Effects of different TLR Agonists on *in vivo* cDC1 and *in vivo* cDC2 Activation

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Background

- Mature dendritic cells (DCs) contribute to the proinflammatory regulatory immune system response through antigen uptake, antigen presentation, cross-presentation, and T cell priming.
- Classical DC differentiation is largely promoted *in vivo* by the fms-like tyrosine kinase 3 ligand (Flt3L).
- Toll-like receptors (TLRs) including TLR3 and TLR7/8 activate multiple arms of the immune response and promote the activation of type 1 and type 2 classical dendritic cells (cDC1s and cDC2s, respectively).
- cDC1s are XCR1+ and are known to respond to TLR3 stimulation; cDC2s are CD172α+ and are known to respond to TLR7/8 stimulation.
- It is unknown to what extent cDC1s and cDC2s can be activated by unconventional TLR stimulation, and whether combinational treatment will alter their optimal activation state.
- Immune responses are mediated by costimulatory molecules CD40, CD80, CD86, which regulate antigen-specific T cell[1] responses, and MHC I and MHC II, which mediate antigen presentation[2], and can be upregulated in response to TLR stimulation .

Hypothesis

We hypothesize that the Poly I:C will more efficiently activate cDC1s, and Imiquimod more effectively activate cDC2s.
Additionally, combination treatment will lead to comparable levels of activation as individual agonist treatment.

Methods

Flt3L hydrodynamic gene transfer. Flt3L was injected into two mice via large volume tail vein injection to generate *in vivo* cDC1s and *in vivo* cDC2s. 8 days later, mice were euthanized by CO_2 asphyxiation and cervical dislocation, and splenocytes were harvested and prepared for cell sorting.

Fluorescence-activated cell sorting (FACS). Prior to the cell sort, the *in vivo* samples were enriched to remove B cells, NK cells and erythrocytes from the sample[4] using magnetic beads that negatively selected these populations. Cells were run through a magnetic column to remove CD19+ (B cells), NK1.1+ (NK cells), and Ter119+ (Erythrocytes) cells. Pure populations of cDC1s (CD11c+, CD103+, CD24-, CD172α-, B220-) and cDC2s (CD11c-, CD103-, CD24+, CD172α+, B220-) were sorted using FACS.

DC stimulation. The cDC1 population were cultured with complete media that was supplemented with hFlt3-L (50ng/ml) and mGM-CSF (2ng/ml)[5]. The cDC1s and cDC2s were then plated at 5x10^5 cells/ml and stimulated with Poly I:C (20ug/ml), Imiquimod (1 ug/ml), or a combination treatment of Poly I:C and Imiquimod for 16, 20 and 24 hours.

Methods (continued)

Flow Cytometry. DC costimulatory expression levels of CD40, CD80, CD86, MHC I, and MHC II were examined post-stimulation using an extracellular stain. The method of staining is similar to FACS sorting, but cells were fixed with 4% formaldehyde prior to analysis. Activation was quantified using flow cytometry using the X20 LSR Fortessa.

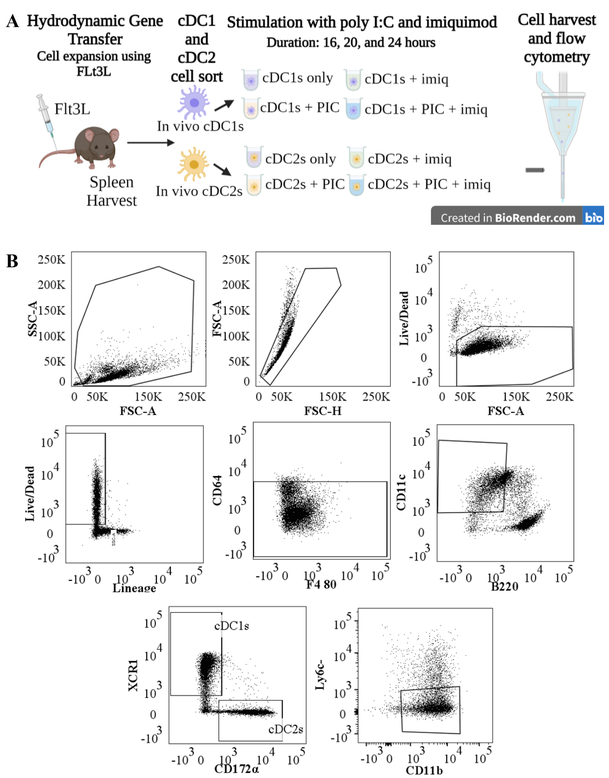

Figure 1. Experimental design. **(A)** cDC1s and cDC2s were generated *in vivo* by utilizing Flt3L hydrodynamic gene transfer via large volume tail vein injection. Splenic cells were harvested eight days later. DCs were sorted using fluorescence-activated cell sorting (FACS), then cDC1s and cDC2s were stimulated with Poly I:C, Imiquimod, or a combination of both agonists. After stimulation, DC costimulatory expression levels were measured at 16, 20, and 24 hours post-stimulation using flow cytometry. **(B)** Gating strategy for *in vivo* generated cDC1 and cDC2 purification.

