Supporting Information for

Spatial separation of the processing and MHC class I loading compartments for cross-presentation of the tumor-associated antigen HER2/*neu* by dendritic cells

Renato B. Baleeiro, [1] René Rietscher, [2] Andrea Diedrich, [3] Justyna A. Czaplewska, [4,5] Claus-Michael Lehr, [2, 6] Regina Scherließ, [3] Andrea Hanefeld, [7] Michael Gottschaldt, [4,5] Peter Walden [1§]

[1] Department of Dermatology, Venerology and Allergy, Charité – Universitätsmedizin Berlin, Berlin, Germany;

[2] Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz Centre for Infection Research (HZI), Saarland University, Saarbrücken, Germany;

[3] Department of Pharmaceutics and Biopharmaceutics – Kiel University, Kiel, Germany;

[4] Laboratory of Organic and Macromolecular Chemistry (IOMC), Friedrich Schiller University Jena, Jena, Germany;

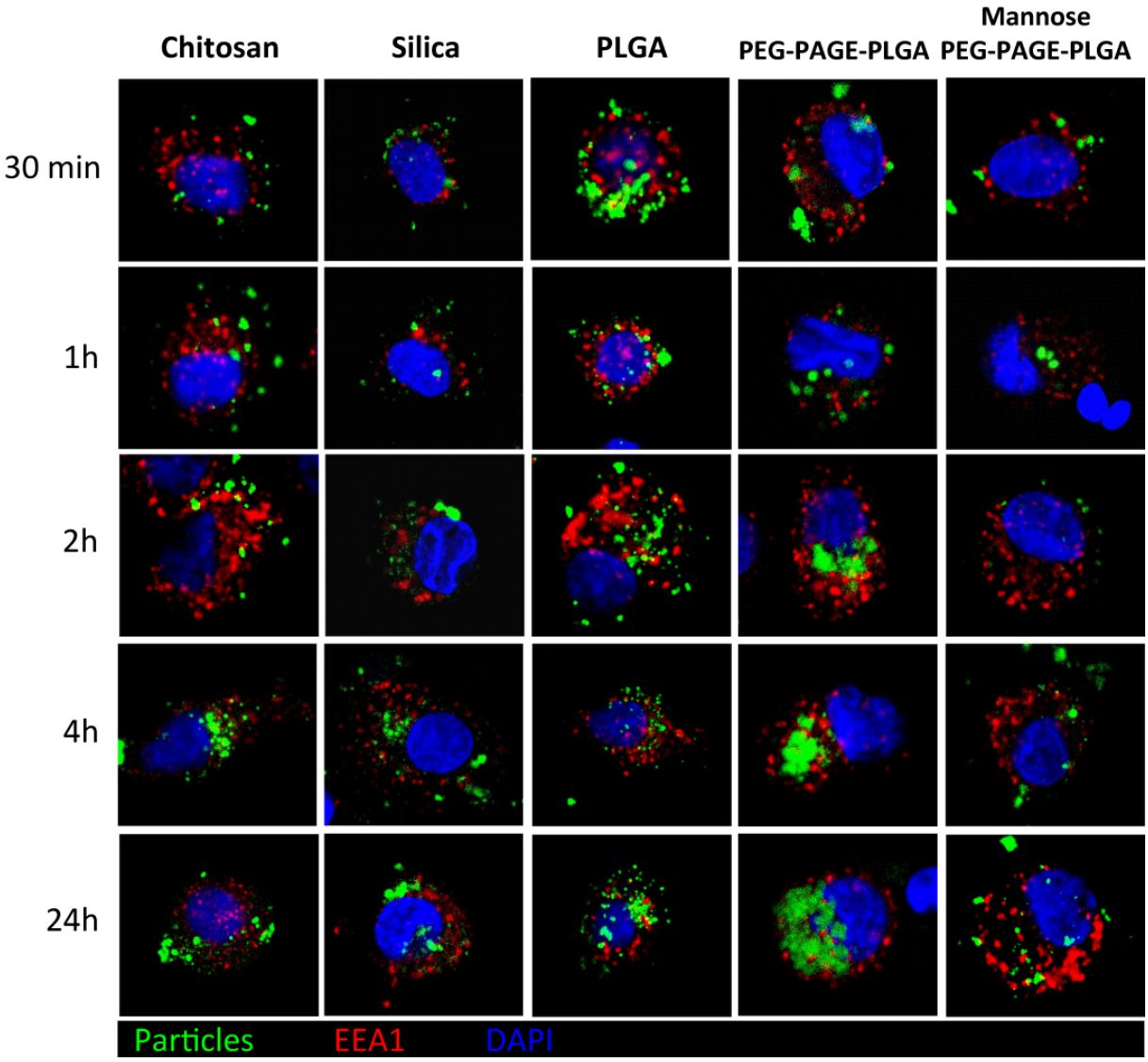
[5] Jena Center for Soft Matter (JCSM), Friedrich Schiller University Jena, Jena, Germany;

