

# Acutely HIV<sub>III</sub>B- Infected CD4+ T Cells and Chronically-Infected HIV<sub>III</sub>B- H9 Cells Preferentially Conjugate and Subsequently Fuse With Plasmacytoid Dendritic Cells

Evans S. Jacobs<sup>1,2</sup>, Thaddeus C. George<sup>3</sup>, Sukhwinder Singh<sup>1</sup>, Richard Wnek<sup>1,4</sup>, Paul Fischer<sup>4</sup>, Shila K. Nordone<sup>5</sup>, Greg Dean<sup>5</sup>, and Patricia Fitzgerald-Bocarsly<sup>1,2</sup> <sup>1</sup>UMDNJ-New Jersey Medical School- Department of Pathology, <sup>2</sup>UMDNJ-Graduate School of Biomedical Sciences, <sup>3</sup>Amnis Corporation, Seattle, Washington, <sup>4</sup>Dept. of Target Validation, Merck Research Laboratories, Rahway, NJ, <sup>5</sup>Dept. of Molecular Biomedical Science, College of Veterinary Medicine, North Carolina State University

## ABSTRACT:

Plasmacytoid Dendritic Cells (pDC) produce copious amounts of IFN- $\alpha$  in response to TLR-7 or -9 stimulation and serve as a link between the innate and adaptive immune systems. In HIV infection, there is a gradual depletion of pDC as well as a functional deficiency in remaining circulating pDC. The mechanism of pDC depletion is not yet understood. We have demonstrated using imaging flow cytometry (ImageStream<sup>®</sup>) that pDC preferentially take up membrane and cytoplasm from HSV and Influenza infected cells in an endocytic process called "nibbling" that results in IFN- $\alpha$  production by the pDC. Using acutely HIV<sub>III</sub>B infected CD4 T cells or chronically HIV<sub>III</sub>B infected H9 cells, pDC preferentially form conjugates with HIV<sub>III</sub>B infected vs uninfected T cells, but, rather than being nibbled, the majority of these encounters lead to pDC/infected T cell fusions. Acutely infected CD4 T cells but not chronically-infected H9 cells stimulated pDC to produce IFN- $\alpha$ , albeit at lower levels than other TLR7/9 inducers. Fusions were blocked with the addition of the specific fusion inhibitors (T-20 or AMD-3100), but there was no increase in conjugate formation or uptake of material. We hypothesize that this successful subversion of pDC nibbling and IFN- $\alpha$  production by HIV-infected cells contributes to loss of pDC and the deficient IFN- $\alpha$  production seen in HIV-infected patients. Supported by AI26806.

## INTRODUCTION:

Plasmacytoid dendritic cells (pDC) are CD123<sup>+</sup>, HLA-DR<sup>+</sup>, CD11c<sup>-</sup>, BDCA-2<sup>+</sup> (CD303), BDCA-4<sup>-</sup> (CD304), and represent 0.1-0.5% of PBMC. They are the primary IFN- $\alpha$  producing cell in the blood (up to 3-10 pg/cell) in response to enveloped virus and synthetic TLR7 and TLR9 ligands and can present antigen to T-cells to prime the adaptive immune response. pDC numbers and function become progressively deficient in patients with HIV infection, with deficient pDC IFN- $\alpha$  production associated with progression to opportunistic infections. However, the mechanisms contributing to the loss of pDC in the peripheral blood and the dysfunction of remaining pDC have not been determined. Although pDC respond vigorously to stimulation with non-cell associated virus with IFN- $\alpha$  production, in vivo, pDC are likely to encounter virus in the context of a virus-infected cell. It has previously been shown that myeloid dendritic cells (MDC) are able to take up material by live cells in a process that has been termed "nibbling". Our lab has found that pDC can nibble live cells and that pDC but not MDC preferentially nibble antigen from live virus-infected cells, resulting in IFN- $\alpha$  production. In this study we evaluated the interaction of pDC with free HIV virions and both chronically HIV-infected H9 cells and acutely-infected CD4 T cells.

## MATERIALS AND METHODS:

**Cells and Reagents:** Chronically HIV-infected H9 cells and uninfected controls were obtained from the AIDS Research Reagent Program. CD4 T-cells were positively selected from fresh PBMC using the Miltenyi CD4 T-cell isolation kit. They were maintained in complete T-cell medium and supplemented with 200 IU/mL rhIL-2 (AIDS Research Reagent Program) and activated for 3 days with 10  $\mu$ g/mL PHA. CD4 T-cells were infected with 10<sup>7</sup> TCID<sub>50</sub> HIV<sub>III</sub>B by inoculation at 120xCO<sub>2</sub> for 2 hrs and then incubated at 37°C 5% CO<sub>2</sub> for 5-7 days. Cells were harvested for target assay when >10% of the cells had down regulated CD4. Soluble CD4 was used at 100 $\mu$ g/mL. AMD3100 was used at 50 $\mu$ g/mL. T20 was used at 100ng/mL.

