Differences in *In Vivo* and *In Vitro* cDC1 Maturation After Stimulation with Different TLR Agonists

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Background

- Dendritic cells (DCs) are efficient antigen-presenting cells and mediate adaptive immune responses
- *In vivo* type 1 conventional dendritic cells (cDC1s) are found throughout the body, eliciting immune functions important for immunity against cancer and other foreign antigens
- The Watowich lab has pioneered an *in vitro* cDC1 culturing method that generates high cell yields utilizing Granulocyte-macrophage colony-stimulating factor (GM-CSF) and FMS-like tyrosine kinase 3 ligand (Flt3L) cytokines to induce CD103$^+$ cDC1 maturation, which has been shown to elicit antitumor T cell immunity (Chrisikos)
    - GM-CSF is known to induce production of cDC1s when included as part of *in vitro* cultures and limits production of other DC subsets when combined with Flt3 Ligand in culture (Chrisikos)
- Toll-like Receptors (TLRs) mediate signaling pathways for the maturation of immune cells (Chrisikos), and have differing effects on DC maturation and antigen presentation
    - TLR3 is expressed on cDC1s (Chrisikos) and is known to be activated by the ligand Polyinosinic:polycytidylic acid (Poly I:C) (Chrisikos)
    - Imiquimod is an agonist for TLR 7/8, and is not known to have high expression levels on cDC1s (Barut)
- It is unknown whether these cDC1 populations mature and behave similarly when stimulated with different TLR agonists as they are generated in different conditions

Hypothesis

We hypothesize that *in vivo* and *in vitro* cDC1s will mature with similar kinetics and overall activation when stimulated with Poly I:C, a TLR3 agonist, and have minimal activation when stimulated with Imiquimod, a TLR7/8 agonist.

Methods

Generation of *In Vivo* cDC1s and Enrichment

8 days prior to purifying the *in vivo* cDC1 cells, we enriched the cDC1 population by performing hydrodynamic gene transfer (HGT), where we inject plasmid encoding Flt3L into the mice within 2 ml volume of phosphate buffer saline (PBS), After 8 days, we harvested the splenocytes and enriched the sample for DCs using magnetic beads to carry out a negative selection by using anti-CD19 (B cells), anti-Ter119 (erythroid cells) and anti-NK1.1 (NK Cells) and running the sample through a magnetic column.

Generation of *In Vitro* cDC1s

16-21 days prior to purifying the *in vitro* cDC1s, we harvested bone marrow from mice and utilized the Watowich lab's method of culturing *in vitro* cDC1s. The bone marrow was harvested from the femur and tibia of mice. Cells were plated at 1x10^6 cells/ml in complete media with 50 ng/mL of Flt3L and 2 ng/mL of GM-CSF. At day 5 post-harvest, cells were supplemented with additional complete media, and at day 9 post-harvest the cells were expanded by re-plating cells at 3x10^5 cells/mL with 50 ng/mL of Flt3 Ligand and 2 ng/mL of GM-CSF for an additional 6-10 days.

TLR Agonist Stimulation

We plated untreated cDC1s, cDC1s + Poly I:C, cDC1s + Imiquimod, and cDC1s + combination treatment for each cDC1 subset for 16, 20, and 24 hours. 100 uL of cells were plated in complete media at 5x10^5 cells/ml with 2 ng/mL GM-CSF and 50 ng/mL Flt3L. Poly-I:C treated samples were plated at 20 ug/mL and Imiquimod was plated at 1 ug/mL.

Methods (continued)

Flow Cytometry/FACS

We used fluorescence activated cell sorting (FACS) to purify cDC1 populations *in vivo* and *in vitro*. To measure maturation, we stained for MHC I and MHC II (Rock). We also stained for costimulatory molecules CD40, CD80, and CD86 involved in T cell activation (Elgueta; Slavik). Samples were run on the Fortessa LSR X20 flow cytometer for analysis.

