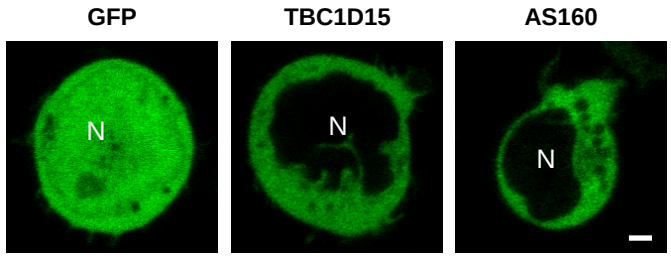
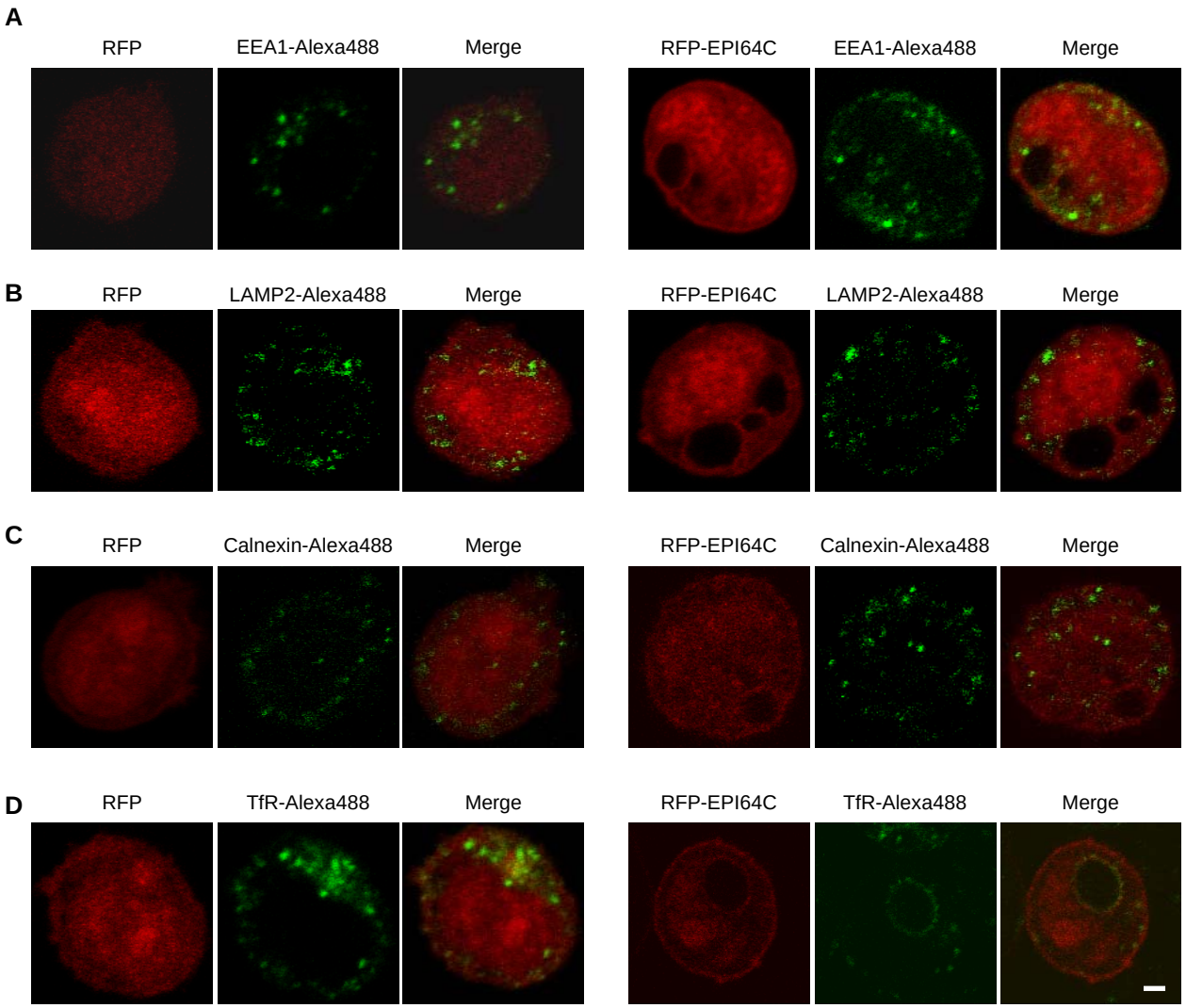
Supplemental Movie Legend.