[6] Biopharmaceutics and Pharmaceutical Technology, Saarland University, Saarbrücken, Germany;

[7] Merck KGaA, Darmstadt, Germany

[§] Corresponding Author: peter.walden@charite.de

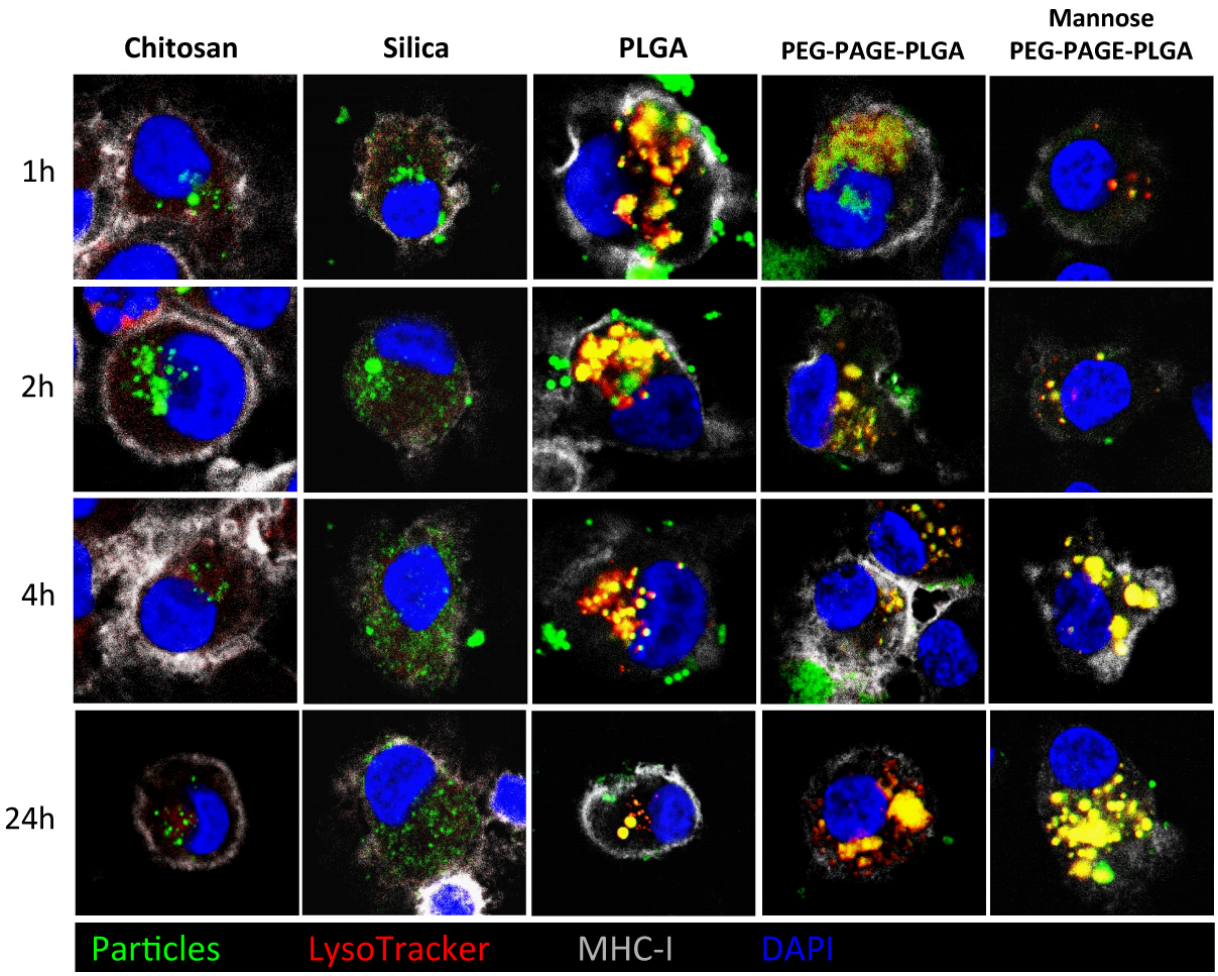
Supplementary Figures



Supplementary Figure 1

Routing kinetics of NPs into early endosomes.