**Preparation of PBMC and Enrichment:** PBMC were isolated by Ficoll-Hypaque gradients from fresh heparinized peripheral blood obtained with informed consent from healthy donors. pDC were negatively selected from PBMC using the Miltenyi pDC isolation kit. Purity was typically >70%.

**IFN- $\alpha$  ELISA:** PBMC were stimulated overnight with HIV or HIV-infected cells and supernatants were analyzed by ELISA using BenderMed Systems IFN- $\alpha$  module set.

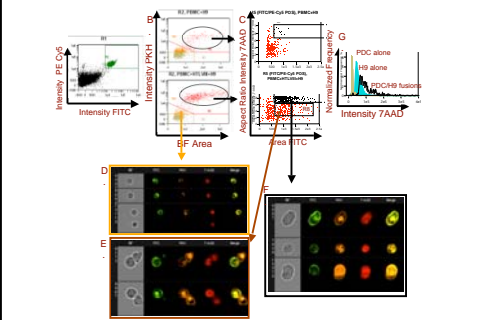
**PKH/Dil labeling:** Infected and uninfected targets were labeled using the Sigma Fluorescent Cell Linker Kits for PKH67 and PKH26 or the Invitrogen Vybrant<sup>®</sup> cell labeling kit.

**Flow Cytometry and intracellular detection of IFN- $\alpha$ :** pDC within PBMC were identified by labeling with BDCA-2 PE or FITC, and CD-123 PE. For intracellular flow, PBMC were incubated with the indicated stimuli and incubated at 37°C, 5% CO<sub>2</sub> for 4 hours before addition of Brefeldin A (5  $\mu$ g/mL, Sigma). After 2 more hrs, pDC were stained and the cells were fixed overnight. Cells were permeabilized and stained with biotinylated anti-IFN- $\alpha$  (PBL clone MMHA2) followed by avidin-PE-Cy7, and acquired using a FACS Calibur and analyzed using Cell Quest software (BD). Additionally, cells were incubated overnight at 37°C, 5% CO<sub>2</sub> after which supernatants were analyzed for IFN- $\alpha$  production by ELISA.

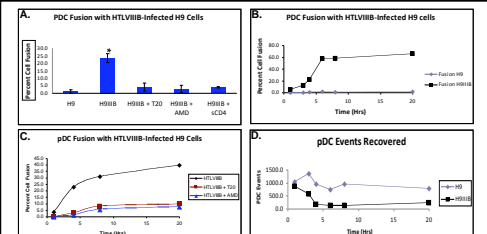
**ImageStream data acquisition and analysis:** For visualization of the interaction between pDC and infected cells, we utilized the ImageStream multispectral imaging flow cytometer (Amnis Corporation Seattle, WA). pDC enriched PBMC (1-2 X 10<sup>6</sup> cells/sample) were co-cultured 1:1 with uninfected or infected cells labeled with the PKH67 Green, PKH 26 Red or Dil in 5 mL round bottom tubes for 2-4 hours at 37°C, 5%CO<sub>2</sub>. Cells were then stained with BDCA2 and DRAQ5.

**CLICK-IT Labeling:** Edu was added to target cell cultures at 1 $\mu$ g/mL and cultured overnight. Then immediately prior to acquisition of Amnis ImageStream<sup>®</sup> samples were stained according to manufacturers protocol.

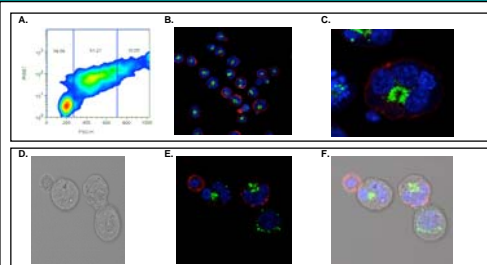
**Confocal imaging:** Samples were recovered from the ImageStream and gated for pDC and sorted for large, PKH-positive cells and analyzed by confocal microscopy (Zeiss 410).



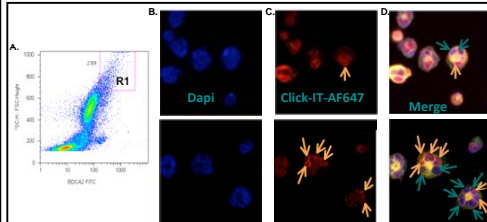
**Figure 2. pDC fuse with HTLV<sub>III</sub>B-infected H9 cells.** PKH-26-labeled H9 cells were washed vigorously and co-cultured with pDC enriched PBMC for 4 hours. Cells were analyzed by Amnis Image Stream for single pDC (low BF area) events where some events were single pDC (R3 B upper and lower) and other that containing PKH labeled membrane (R4, B upper and lower) and high BF area pDC events (R5, B upper and lower). Conjugated events were located by further analyzing large area pDC events for low circularity (aspect ratio below 0.6) events with high FITC area (R8, C upper and lower). pDC-H9 fusions were located by further analyzing large area pDC events for large circular (aspect ratio close to 1) events with high FITC area (R7 C upper and lower). Representative images of single pDC with internalized PKH (D) and pDC-H9 conjugates (E) and pDC-H9 fusions (F) are shown. pDC-HTLV<sub>III</sub>B-H9 samples were analyzed for DNA content (G), where single pDC events are gold, non-pDC associated H9 are green and pDC-H9 fusions are black.