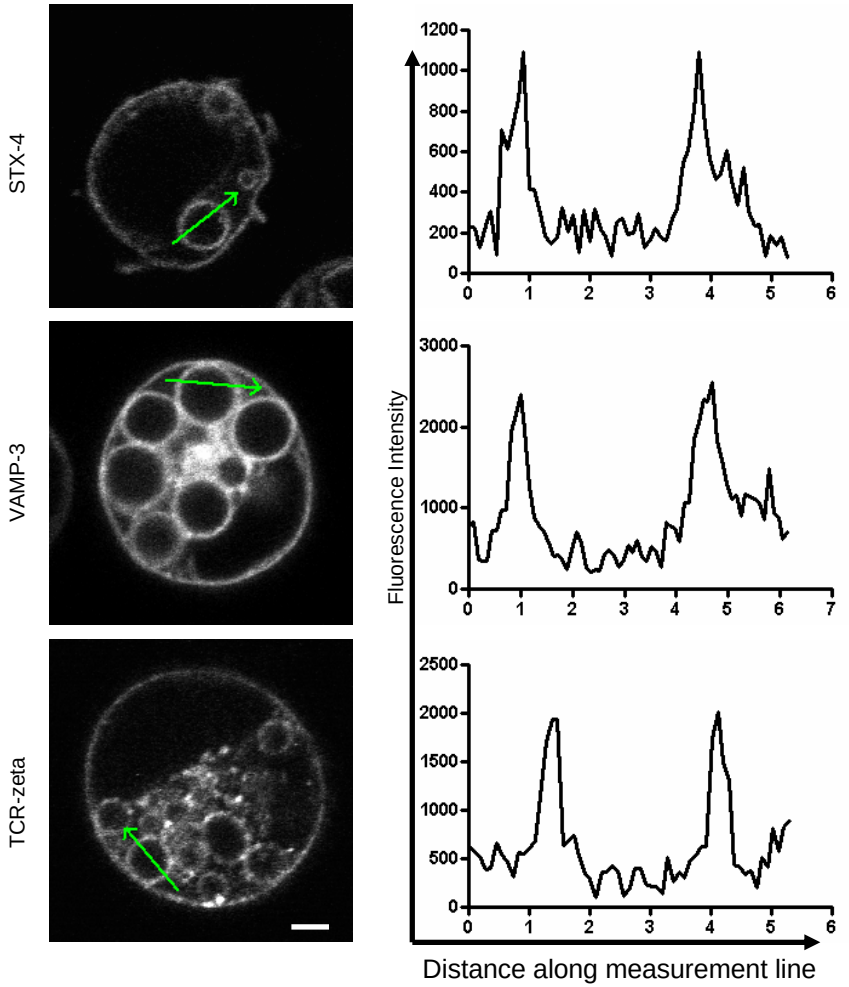
Transfected T cells were dropped onto SEE superantigen-pulsed Raji B cells (approximate location indicated by circle). Maximum-intensity projections from Z-stacks (17 slices, 1 μm apart) were compiled at each time-point and sequenced into a movie with IPLab (Scanalytics). The zero time-point represents the initiation of imaging, which was prior to the first contact between the T cell and the APC. The movie speed is 100x real time. Scale bar 5μm.



Supplemental Figure 1. Control RabGAPs do not induce large vacuoles
Transfection of two TBC-containing proteins (AS160, TBC1D15) under conditions identical to those used for EPI64C (Fig 1A) does not induce large vacuoles. Bar 2µm.

