

# 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<sup>1</sup> responses, and MHC I and MHC II, which mediate antigen presentation<sup>2</sup>, 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

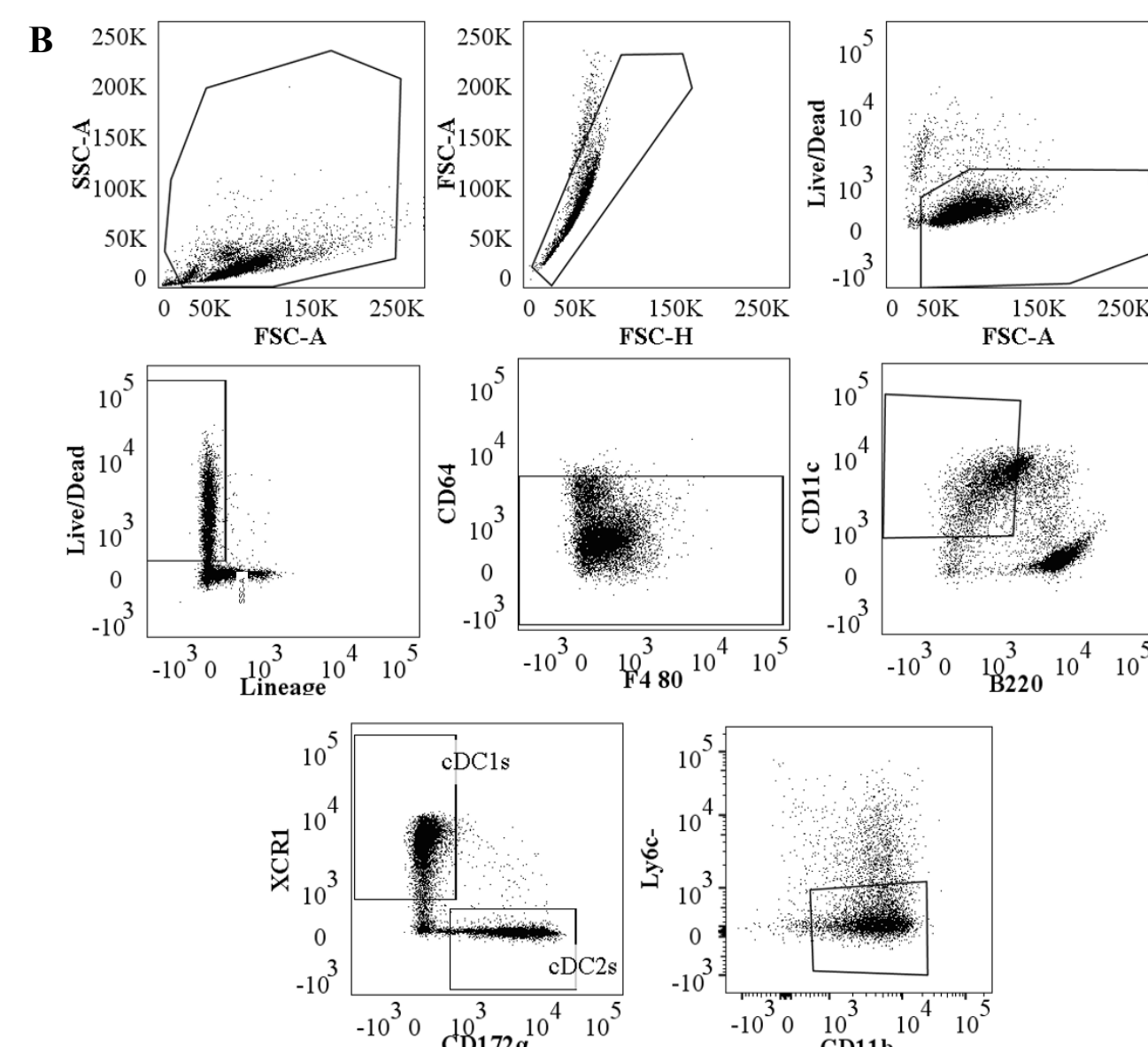
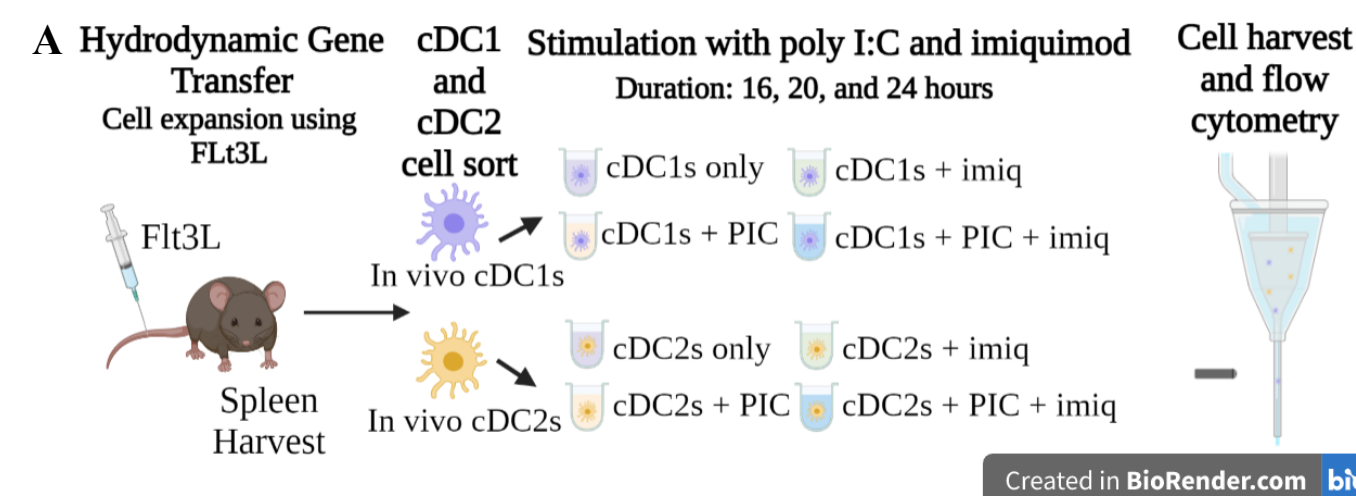
**Flt3L hydrodynamic gene transfer.** Flt3L was injected into two mice via large volume tail vein injection to generate *in vivo* cDC1s and *in vivo* cDC2s. 8 days later, mice were euthanized by CO<sub>2</sub> asphyxiation and cervical dislocation, and splenocytes were harvested and prepared for cell 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<sup>4</sup> 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)<sup>5</sup>. The cDC1s and cDC2s were then plated at 5x10<sup>5</sup> 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.