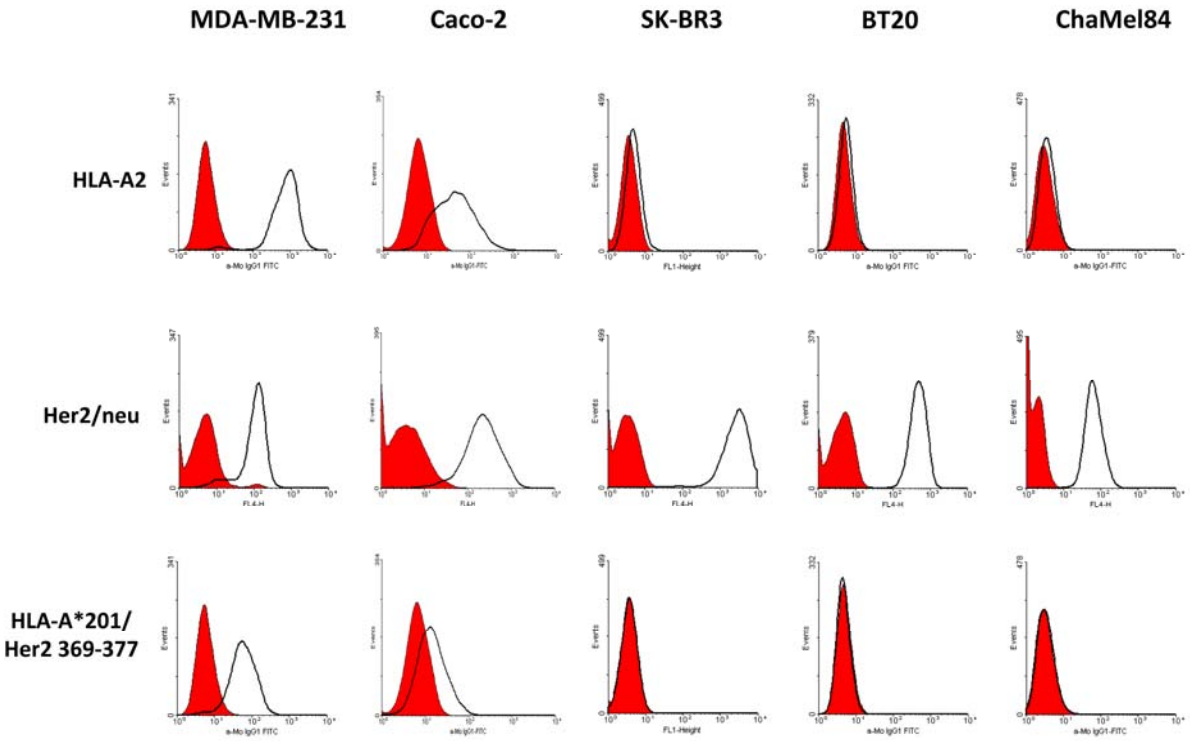
Confocal images showing DCs loaded with the indicated fluorochrome-labeled NPs (green) and stained with EEA1 (red; for early endosomes labeling), and DAPI (blue; for nuclei labeling). Confocal micrographs were taken with a 63x oil objective lens.



Supplementary Figure 2

Kinetics of routing NPs into acidic compartments.

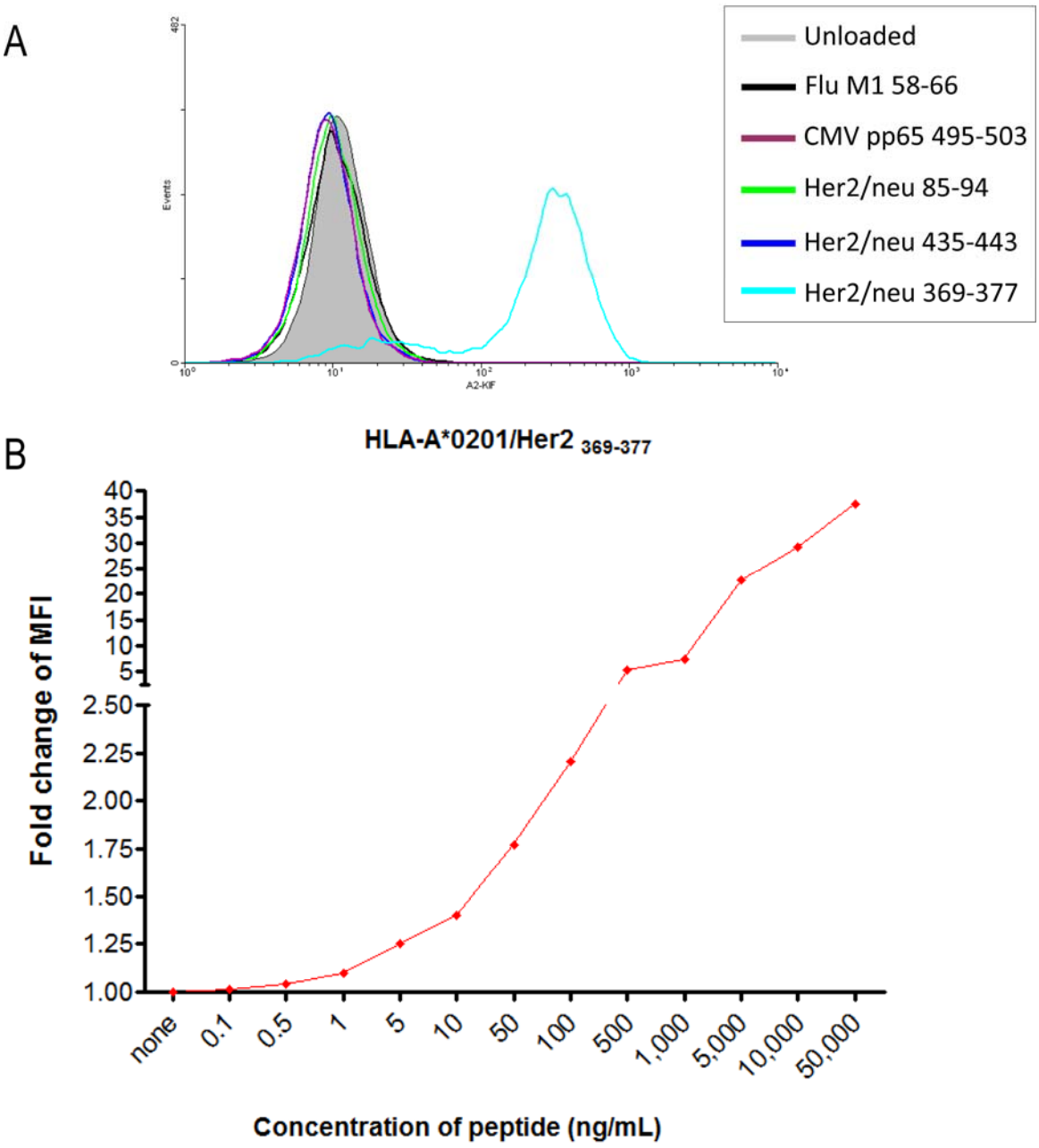
Confocal images depicting DCs loaded with fluorochrome-labeled NPs (green) and stained with LysoTracker Red DND-99 (red; for late/acidic endosomes), Alexa fluor 647-labeled MHC-I (gray; for cell membrane) and DAPI (blue; for nuclei). NPs inside acidic compartments appear in yellow. Confocal micrographs were taken with a 63x oil objective lens.