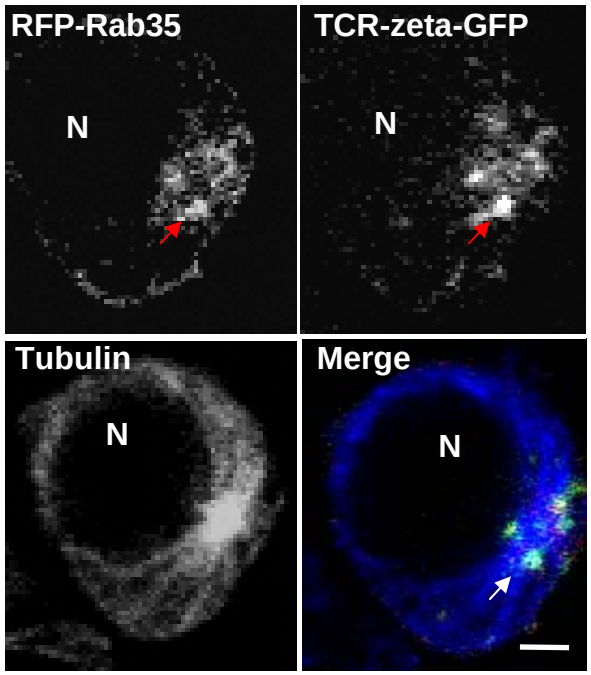


Supplemental Figure 2. **TfR but not markers of other compartments are located in EPI64C-induced vacuoles.** This figure provides additional one color images and controls for Fig 1c. Jurkat cells were transfected with mRFP or mRFP-EPI64C and stained with (A) anti-EEA1, (B) anti-LAMP2, (C) anti-Calnexin, (D) anti-TfR respectively. Single color, merged confocal images showed the localization of EEA1, LAMP-2, Calnexin, TfR (Green). Bar 2µm.

