/cell from 40 cells analysed in 3 independent experiments. R59949 alone had no effect on the number of $CD63^+$ or $LBPA^+$ intracellular vesicles**.**

Supplementary Fig. S5. A) Cells were transfected with different GFP fusion constructs (GFP-VPS4wt, GFP-VPS4EQ and GFP-DGKα), stimulated with CCh (8 h) and stained with anti-LBPA, and imaged by confocal microscopy. Z-projection images of the GFP-containing construction and LBPA staining are shown to visualize the effect of the different constructions on the subcellular distribution of LBPA. B) Cells expressing GFP-VPS4EQ mutant together with CFP-CD63, were stimulated for 8 h with CCh and stained with anti-LBPA (red) as indicated in Material and Methods. Cells were imaged by confocal microscopy and a representative, single optical section (0.4 μm thick) is shown in the upper row. The lower row shows the merged images, with coincident labelling appearing yellow, pink and sky blue, respectively. C) The vesicle diameter distribution analysis corresponding to the experiment summarized in Fig. 6C was performed as indicated in Material and Methods. The distribution is represented as a histogram of the average (±SD) vesicle number per cell versus the indicated vesicle diameter classes.

Supplementary Fig. S6. Raji B cells labelled with CMAC (R) were pulsed with SEE and synapses with Jurkat cells expressing GFP-CD63 (J) were formed as indicated in Material and Methods. Four hours after synapse formation, snapshots (transmittance plus CMAC, GFP-CD63) and videos (GFP-CD63, see Video 5 synapse) were recorded as indicated in Material and Methods. In the middle panel, the first frame corresponding to video 5 is shown, and one synapse area is seen at the upper left side of the Jurkat cell. In the right side panel, trajectories followed by GFP-CD63 vesicles from video 5 are plotted.