Supplementary Figure 3

Specificity of the antibody RL1S for the complex HLA-A*0201/HER2/*neu*$_{369\text{-}377}$.

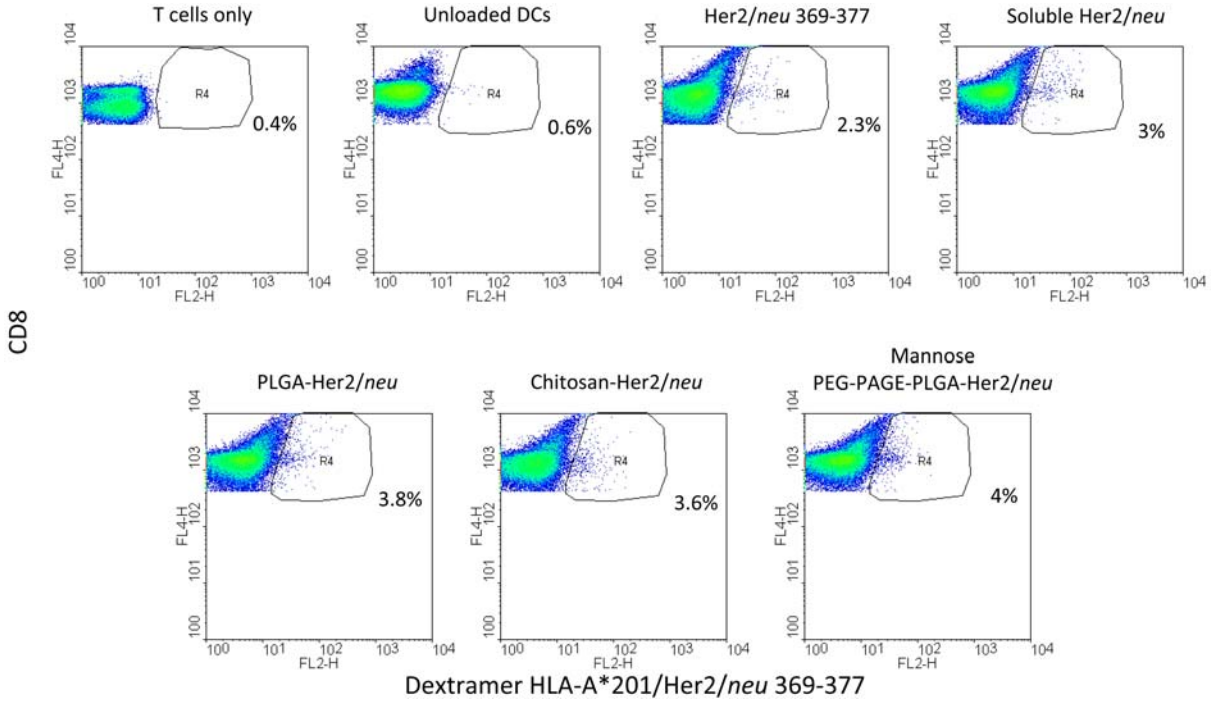
Tumor cells lines were checked for their expression of HLA-A2 (upper row), HER2/*neu* (intermediate row) and the complex HLA-A*201/HER2/*neu*$_{369\text{-}377}$ (lower row). Only tumor cell lines expressing both HER2/*neu* and HLA-A2 stained positive for the complex HLA-A*201/HER2/*neu*$_{369\text{-}377}$. Red-filled histogram= unstained cells; Empty histogram= cells stained with the indicated antibody.