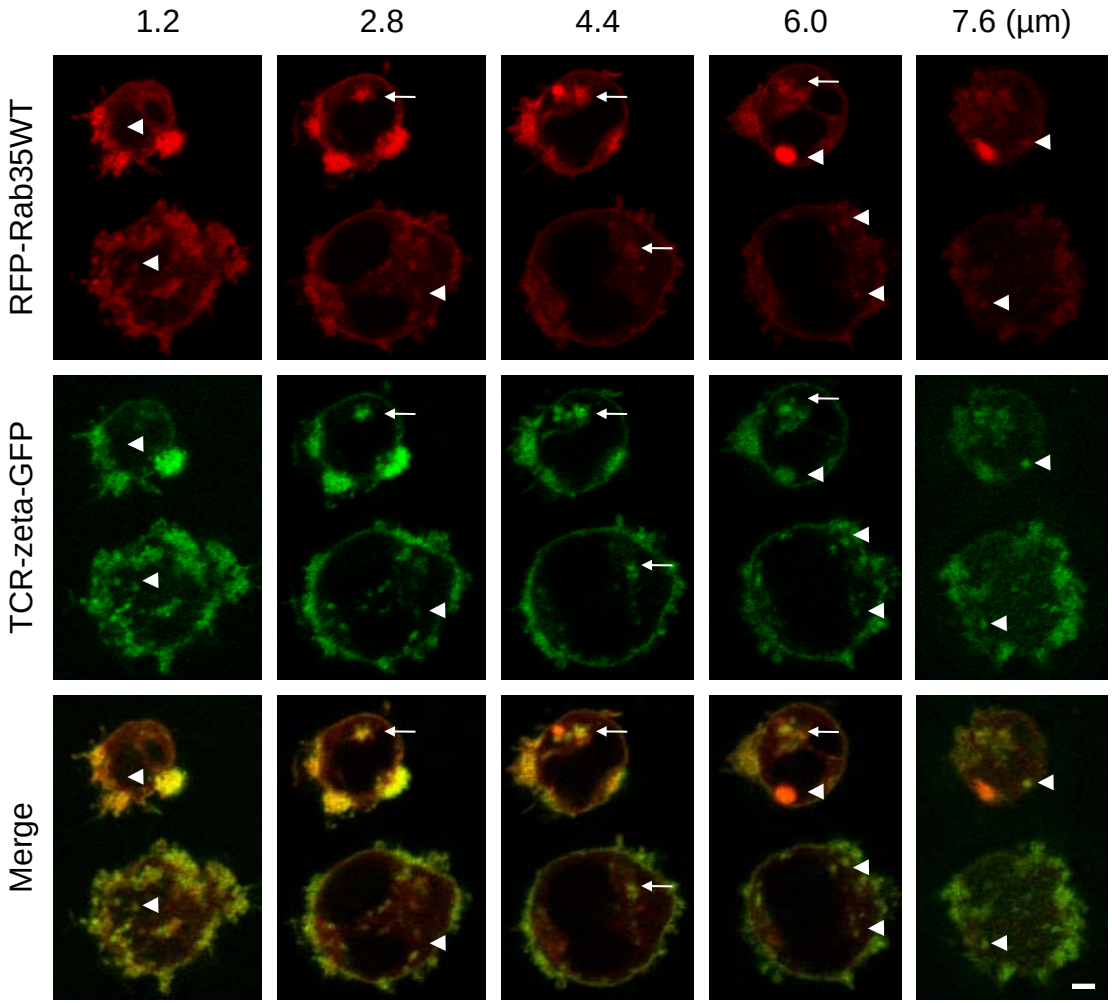


Supplemental Figure 3. TCRzeta, VAMP-3 and STX-4 are enriched in the vesicle membrane

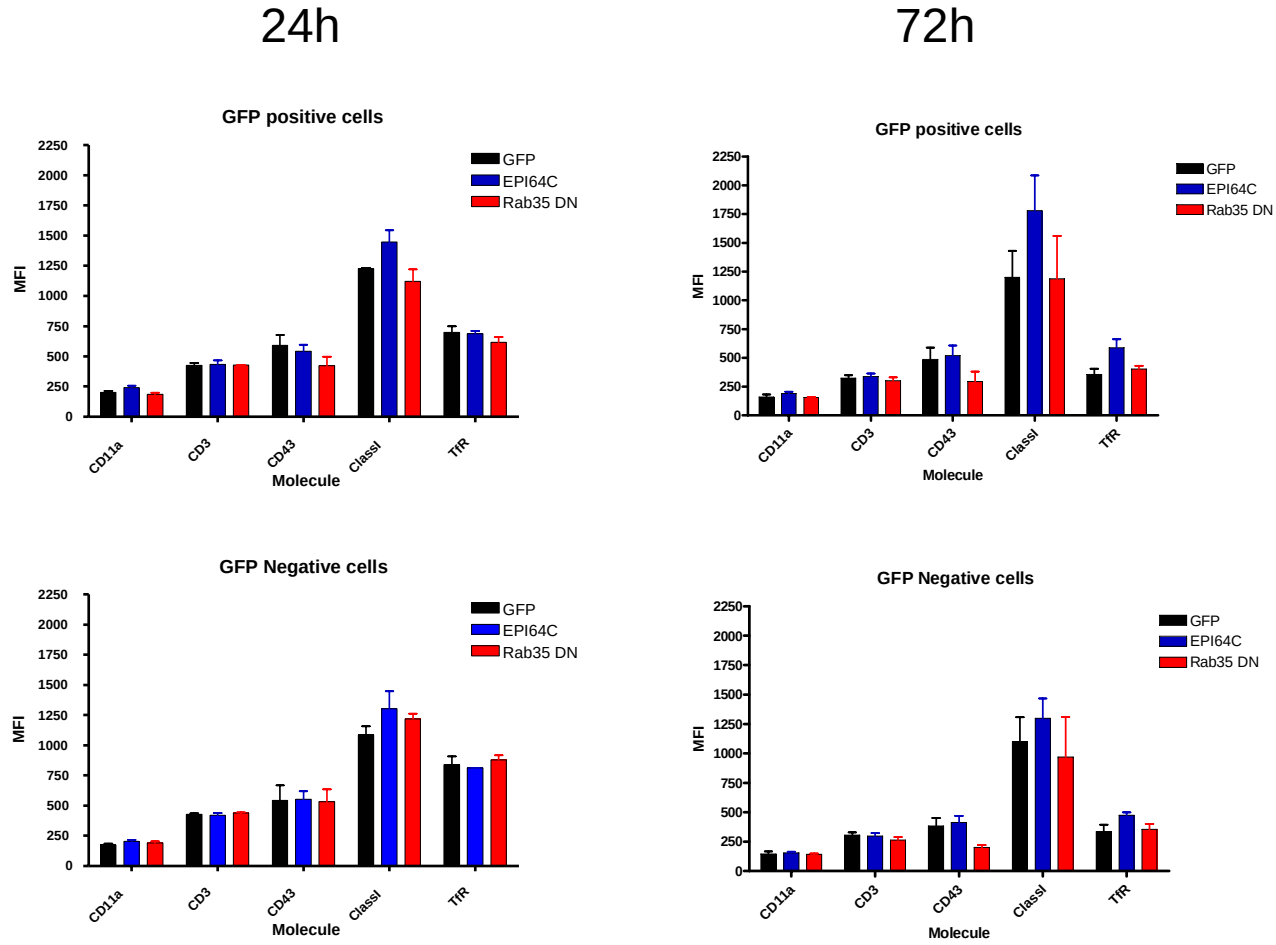
Localization of the GFP-tagged constructs from Fig. 1D is shown in the grayscale panels. Graphs on right represent quantitative analysis of GFP intensity along a line through a representative vacuole that is bounded by cytoplasm on both sides (at the location shown by green arrow on corresponding image). Bar 2µm



