the internalization of the receptor almost perfectly correlated with the internalization of the ligand, indicating an interdependence of both processes (Fig. 4B). However, as the incubation continued, the correlation coefficient decreased significantly (data not shown), indicating that internalization proceeded very quickly within the first 7-15 min and was residual afterwards. Furthermore, when the co-localization score of the ligand and the receptor was assessed, we observed that it was maximal at the baseline (t=0 min) and very quickly decreased once both the ligand and the receptor internalized (Fig. 4C), indicating that ligand and receptor dissociate in the endocytic route at a certain time-point. We also assessed the amount of ligand and receptor left at the end of the experiment and observed that although the receptor signal decayed to approximately 50-60% of the starting amount of material, in the case of the ligand it was below 20% (Fig. 4D), indicating that the ligand is processed at a fast ratio and that part of the receptor is targeted for degradation.

**Figure 4.** DC-SIGN and its cargo quickly dissociate upon internalization. (A) Time-course of the median internalization score of DCs triggered with AF405-labeled-AZN-D1 and stained intracellularly against DC-SIGN (n > 5000). (B) Scatter plot of the internalization scores of both ligand and receptor 7.5 min after triggering with AF405-labeled-AZN-D1 (n > 5000). (C) Time-course of the median co-localization of AF405-labeled-AZN-D1 and DC-SIGN (n > 5000). (D) Time-course of the fluorescence signal intensity of both AF405-labeled-AZN-D1 and DC-SIGN (n > 5000).

**DC-SIGN directs its cargo through the classical endocytic route and to the ER**

In order to investigate the fate of both ligand and receptor, we measured the co-localization scores of both ligand (AZN-D1) and receptor with several antibodies commonly used to track endocytic compartments. We first validated the assay by comparing the colocalization scores obtained by imaging flow cytometry with CLSM images after one hour of triggering with AZN-D1 and found an excellent correlation between both techniques (Fig. 5A). From these data we could conclude that upon
Figure 5. Intracellular routing of DC-SIGN and its ligand. (A) CSLM images showing a representative example (n = 10 cells) of DCs incubated with AF405-labeled-AZN-D1 (pseudo-colored in red) for 30 min at 37 °C and intracellular stainings against EEA1, LAMP1, HLA-DM, PDI, rab5, rab7, rab11 and TGN46 (pseudo-colored in green). Below each marker column, the percentage of co-localization in both CSLM and imaging flow cytometry are shown. (B) Time-course of the co-localization scores of AF405-labeled-AZN-D1 or DC-SIGN with EEA1, LAMP1, HLA-DM, PDI, rab5, rab7, rab11 and TGN46 (n > 5000). Means ± SEM
internalization AZN-D1 shows a good colocalization with EEA1 and LAMP (early endosomes and lysosomes, respectively), a moderate colocalization with rab7 (late endosomes), rab11 (slow recycling endosomes), HLA-DM (MHC class II compartment) and PDI (ER), and a poor colocalization with TGN46 (trans-Golgi network), and rab5 (endocytic vesicles). These data indicate that several internalization routes are followed upon internalization, targeting classical DC endocytic compartments, such as the early and late lysosomes, necessary for proper antigen-processing prior to MHC class II presentation, but also other compartments that may be involved in cross-presentation (ER). Interestingly, transport to the ER appears to be independent of retrograde transport through the trans-Golgi network. Other endosomal compartments (rab7+ and rab11+) appeared to be involved as well, probably as intermediate or storage compartments.

We then studied the co-localization scores of these markers with either the ligand (AZN-D1) or the receptor. For the detection of the receptor we either used our rabbit polyclonal anti-DC-SIGN antibody (CSRD) or an antibody against the stalk region (DC-28). Both antibodies detect epitopes that are far apart from the ligand-binding site and therefore do not interfere with the binding of AZN-D1 to its target on DC-SIGN (Fig. 2B). Both ligand and receptor appeared to co-localize evenly with the early endosomal marker EEA1 until approximately 30 min, then colocalization for the receptor decreases dramatically and co-localization with the ligand continues but slowly decays. These results indicate that the receptor accompanies its cargo to early endosomes, dissociates from it and leaves the early endosome. This is supported by the LAMP1 co-localization scores, which show that the ligand reaches the lysosomal compartment, but not the receptor. Also in accordance to this, the MHC class II compartment shows a good co-localization with the ligand but not with the receptor. On the contrary, both ligand and receptor show a moderate level of co-localization with the ER, indicating that transport to this compartment may be mediated by a DC-SIGN-dependent mechanism directly after passage by early endosomes. Interestingly, both rab7 and rab11 show a moderate co-localization with the ligand but a poor co-localization with the receptor, suggesting that routing to these compartments is receptor-independent and may occur after a stay at the early endosomes or the lysosomes. The decay observed for the receptor in Fig. 4D can hardly be explained by lysosomal degradation, since there is very little co-localization of the receptor with the lysosomal marker LAMP1, whereas this is a very likely candidate for the signal degradation observed for the ligand. Since receptor degradation could also occur in the early endosomes, it could be possible that upon internalization to the early endosome, part of the receptor is targeted for destruction by a yet uncharacterized mechanism, while part continues routing to the ER together with the ligand in order to mediate cross-presentation. The fraction of ligand that dissociates from the receptor in the early endosome is then transferred to the lysosomes, as well as to rab7+ and rab11+ compartments.
Figure 6. DC-SIGN is located in transport vesicles, endosomes and ER after internalization. Example electron micrographs of dendritic cells after DC-sign antibody uptake, labeled by anti-mouse-10 nm gold. Gold label was observed at (A) the plasma membrane and omega-shaped pits, (B) small transport vesicles, (C-E) multivesicular endosomes and (F) ER (arrows indicate labeling of transport vesicles, arrowheads indicate labeling of ER; Endosomes are indicated with “e”). All scale bars represent 200 nm.
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The findings obtained by imaging flow cytometry and CLSM were confirmed by immuno-electron microscopy (Fig. 6). At early time-points it was possible to observe gold label still at the membrane and associated to omega-shaped pits, indicating active receptor-mediated internalization still occurring (Fig 6A). Gold label was also clearly observed on small transport vesicles (Fig. 6B), multivesicular bodies (Fig. 6C-E), endosomes (Fig. 6F) and the ER (Fig. 6F).

These data demonstrate an internalization model in which DC-SIGN mediates the internalization of the cargo into early endosomes where the receptor-ligand complex dissociates. Released cargo continues its way to lysosomes by the maturation of early endosomes, while a fraction of receptor-ligand complexes translocate by a yet uncharacterized mechanism to the ER.

![Image of graphs and data](image_url)

**Fig 7.** The internalization and routing of DC-SIGN is mediated by its dileucine motif. (A) Time-course of the fluorescence signal intensity of AF405-labeled-AZN-D1 in the different DC-SIGN-transduced cell lines. (B) Co-localization scores of AF405-labeled-AZN-D1 with EEA1 at either 60 or 180 min after triggering. (C) Co-localization scores of AF405-labeled-AZN-D1 with LAMP1 180 min after triggering. (D) Co-localization scores of AF405-labeled-AZN-D1 with PDI 180 min after triggering. (E) Co-localization scores of AF405-labeled-AZN-D1 with rab11 180 min after triggering. Means ± SEM (n > 5000). *, p < 0.01 compared to WT cells.