Supplementary Figure 4

Specificity and sensitivity of the antibody RL1S for the complex HLA-A*0201/HER2/*neu*$_{369\text{-}377}$.

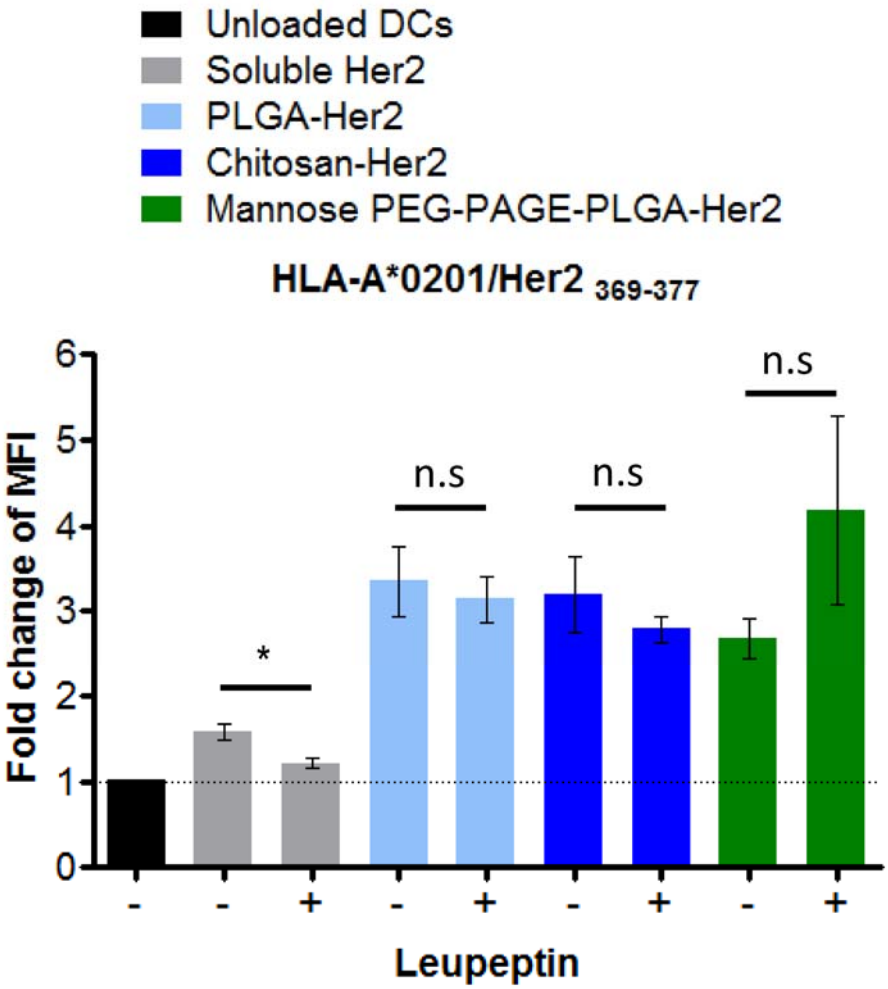
(**A**) TAP-deficient T2 cells were pulsed for 4 hours with the following HLA-A2-binding peptides at 50 µg/mL: Flu M1$_{58\text{-}66}$, CMV pp65$_{495\text{-}503}$, HER2/*neu*$_{85\text{-}94}$, HER2/*neu*$_{435\text{-}443}$ and HER2/*neu*$_{369\text{-}377}$, or left untreated (unloaded). Then, the cells were labeled with the mouse IgG1 antibody with specificity for the complex HLA-A*0201/HER2/*neu*$_{369\text{-}377}$ (clone RL1S), followed by a FITC-labeled goat anti-mouse IgG1, as secondary antibody, and analyzed by flow cytometry. The cells pulsed with peptides other than HER2/*neu*$_{369\text{-}377}$ display fluorescence intensity similar to the untreated cells, whereas the cells pulsed with HER2/*neu*$_{369\text{-}377}$ display higher fluorescence (blue line), which shows its specificity for the complex HLA-A*0201/HER2/*neu*$_{369\text{-}377}$. (**B**) T2 cells were pulsed for 4 hours with titrated doses of the peptide HER2/*neu*$_{369\text{-}377}$. Then cells were labeled with anti-HLA-A*201/HER2/*neu*$_{369\text{-}377}$ and analyzed by flow cytometry as described for (**A**).



Supplementary Figure 5

Immunostimulatory capacity of DCs loaded with HER2/*neu* in different formulations.