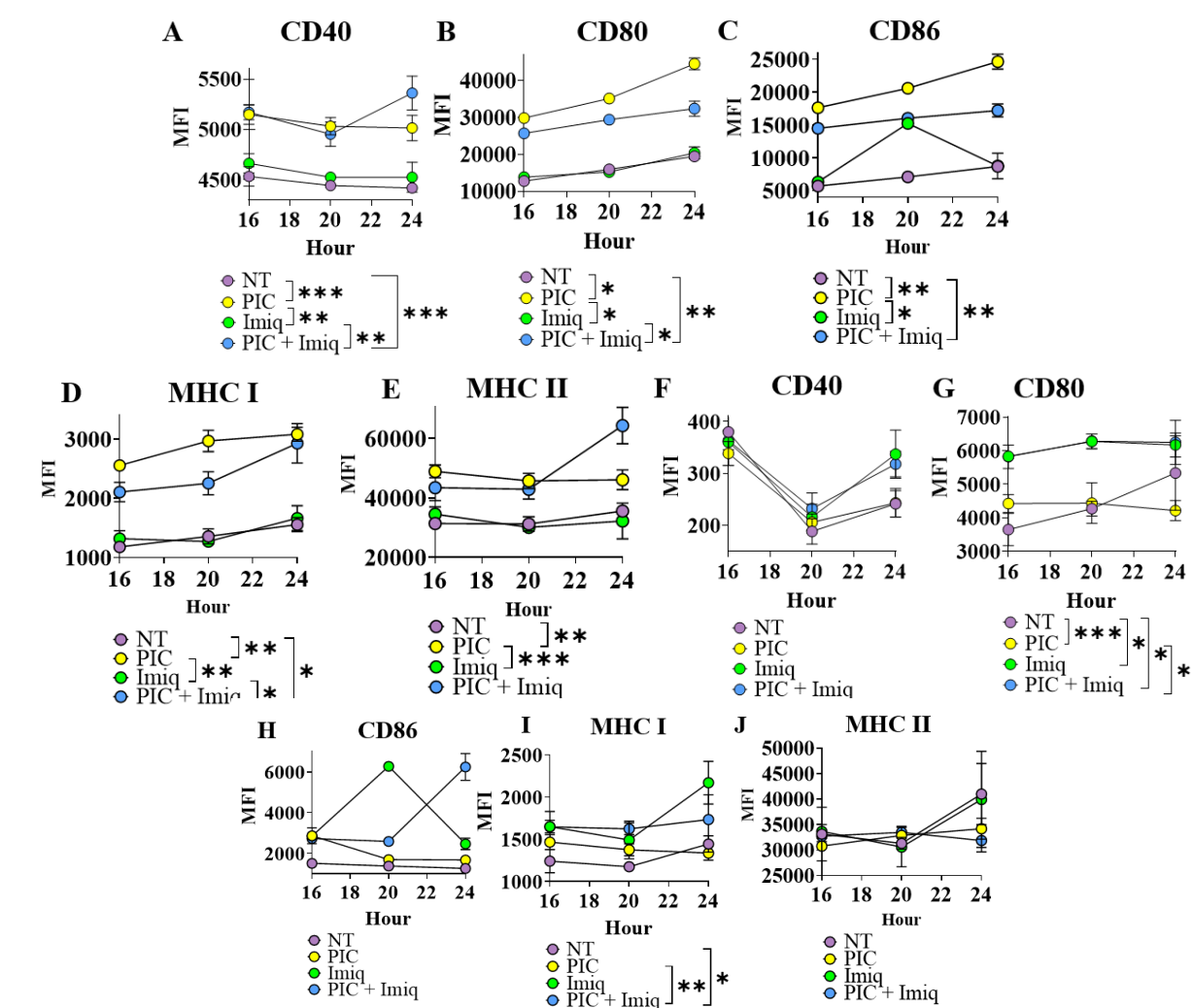
## Methods (continued)

