



# Plasmacytoid Dendritic Cells Are Dispensable For Antiviral Immune Responses Following Cationic-Lipid-DNA-Complex (CLDC) Adjuvanted Influenza Vaccination



T. D. Giffon<sup>1</sup>, D. K. Hong<sup>1</sup>, L. Winestone<sup>1</sup>, J. Fairman<sup>2</sup>, and D. B. Lewis<sup>1</sup>  
<sup>1</sup>Dept. of Pediatrics and Program in Immunology, Stanford University School of Medicine, Palo Alto, CA, <sup>2</sup>Juvaris BioTherapeutics, Burlingame, CA

Thierry D. Giffon, Ph.D.  
 Department of Pediatrics, CCSR 2100  
 Stanford School of Medicine  
 269 Campus Drive, Palo Alto, CA 94043  
[tgiffon@stanford.edu](mailto:tgiffon@stanford.edu)  
 (650) 498-4596



## INTRODUCTION

Cationic-Lipid DNA complex (CLDC) is a novel adjuvant consisting of cationic liposomes combined with a non-coding DNA plasmid. Inactivated influenza A virus adjuvanted with CLDC elicits high levels of influenza-specific antibodies and T cells. The DNA component of this vaccine is a plasmid that lacks a cDNA segment and which stimulates leukocytes by both unmethylated CpG DNA-dependent and -independent mechanisms. CLDC administration *in vivo* induces high levels of cytokines that promote Th1 immunity (e.g., IL-12p70), type I interferons (IFN- $\alpha$ s and IFN- $\beta$ ), and IFN- $\gamma$ . At least part of this stimulation is likely to be dependent on triggering of Toll-like receptor (TLR)-9. Plasmacytoid dendritic cells (pDCs), which express particularly high levels of TLR-9, and conventional dendritic cells (cDCs) have been shown to interact after unmethylated CpG DNA administration to provide an optimal innate immune response, e.g., IL-12p70 production. pDCs, which are a major source of circulating type I IFNs after unmethylated CpG administration *in vivo*, have also been shown in some viral infection models to promote responses mediated by NK cells, cytotoxic T lymphocytes (CTL), Th1 cells, and B cells. Here we investigate the impact of pDC depletion on CLDC/influenza vaccine-induced adaptive immune responses.

## EXPERIMENTAL DESIGN

**Plasmacytoid Dendritic cell depletion:** C57BL/6 mice received 2 intraperitoneal (i.p.) injections of 100  $\mu$ g of rat anti-mouse PDCA-1 antibody (Miltenyi Biotec) or control rat IgG 24 hours apart.

**Flow cytometric detection of pDCs:** Whole blood, spleen and lymph nodes (pooled axillary, brachial and inguinal node tissue) were harvested from the 2 groups of mice. Single cells suspensions were prepared and stained in PBA (PBS, 1% BSA, and 0.1% sodium azide) with antibodies to PDCA-1 (Miltenyi Biotec), CD11c and CD45R/B220 (BD). Samples were read on a FACSCalibur (BD) and analyzed using FlowJo software (Tree Star, Inc.). Dead cells were excluded from the analysis with size scatter and 7-amino actinomycin D (AAD) exclusion.

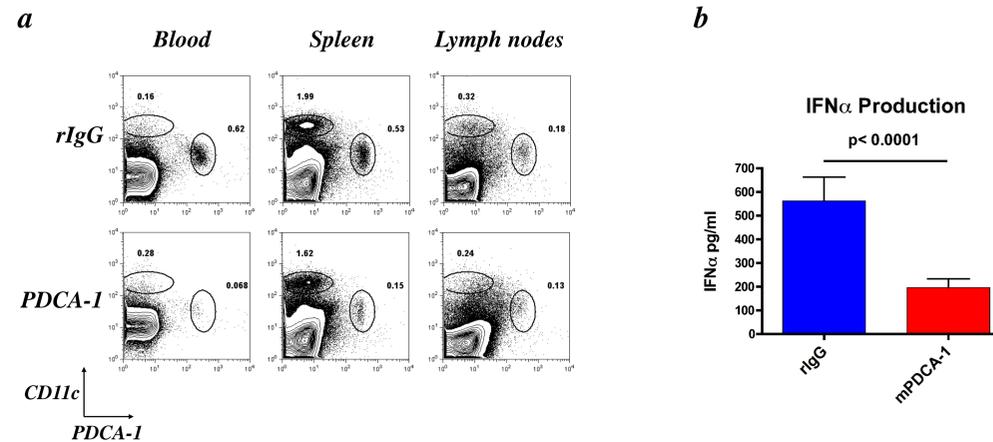
**Functional test of pDC depletion:** We injected anesthetized mice with 200  $\mu$ l containing 5.0  $\mu$ g of PS-CpG ODN1826 (5'-TCCATGACGTTCCCTGACGTT-3', Sigma-Genosys) complexed with the cationic lipid DOTIM (octadecenoyloxy[ethyl-2-heptadecenyl-3-hydroxyethyl] imidazolium chloride, Avanti Polar Lipids Inc.). 30  $\mu$ l DOTIM were mixed with 5.0  $\mu$ g ODN in 170  $\mu$ l 10% sucrose solution, incubated 20 min then injected intravenously in the tail vein. Blood samples were collected by tail clippings at 6 hours. The IFN $\alpha$  was quantified using an ELISA assay (PBL).

**Immunization:** Twenty four hours after the last injection of control or depleting antibodies, we injected i.p. 5.0  $\mu$ g of purified HKx31 influenza virus (Charles River) adjuvanted with CLDC. Tail vein blood samples were obtained at the indicated time points.