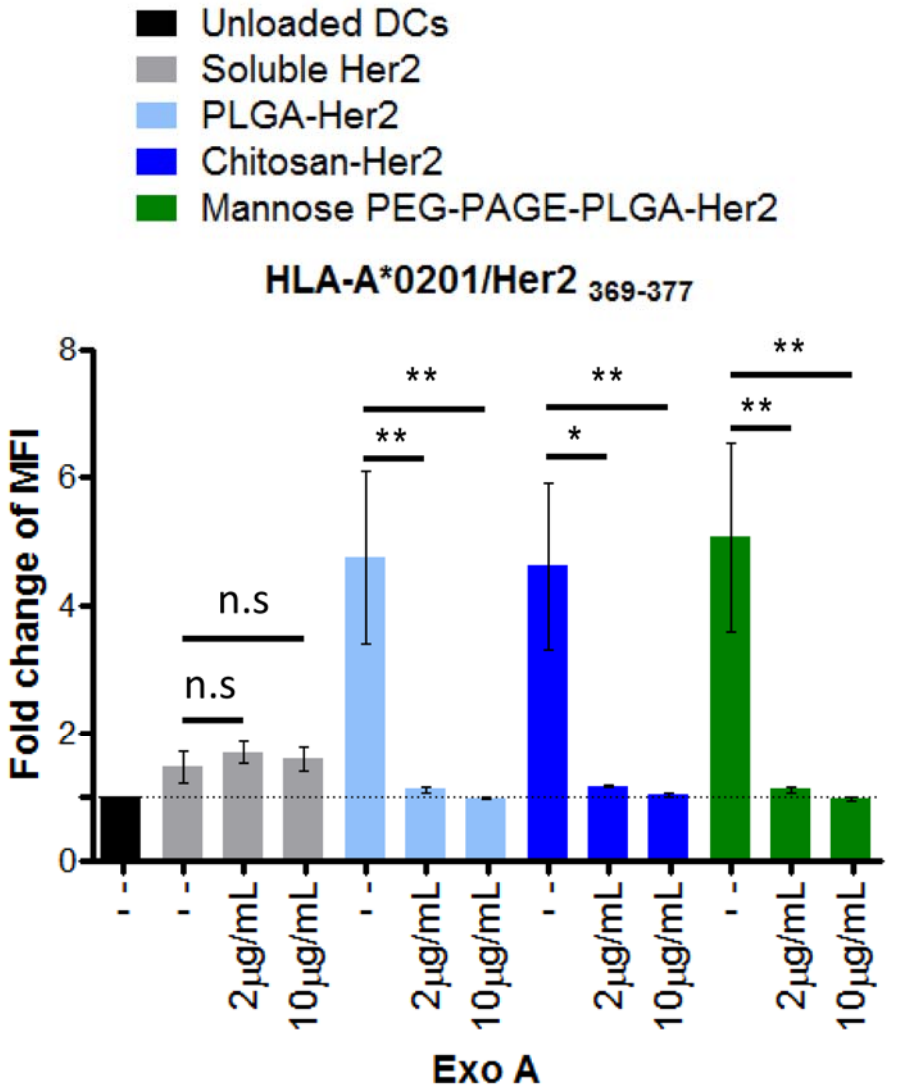
iDCs were incubated for 24 h with soluble HER2/*neu* (50 µg/mL) or NP-HER2/*neu* (5 µg/mL). Soluble HER2/$neu_{369\text{-}377}$ peptide (50 µg/mL) was incubated for 2 h. After incubation, DCs were washed and co-cultured with autologous $CD8^+$ T cells. Seven days later, $CD8^+$ T-cell expansion was evaluated by specific HLA-A*201/HER2/$neu_{\ 369\text{-}377}$ dextramer staining. Data are representative of 6 independent experiments performed with cells from 6 different donors.



Supplementary Figure 6

Effect of leupeptin on the cross-presentation of HER2/*neu*$_{369\text{-}377}$.