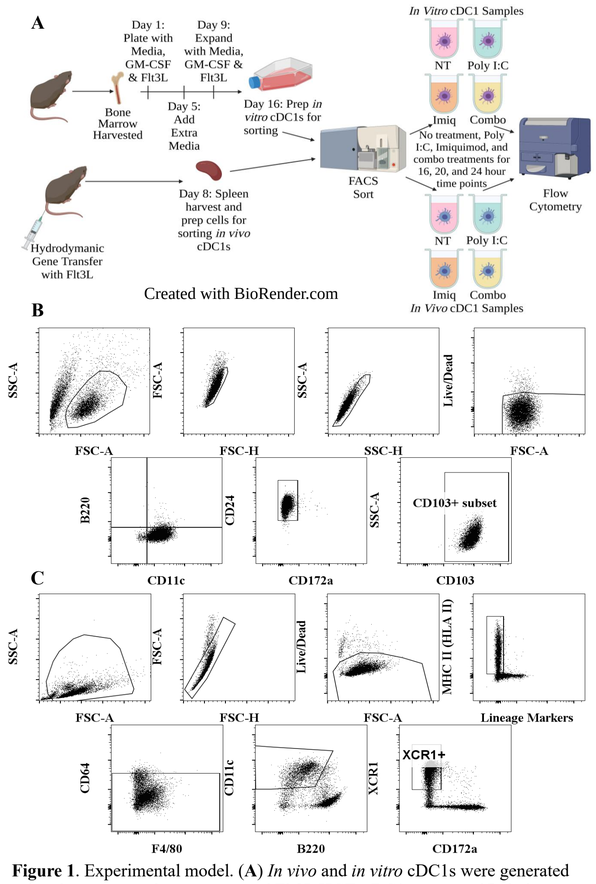

Figure 1. Experimental model. (**A**) *In vivo* and *in vitro* cDC1s were generated using the *in vitro* culture system and Flt3L HGT. cDC1s were purified via FACS sorting and stimulated with TLR agonists for 16, 20 and 24 hours. Cells were stained for costimulatory markers and analyzed via flow cytometry. (**B**) *In vitro* and (**C**) *in vivo* cDC1 purification gating strategy.

Results

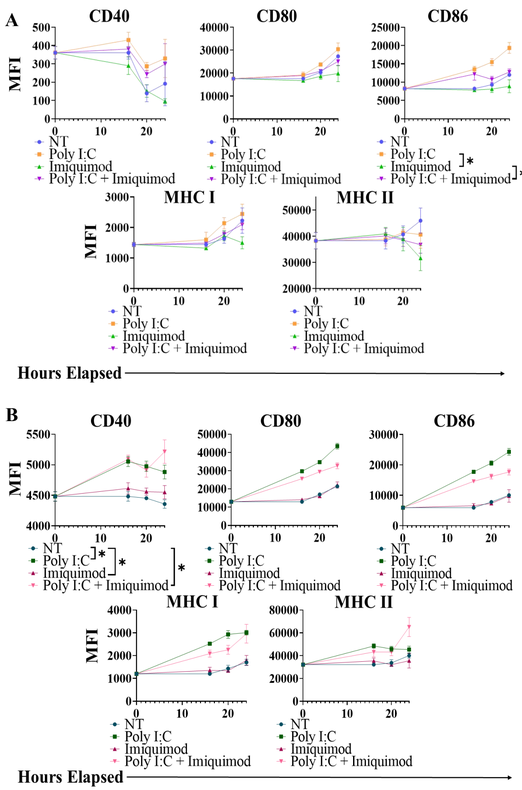

Figure 2. Observable differences in cDC1 subset maturation are highest 24-hours post TLR agonist stimulation. (**A-B**) Time course of CD40, CD80, CD86, MHC I, MHC II expression levels at timepoints 16, 20, and 24 hours treated with Poly I:C, Imiquimod, and Poly I:C + Imiquimod combination treatment. For (**A**) *in vitro* cDC1s and (**B**) *in vivo* cDC1s.

Both cell types showed the greatest upregulation of maturation markers 24 hours post-TLR stimulation. MHC I expression is most upregulated by Poly I:C and combination treatment in *in vivo* cDC1s but not upregulated in *in vitro* cDC1s. MHC II expression showed similar upregulation to MHC I for both cell types. CD80 expression for both cell types was upregulated by Poly I:C and combination treatment, however, upregulation of CD80 is significantly greater for *in vivo* cDC1s.

Results (Continued)

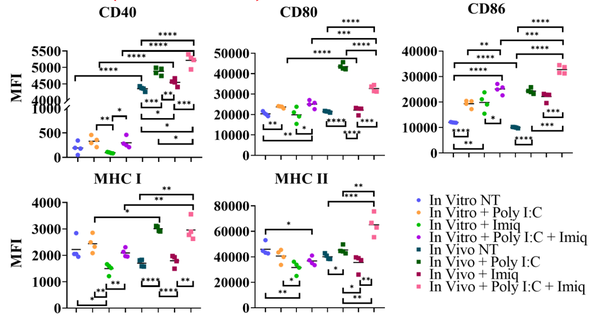

Figure 3. Differences in cDC1 subset activation at 24 hours post TLR agonist stimulation. CD40, CD80, CD86, MHC I, and MHC II maturation marker expression at 24 hours for non-treated, Poly I:C treated, Imiquimod treated, and combination treated *in vitro* and *in vivo* cDC1s.

Interestingly, basal CD86 expression was found to be greater for *in vitro* cDC1s compared to *in vivo* cDC1s. Both cell types upregulate CD86 expression upon stimulation with all the tested agonists. CD40 expression was not upregulated for *in vitro* cDC1s stimulated by TLR agonists, while both TLR agonists upregulated CD40 expression for *in vivo* cDC1s.

Conclusions

These data indicate differences in the maturation of *in vitro* and *in vivo* cDC1s over a 24-hour period in stimulated and non-treated conditions. *In vivo* cDC1s were more susceptible to maturation when stimulated with Poly I:C and Imiquimod, but typically have a lower basal level of maturation compared to *in vitro* cDC1s. These data provide valuable insights in how long-term immune responses may alter between cDC1 subtypes, with future work involving measuring differences in subsequent T cell responses and adaptive immune responses.

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