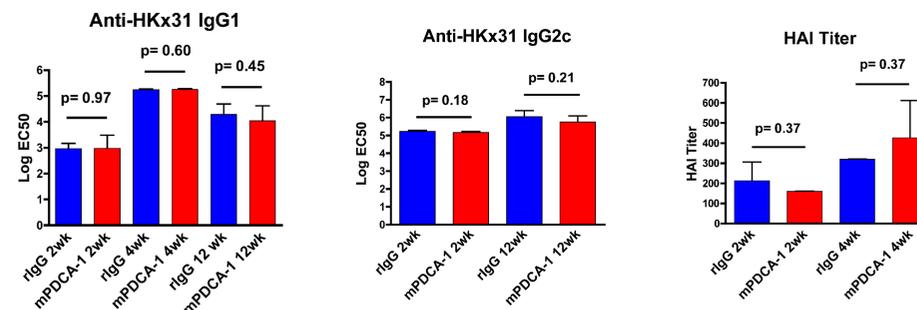
**Detection of HKx31-specific antibodies:** Briefly, plates were coated overnight with 1.0  $\mu$ g/ml purified inactivated HKx31 virus in PBS. After blocking in diluent assay (10% FCS in PBS), ten-fold serial dilutions of sera were incubated for 2 hours, washed, and specific isotypes were detected with anti-mouse-IgG1 and -IgG2c HRP-conjugated antibodies (Southern Biotech). TMB substrate was used for the final detection step. Plates were read at 450 and 570 nm. HAI titers were performed using standard methods.

**IFN- $\gamma$  T-cell secretion assay:** Single-cell suspensions were prepared from spleens harvested 4 weeks post-immunization. Splenocytes were cultured at  $2 \times 10^6$  cells/ml, and stimulated with 1.0  $\mu$ g/ml purified inactivated HKx31 virus or 20 HA units live HKx31 virus. Culture supernatants were harvested at 24, 48, 72 and 96 hours post-stimulation, and the levels of IFN- $\gamma$  were quantified using an ELISA assay (BD Biosciences) following the manufacturer's conditions.

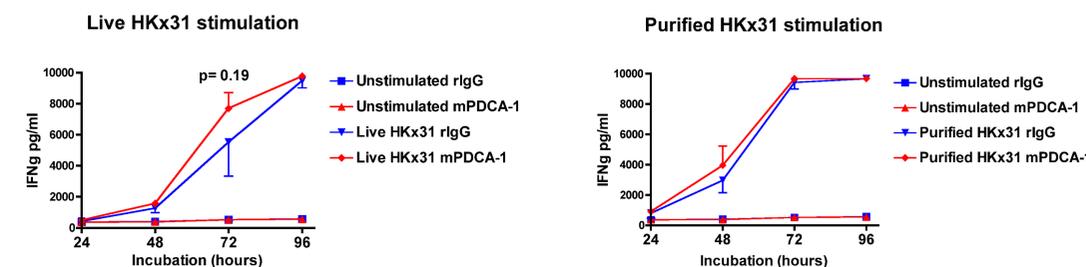
## RESULTS



**Figure1: *In vivo* pDC depletion.** a- Flow cytometric analysis analysis of mice injected with anti-mouse PDCA-1 or rat IgG antibodies. The depletion resulted in a 75 to 90% decrease in pDCs. b- IV injection of a CpG ODN resulted in a 60-70% decrease in IFN- $\alpha$  in the group injected with the anti-mPDCA-1 antibody.



**Figure2: Post-immunization antibody responses.** There were no statistical differences in anti-HKx31 IgG1 or IgG2c isotype levels at any time point tested after one immunization. The level of protective antibodies (HAI titers) were similar at 2 and 4 weeks post-immunization for the pDC-depleted and non-depleted groups.



**Figure3: *In vitro* splenocyte stimulation for IFN- $\gamma$ .** There were no statistical differences in the amounts of IFN- $\gamma$  secreted after activation with either live or purified HKx31 influenza virus in cultures of splenocytes harvested from pDC-depleted or non-depleted mice. The spleens were harvested at 4 weeks post-immunization.

## SUMMARY

- Our *in vivo* pDC depletion protocol results in a 75 to 90% decrease in pDCs numbers.
- Despite this depletion, there were no significant differences in antibody responses in the 2 groups of mice, as seen by equivalent influenza-specific IgG1 and IgG2c titers as well as comparable levels of protective antibodies, as assessed by HAI.
- Upon stimulation of splenocytes with live or purified inactivated influenza virus, there is no statistical differences in the level of IFN- $\gamma$  secreted between the two groups of mice. Our previous work has found that this secretion is mediated mainly by CD4 T cells.

## CONCLUSIONS

Despite a substantial *in vivo* decrease in pDCs following antibody administration as evaluated by flow cytometry, we did not see an impact of this depletion on influenza-specific B-cell or T-cell adaptive immune responses following immunization with CLDC/HKx31 influenza A. This suggests that pDC-derived IFN- $\alpha$  is not a limiting factor for a robust immune response induced by CLDC/antigen vaccination. However, we cannot exclude the possibility that the remaining pDCs are still secreting enough type I interferons, as seen in our IFN- $\alpha$  assay, to shape the adaptive immune response. It is also possible that CLDC might be able to induce IFN- $\alpha$  by a pDC-independent mechanism.

## FUTURE DIRECTIONS

- Determine the impact of the pDC depletion on the generation of the memory T-cell and B-cell response
- Determine the importance of type I IFNs in CLDC immunization by evaluating immune responses in type I IFN receptor deficient mice

## ACKNOWLEDGEMENTS

This work was supported by the NIH grants U01 AI-074512 and R41 AI-068260 (to DBL and JF).