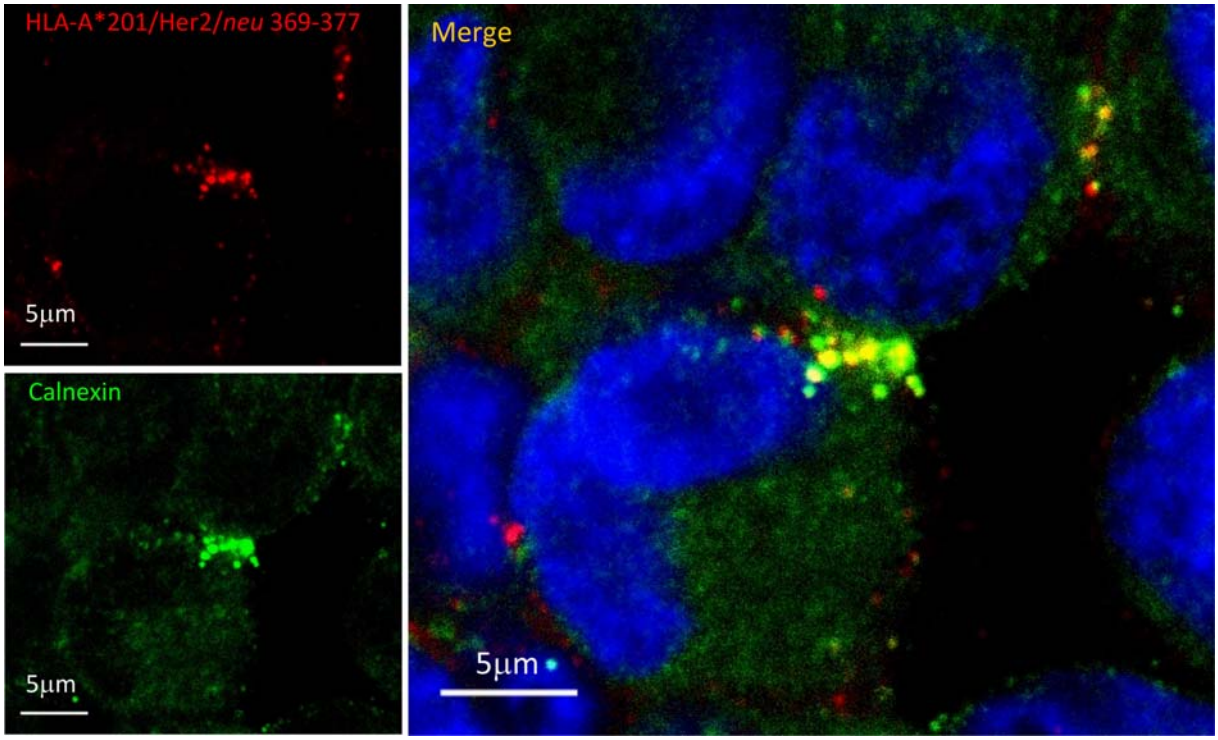
iDCs were incubated for 24 h with 5 µg/mL of HER2/*neu* in the soluble form or encapsulated in NPs. After incubation, cells were harvested and stained for the complexes HLA-A2/HER2/*neu*$_{369\text{-}377}$ and analyzed by Flow Cytometry. To investigate the endosomal processing leading to the cross-presentation, cells were treated with the serine and cysteine protease inhibitor leupeptin at 100 µM 30 min prior to adding the Ag. n= 3; Error bars= Standard error of the mean; n.s nonsignificant, *$p<0.05$ in student´s t-test.



Supplementary Figure 7

Effect of Exo A on the cross-presentation of HER2/*neu*$_{369\text{-}377}$.

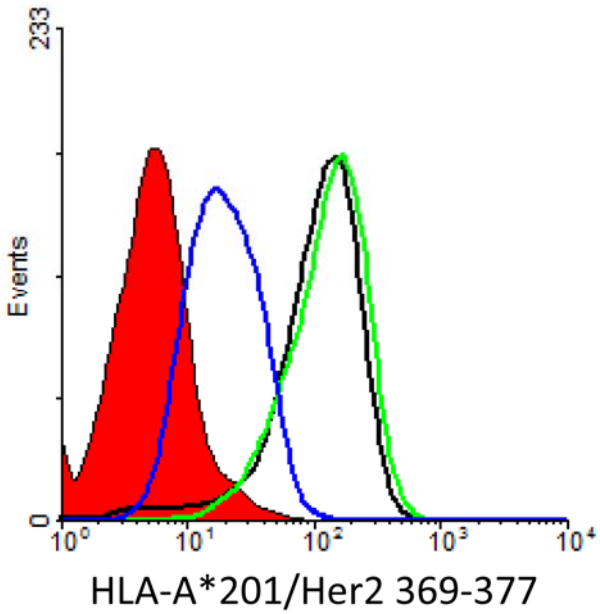
iDCs were incubated for 24 h with 5 µg/mL of HER2/*neu* in the soluble form or encapsulated in NPs. After incubation, cells were harvested and stained for the complexes HLA-A2/HER2/*neu*$_{369\text{-}377}$ and analyzed by Flow Cytometry. To investigate the importance of Sec61 for the cross-presentation, cells were treated with Pseudomonas aeruginosa exotoxin A (Exo A), a Sec61 inhibitor at the indicated concentrations 30 min prior to adding the Ag. n= 4; Error bars= Standard error of the mean; n.s nonsignificant, *p<0.05, **p<0.01 in student´s t-test.



Supplementary Figure 8

Intracellular expression of the complex HLA-A*201/HER2/*neu*$_{369\text{-}377}$ by the cell line MDA-MB-231.

