Reviewer's report

Title: Doxorubicin-enriched, ALDHbr mouse breast cancer stem cells are sensitive to oncolytic herpes simplex virus type 1

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Reviewer: Samuel Rabkin

Reviewer's report:

This manuscript describes the isolation of cancer stem cells (sorting for ALDHbr cells) from the mouse 4T1 cell line. A number of papers have already described isolating 4T1 cancer stem cells. These cells are then used to test infectivity with oncolytic HSV and sensitivity to doxorubicin. Unfortunately, the actual sensitivity of the ALDHbr and lo cells to killing by doxorubicin or oncolytic HSV is never tested. The change in the % of ALDHbr cells after doxorubicin, and not oncolytic HSV treatment in vitro is not recapitulated in vivo for unknown reasons. While the manuscript focuses on cancer stem cells, the tumor model is generated from unselected, classically cultured 4T1 cells and it is not clear how or whether cancer stem cells in the tumor play any role in the efficacy seen with the combination treatment of doxorubicin and oncolytic HSV.

Major compulsory revisions:

1. In Fig 1C, is this the total number of mammospheres in a plate with 105 cells? If so the efficiency is exceeding low. If not, what is the mammosphere efficiency (spheres/cells plated)? It is described as ± SEM, yet there are no error bars in the figure. How can SEM be determined from duplicate wells?

2. In Fig 3C, the ALDHlo cells seem to be less well infected than the ALDHbr cells (ie., MOI=0.1), as opposed to similar, is this reproducible or due to image selection? What is the actual infectivity of ALDHbr and lo cells (%GFP-positive cells)? Previous studies with oncolytic HSV and 4T1 suggest that it is poorly permissive to HSV replication.

3. The in vitro experiments (pg 15) don't measure cytotoxicity, so it is not possible to conclude whether oncolytic HSV kills any cells (unlikely at this early time point). What is the cytotoxicity of oncolytic HSV in the 4T1 cells (ALDHbr or lo)? How was the dose of doxorubicin selected and what is the cytotoxicity (survival/proliferation assay) of this dose in ALDHbr and lo cells?

4. The explanation for the high %ALDHbr in Fig 5B (pg 17) doesn't make sense as the tumors were harvested at day 11, closer to 1 week than to 3 weeks, and still much higher than in Fig 5C. What was the size of the tumors harvested in Fig 5B? OV treatment reduced the %ALDHbr compared to DOX, yet the tumor sizes look the same in Fig 6A. Was there a difference in the DOX or OV sensitivity of ALDHbr cells isolated from tumors versus in culture? Oncolytic HSV has been reported to induce a robust anti-tumor immune response in the 4T1 model, could
this account for the effect on ALDHbr cells?

5. There are a number of papers describing oncolytic HSV treatment of 4T1 tumors, especially from the lab of X. Zhang. The efficacy of HSV1-hGM-CSF should be discussed in comparison to these other papers, including ref 25.

Minor Essential Revisions:
1. A description of the oncolytic HSVs should be provided, including their mutations and transgene structure. The reference listed (33) is not in PubMed, in English, nor full text available and the title indicates HSV-2 not HSV-1.

2. This is not really a metastatic breast cancer model (pg 10 title), but a subcutaneous implant model.

3. There have been a couple of recent papers that describe 4T1 cancer stem cells (Matilainen, H et al, ’12), including with aldefluor (Park SJ et al, ’11), which should be referenced.

4. Describe in Fig 1A legend what the 2 left panels represent. What percent of cells are ALDHbr?

5. "GFP was detected in cells of both the outer and inner spheres" (pg 14). There is no way to know whether the fluorescence is coming from inner or outer cells without confocal microscopy or sectioning. Therefore this conclusion is not supported by the data. At an MOI=1, the number of infected cells looks very small, so that the mammospheres are not "effectively" infected.

6. Fig 5A legend does not indicate what the upper panels (red) are and the labeling in the figure is small and not very clear. The percent of CD45- cells in each of the groups should be indicated on the figure or in the figure legend.

7. Why was OV administered after DOX (Fig 6)?

8. The title is misleading and should be changed, "doxorubicin-enriched, ALDHbr mouse breast cancer cells" were never examined and the "sensitivity" only relates to infection and not killing.

9. The supplemental material is not referenced in the text and therefore is not really supplemental but additional. Why is it not included in the manuscript?

Discretionaly Revisions:
1. It is not clear that the differences reported between ALDHbr and lo cells in vitro are related to the results in vivo. Even if OV does kill ALDHbr cells, it is not sufficient to cure the mice. Is there a difference in tumors generated from ALDHbr cells (Table 1) versus unsorted 4T1 cells (as in Fig 5, 6)?

Level of interest: An article of importance in its field

Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:
I declare that I have no competing interests.