**Checkpoint inhibitor administered either systemically or subcutaneously enhances the anti-tumor immunity of SD-101 delivered by PEDD device in treating Liver Metastasis**

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Word Count: 385/400

**Background:** The success of checkpoint inhibitors (CPIs) in treating liver tumors is limited in part due to the distinctive biology of intrahepatic myeloid-derived suppressive cells (MDSCs). We have reported in a phase 1 trial that infusion of SD-101, a class C TLR9 receptor agonist, by PEDD in combination with systemic CPI promotes MDSC depletion and broad immune stimulation, in association with encouraging clinical outcomes. Delivery of SD-101 via PEDD for liver tumors is intended to improve responsiveness to a systemic CPI. Currently, CPIs are delivered intravenously and there is growing interest in subcutaneous (SQ) administration. We compared SD-101 via PEDD with systemic or SQ CPI delivery in a murine model of liver metastases (LM).

**Methods:** LM model was developed by injecting MC38-Luc cells via the spleen of 8-12 weeks old male C57/BL6 mice followed by splenectomy. After a week, fluorescently labelled SD-101 (10μg/mouse) was delivered via PEDD with anti-PD-1 delivered either via SQ or intraperitoneally (Sys). Tumor burden was monitored by bioluminescence. Serum cytokine levels were analyzed by Luminex. Tissues were harvested on D3 or D10 to isolate CD45+ cells, with RNA subjected to Nanostring analysis (D3), and flow cytometry (FC) to interrogate immune cell populations (D10). For Nanostring analysis, the innate immune panels were selected and for FC, MDSCs (CD11b+Gr1+), B cells (B220+), T (CD3+) cells and M1 (F4/80+CD38+Egr2+) macrophages were quantified.

**Results:** SD-101 via PEDD in combination with either SQ or Sys anti-PD-1 antibody delivered significantly and equivalently reduced LM progression (Figure 1). Moreover, reduction of MDSCs with increase in B, T and M1 macrophages within the LM were observed, irrespective of CPI route (Table 1). IFNγ (p<0.05) and IP10 (p<0.01) significantly increased in the circulation of mice that received SD-101 as compared to the vehicle control. Nanostring analysis revealed that monotherapy and combination treatment inhibited myeloid cells differentiation and maintenance, angiogenesis, and increased cytokine, lymphocyte activation and TLR signaling pathways. The combination of SD-101 and CPI enhanced the survival of mice as compared to monotherapy or Veh control, irrespective of delivery route.

**Conclusion:** SD-101 administered via PEDD in the murine model promoted potentially favorable immune changes in LM, and addition of CPI improve tumor control, along with survival times. The SQ route of CPI delivery was equivalent to Sys in the murine model, suggesting the SQ CPI may be a rational choice for combination with PEDD of SD-101 for liver tumors.
### Table 1:

<table>
<thead>
<tr>
<th>Cell Types (%)</th>
<th>Treatment, p value vs. Veh</th>
<th>Veh</th>
<th>SD-101</th>
<th>SQ (SD-101+ anti-PD-1)</th>
<th>Sys (SD-101+ anti-PD-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDSC (CD11b+Gr1+)</td>
<td></td>
<td>25.95±4.1</td>
<td>15.67±2.6, &lt;0.01</td>
<td>8.29±1.4, &lt;0.01</td>
<td>10.88±2.2, &lt;0.01</td>
</tr>
<tr>
<td>B (B220)</td>
<td></td>
<td>8.9±1.6</td>
<td>17.16±1.3, &lt;0.05</td>
<td>17.86±1.8, &lt;0.001</td>
<td>17.44±1.3, &lt;0.01</td>
</tr>
<tr>
<td>T cells (CD3)</td>
<td></td>
<td>6.35±1.2</td>
<td>11.03±1.2, &lt;0.05</td>
<td>13.27±2.6, &lt;0.05</td>
<td>13.92±2.06, &lt;0.05</td>
</tr>
<tr>
<td>M1 Macrophages</td>
<td></td>
<td>12.78±4.34</td>
<td>30.34±5.6, &lt;0.05</td>
<td>28.01±2.7, &lt;0.05</td>
<td>29.83±14.9, &lt;0.05</td>
</tr>
</tbody>
</table>

### Figure 1: Tumor Progression

![Tumor Progression Graph](chart.png)

- *p<0.05
- *p<0.01
- **p<0.001

2-way ANOVA followed by Tukey’s post hoc test:

- Veh vs SD-101 groups:
- SQ vs SD-101 groups:
- Sys vs SD-101 groups:

All p values are for respective data points versus control.