**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.

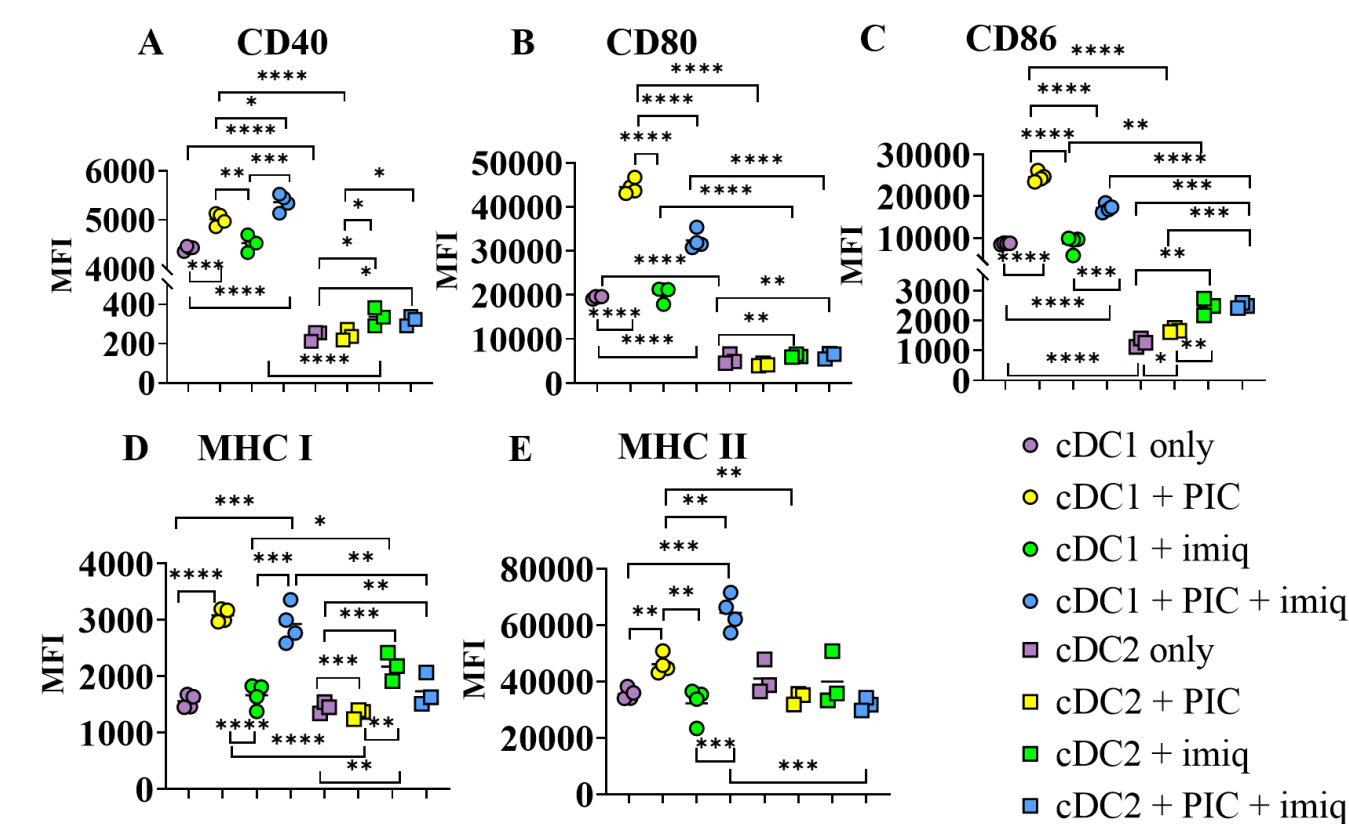


**Figure 1.** Experimental design. (A) cDC1s and cDC2s were generated *in vivo* by utilizing Flt3L hydrodynamic gene transfer via large volume tail vein injection. Splenic cells were harvested eight days later. DCs were sorted using fluorescence-activated cell sorting (FACS), then cDC1s and cDC2s were 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.

## References

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