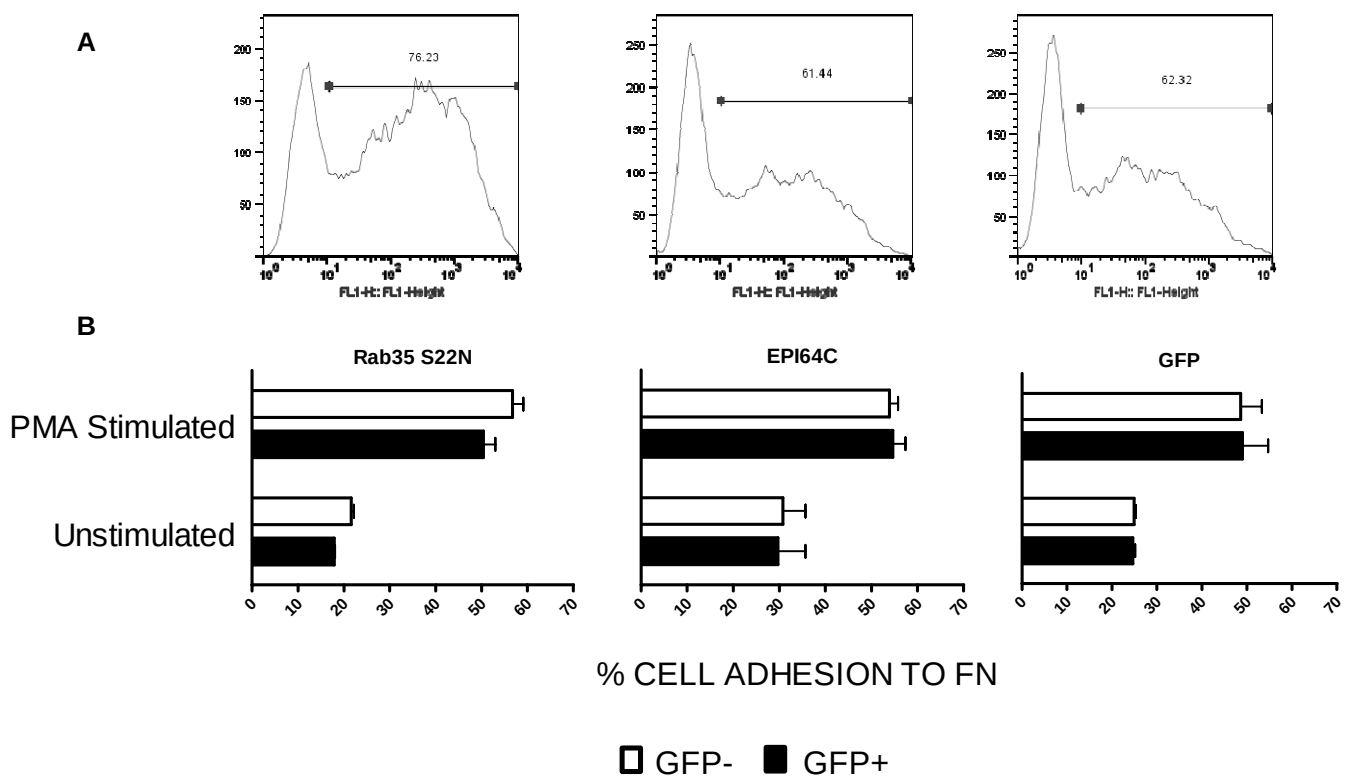
Supplemental Figure 4. IntracellularRab35 localization includes a peri-centriolar compartment. Detection of MTOC by anti-alpha-tubulin Ab in Jurkat cells transfected with TCR-zeta-GFP and mRFP-Rab35. The single large amorphous colocalization of TCR and Rab35 is in immediate proximity to the MTOC. Arrow highlights a discrete vesicle close to that region with colocalized TCR-zeta and Rab35. Bar 2µm.