Results

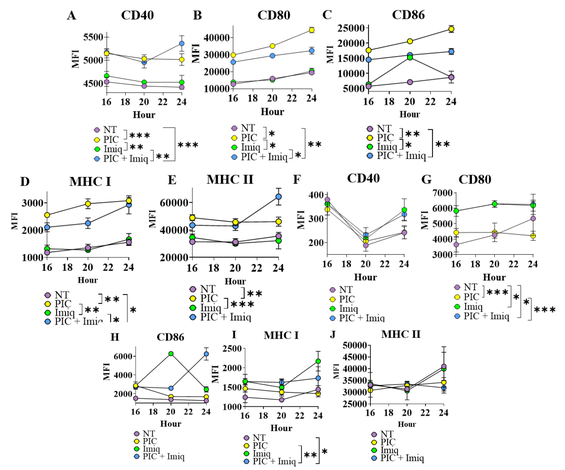

Figure 2. Observable cDC1 and cDC2 activation is highest 24hrs post-stimulation. **(A-J)** Expression of costimulatory molecules CD40, CD80, CD86, MHC I, and MHC II following stimulation with Poly I:C, Imiquimod, and the combination treatment at 16, 20, and 24 hours of **(A-E)** cDC1s and **(F-J)** cDC2s.

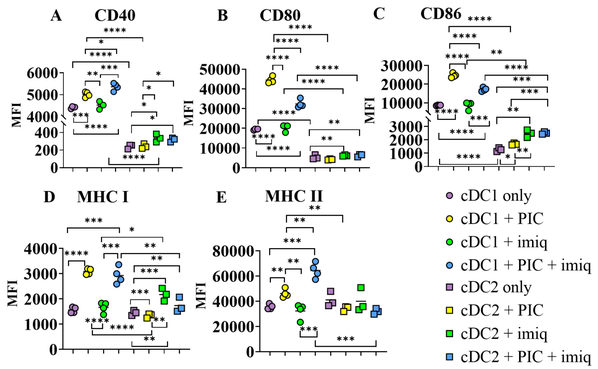

Figure 3. Differences in cDC1 and cDC2 activation states at 24 hours. **(A-E)** *in vivo* cDC1 and *in vivo* cDC2 expression of costimulatory molecules CD40 **(A)**, CD80 **(B)**, CD86 **(C)**, MHC I **(D)**, and MHC II **(E)** at 24 hours after stimulation with Poly I:C, Imiquimod, and the combination treatment.

Results (continued)

Our studies demonstrated that costimulatory expression increased over time for both cell types, and *in vivo* cDC1s and cDC2s were most activated at 24 hours post-stimulation. cDC1s expressed higher basal levels of costimulatory molecules compared to cDC2s. cDC1s were most activated after stimulation with Poly I:C, indicated by significantly higher expression of CD40, CD80, CD86, MHC I and MHC II after 24 hours, while Imiquimod failed to significantly increase cDC1 activation. Interestingly, combination treatment, for some maturation markers, showed lower activation compared to Poly I:C alone. Poly I:C did not increase cDC2 activation; However, Imiquimod or combination treatment significantly increased cDC2 activation, with upregulation of CD40, CD80, CD86 and MHC I at 24 hours post-stimulation.

Conclusions

This data demonstrates that cDC1s and cDC2s are largely activated 24 hours after TLR agonist stimulation. Poly I:C is most effective for promoting cDC1 activation, while the combination of Poly I:C and Imiquimod is most effective for activating cDC2s. Thus, it is ineffective to use Imiquimod to promote cDC1 activation, although there is a potential for Poly I:C, which was previously not believed to promote robust cDC2 activation, to increase cDC2 maturation in conjunction with Imiquimod. Further research would investigate the communication between T cells, cDC1s, and cDC2s after dendritic cell stimulation with Poly I:C and Imiquimod to elucidate the effects of different agonists on the quality of the immune response.

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