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Reply to "Can HIV-1 Entry Sites Be Deduced by Comparing Bulk Endocytosis to Functional Readouts for Viral Fusion?"

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With this letter, we would like to respond to the letter by Marin and Melikyan (1) critiquing our recently published work on the contribution of endocytotic uptake to productive HIV-1 infection of T-cell lines and primary T cells (2). On the basis of the observation that these cell types displayed CD4- and Env-mediated HIV-1 particle endocytosis, we investigated the contribution of this pathway to productive infection by overexpression of dominant-negative dynamin and temperature shift experiments. Our analyses revealed that endocytosis did not contribute to cytoplasmatic entry and infection in primary $CD4^+$ T cells, SupT1-R5, and CEM-ss cells. These findings disagree with some conclusions drawn by Miyauchi and coworkers (3). These authors had reported that HIV-1 cell entry occurs exclusively via endocytosis, whereas entry at the plasma membrane does not proceed beyond the hemifusion stage (3).

A response from this group to our recent publication is thus not unexpected. We would like to emphasize, however, that our study did not aim at either validating or refuting published data from other groups. We followed up on our own published work, which had shown that endocytosis contributes to productive HIV-1 entry in HeLa cell-derived model cell lines (4). Our recently published study (2) was aimed at extending this to more physiologically relevant cell types, initially expecting a significant contribution also in these cells.

A major critique by Marin and Melikyan (1) concerns the use of "bulk assays," which they consider outdated compared to analysis of single particle events. On the basis of this assessment, they argue that detecting endosomal HIV-1 particles may not faithfully report productive viral entry. We agree with this conclusion (see below) and actually argue this way in our report, but we disagree with the further arguments and conclusions by Marin and Melikyan. First, it is not appropriate to generally disqualify ensemble measurements as an "outdated" strategy. The choice of experimental method must be guided by the specific scientific problem and not by the age of the method. Considering the example in question, single-virus tracking (SVT) is optimal for studying intracellular localization and kinetics of membrane fusion events but does not allow determining productive infection. The contribution of endocytosis (or plasma membrane fusion) to productive HIV-1 infection of T cells was the topic of our study, however, and SVT cannot answer this question. It is currently not possible to monitor entering HIV-1 particles at least up to the stage of integration in live cells, and correlation of individual fusion events with productive infection of the cell in which the fusion event was detected is therefore technically not feasible at the moment. When considering productive infection, the smallest possible experimental entity is a single cell, and this is what we employed in our study. The statement on bulk assays by Marin and Melikyan appears to mainly refer to the detection of endocytosed HIV-1 particles, but our study actually showed that dominant-negative dynamin blocked endocytosis (i.e., no endosomal particles), while not affecting productive HIV-1 infection; this result clearly shows that endosomal HIV-1 uptake can be nonproductive (as stated by Marin and Melikyan), and our results indicate that this is the case in T-cell lines and primary human T cells.

Furthermore, we consider our findings to be less discrepant to the data shown in the reports from the Melikyan lab than may be assumed from the conclusions. Miyauchi and colleagues (see Fig. 1A in reference 3) showed that endocytosis contributes significantly to HIV-1 fusion in HeLa-derived cell lines by comparing the kinetics of blocking fusion with a 4°C temperature block and a membrane-impermeant fusion inhibitor. This result is in accordance with our prior report analyzing productive HIV-1 entry in HeLa cell-based cell lines (4). A more recent study by de la Vega et al. (5) of the Melikyan research group using the same assay as Miyauchi et al. (3) analyzed the contribution of HIV-1 endocytosis in different cell types. These authors reproduced the results for HeLa-derived cell lines (see Fig. 2A in reference 5). For primary $CD4^+$ T cells (see Fig. 2F in reference 5), however, there was only a marginal—if any—difference between the fusion kinetics of the temperature block compared to the fusion inhibitor block. We interpret this observation to indicate that endocytosis is dispensable for HIV-1 fusion and infection in primary T cells, which is consistent with our data. This leaves only the results for CEM-ss cells, which cannot be easily reconciled: for this cell type, Melikyan and colleagues reported a role of endocytosis for productive HIV-1 fusion, while we do not observe a contribution of endocytosis to productive infection. We cannot explain the reason for this specific discrepancy, but the results from several different approaches and applying different T-cell lines and primary T cells were consistent in our study, supporting the conclusions drawn in our article. Independent of this issue, we consider the results for primary human T cells to be most relevant, and we interpret the data by Melikyan and colleagues to be consistent with our conclusions in this case.

A further concern by Marin and Melikyan referred to possible detrimental alterations of endocytotic processes by lowering the temperature to 22°C. Although this cannot be formally excluded, we did not obtain any indication that endocytosis was altered other than kinetically. Microscopic analyses of both transferrin uptake and HIV-1 endocytosis did not reveal differences with respect to the amount of endocytosed cargo and the subcellular distribution. Fur-

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thermore, blocking endocytosis with dominant-negative dynamin-2 yielded the same conclusions as those of the temperature block experiments.

We thus conclude that endocytosis does not contribute significantly to HIV-1 infection of primary T cells or T-cell lines under the experimental conditions used. Our study compared different T cells and HIV-1 Env variants (including Env from primary isolates) yielding similar results, but we cannot exclude a contribution of endocytosis to productive HIV-1 infection in other cell types (as already observed for model cell lines), for other Env derivatives, or in the case of cell-to-cell spread. This is explicitly stated in our discussion, and we have refrained from an absolute statement regarding the exclusive pathway of HIV-1 cell entry. In contrast, Miyauchi and colleagues made the bold conclusion that HIV-1 entry occurs exclusively from endosomes (3), and our findings clearly contradict this statement.

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