Supplemental Figure 5. Z-series showing that Rab35 colocalizes with TCRz. Confocal images of Jurkat cells co-transfected with RFP- Rab35wt (red) and CD3-zeta-GFP (green) constructs. From bottom (1.2 µm) to top (7.6 µm), five serial sections through cells co-transfected with Rab35wt and CD3-zeta-GFP were analyzed. Colocalization is seen in the pericentrosomal region (arrow) and in isolated vesicles (arrowheads). Bar 2µm.



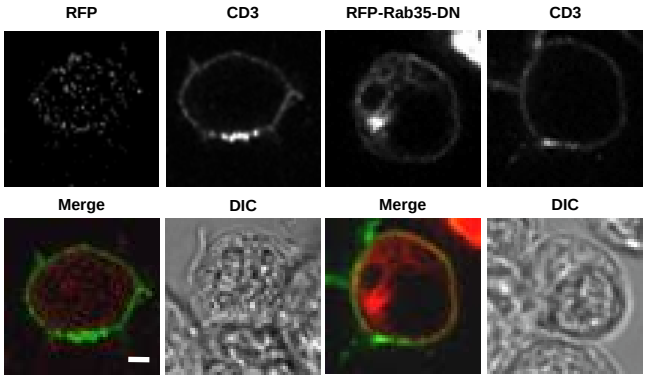
Supplemental Figure 6. EPI64C and Rab35 does not alter surface molecule expression in Jurkat. Jurkat cells transfected with empty vector (GFP), GFP-EPI64c (EPI64C) or GFP-Rab35 DN (rab35 DN) were analyzed by FACS for surface expression of transmembrane proteins 24 and 72h post-transfection. Cells were gated based on GFP expression and the analysis was done comparing MFI in GFP-positive and GFP-negative cells from the same tube and between constructs. Notably the level of surface CD3 expression is not significantly altered by expression of EPI64C or Rab35DN



Supplemental Figure 7. Overexpression of EPI64C and Rab35 S22N does not impair T cell adhesion to FN. Jurkat cells were transfected and analyzed for adhesion to fibronectin 14-18h after transfection. A) Fluorescence histograms the transfected cell populations. B) Comparison of adhesion of GFP-positive vs GFP-negative cells in the same culture of electroporated cells. For each construct adhesion was measured without stimulation or after 10 min PMA treatment.

Methods: Adhesion was measured by flow cytometric comparison of the adherent cells with the total cells as generally as previously described (Ref 1). The only differences were that Jurkat was utilized instead of T cell blasts, 6 wells were pooled per sample instead of 4 and the samples were resuspended in a final volume of 275µl. Each sample was analyzed on the flow cytometer, acquiring at least 30,000 total events**.** Using the gates shown in the corresponding histograms, the percent of adhesion of the GFP+ population (solid bars) was calculated and compared with the percent of adhesion of the electroporated cells that lacked GFP expression (white bars). Results shown are representative of 3 independent experiments

[1]Chan AS, Mobley JL, Fields GB, Shimizu Y. 1997. CD7-mediated regulation of integrin adhesiveness on human T cells involves tyrosine phosphorylation-dependent activation of phosphatidylinositol 3-kinase. J Immunol 159:934-942 (PMID: 9218614)



Supplemental Figure 8. Example of analysis of surface CD3 localization in conjugates. Staining for surface TCR in representative fixed non-permeabilized conjugates between Jurkat cells (transfected with RFP or RFP-Rab35DN) and Raji cells in the presence of superantigen SEE. Bar 2µm.