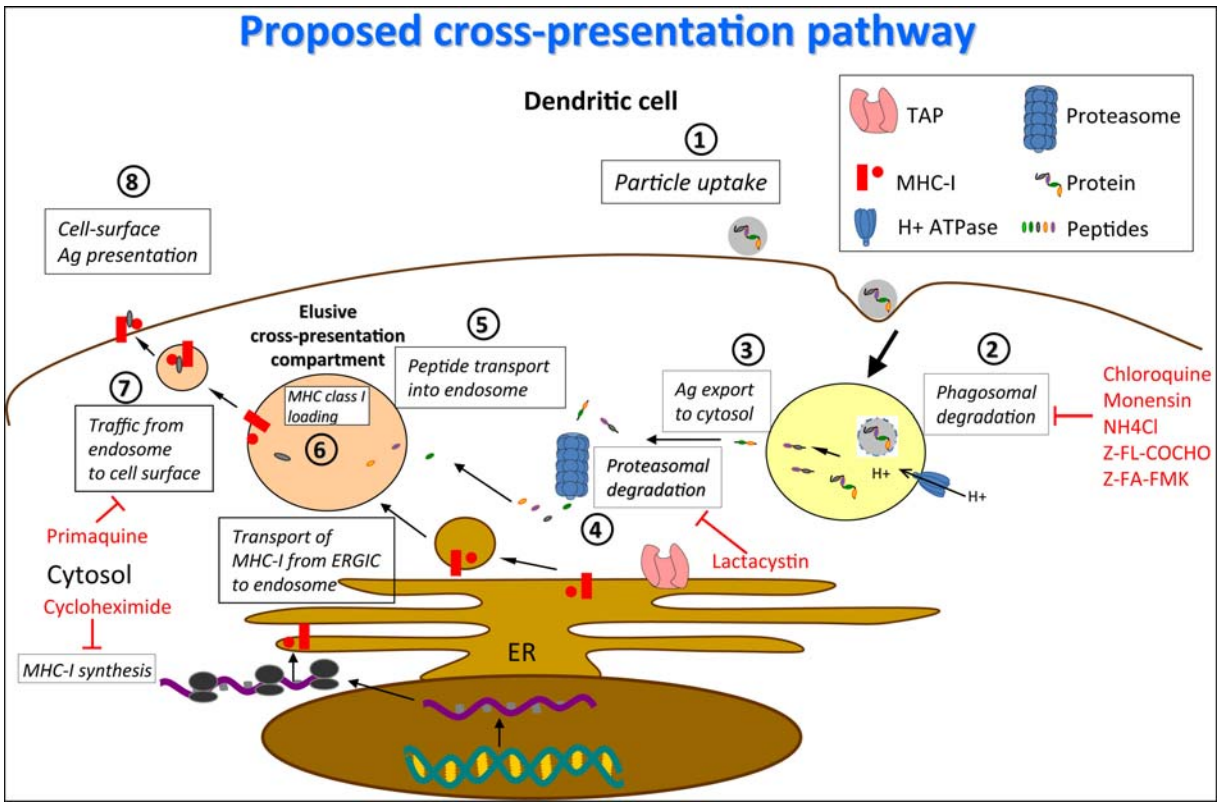
To identify the intracellular localization of the complex HLA-A*201/HER2/*neu*$_{369\text{-}377}$ in a cell line that expresses the protein HER2/*neu* and is HLA-A2+, MDA-MB-231 cells were stained with antibody for HLA-A2/HER2/*neu*$_{369\text{-}377}$ and an antibody for calnexin, a protein expressed in the ER. Cells were counterstained with DAPI for nuclei visualization and examined by confocal microscopy.



Supplementary Figure 9

Effect of Brefeldin A and primaquine on the endogenous presentation of HER2/*neu*$_{369\text{-}377}$ by MDA-MB-231 cells.

To investigate the importance of the secretory pathway via ER-Golgi and endosomal transport for the endogenous presentation of the epitope HER2/*neu*$_{369\text{-}377}$ on HLA-A2, MDA-MB-231 cells were left untreated (black line), or incubated for 24h with Brefeldin A at 1 µg/mL (blue line) or primaquine at 100 µM (green line). After incubation, cells were harvested and surface stained for the complexes HLA-A2/HER2/*neu*$_{369\text{-}377}$ and analyzed by Flow Cytometry. Red-filled histogram= unstained cells.



Supplementary Figure 10

Proposed cross-presentation pathway for HER2/*neu* delivered to DCs in nanoparticles.

The cartoon depicts the proposed cross-presentation pathway following uptake of particulate HER2/*neu* protein by human DCs. ER= Endoplasmic reticulum; ERGIC= Endoplasmic reticulum Golgi intermediate compartment; NH4Cl= Ammonium chloride; Z-FA-FMK= Z-Phe-Ala fluoromethyl ketone; Z-FL-COCHO= benzyl N-[1-[(5-methyl-1,2-dioxohexan-3-yl)amino]-1-oxo-3-phenylpropan-2-yl]carbamate; MHC= Major histocompatibility complex; TAP= Transporter associated with antigen processing.