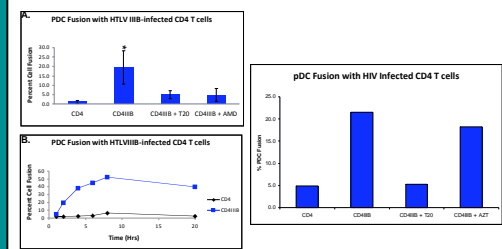
**Figure 3. pDC fuse when coincubated with HTLV<sub>III</sub>B-H9 cells and not when incubated with uninfected H9.** A, Dil-labeled uninfected or HTLV<sub>III</sub>B-infected H9 cells were co-cultured with enriched pDC for 4 hours. Samples were then analyzed for cell-cell fusion on the Amnis ImageStream. (Mean and SD for n=9 HTLV<sub>III</sub>B, n=6 (T20), n=8 (AMD3100), n=3 (sCD4). Anova, with Turkey Post Hoc Test, p<.0001). B, C, Percent of pDC fused increases over time in culture and is blocked by fusion inhibitor T20 or CXCR4 antagonist AMD3100. D, There is a progressive loss of total pDC numbers in samples incubated with HTLV<sub>III</sub>B-infected cells. Samples were incubated or 1,2,4,6,8, and 20 hrs.



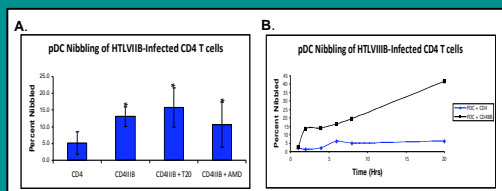
**Figure 4. Confocal imaging of sorted pDC/HIV-H9 fusions.** A, Samples recovered from the ImageStream were gated on pDC, then gated for size and sorted for R3. B, Multinucleated cells from R3 C. 40x zoom on a multinucleated cell showing BDC2 (red), PKH (green) and multiple nuclei (DRAQ5, blue). D, E, F, DIC, fluorescence and merged images from R3.



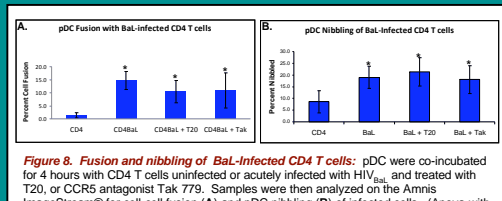
**Figure 5. Confocal imaging of fusions with double labeled nuclei:** Prior to coculture, 1 $\mu$ g/mL Edu was added to culture for incorporation into DNA of actively replicating cells. After coculture cells were labeled with CLICK-IT Alexa-fluor 647 to detect Edu incorporation into HTLV<sub>III</sub>B-infected H9 cells and Dapi. A, pDC events were gated on for size and BDC2 and then sorted on R1. B, All nuclei are Dapi Positive. C, Nuclei originating from HTLV<sub>III</sub>B-infected H9 cells are AF647 positive. D, Fused cells contain multiple nuclei that are either single positive for Dapi (pDC nuclei, blue arrows) or double positive for Dapi and AF647 (H9 nuclei, orange arrow).



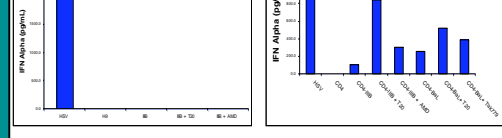
**Figure 6. CD4<sup>+</sup> T cells acutely infected with HTLV<sub>III</sub>B fuse with pDC.** A, Similar to above, Dil-labeled, acutely HTLV<sub>III</sub>B-infected CD4 T cells fused upon co-culture with pDC (Mean and SD N=7, Anova, with Turkey Post Hoc Test, p<.0001). B, Fusions increase with time and also similar to above can be blocked with T20 and AMD3100, however fusion is not dependent of actively replicating virus (C).



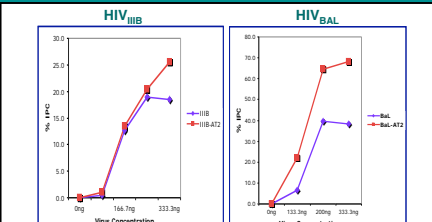
**Figure 7. pDC Nibbling of HTLV<sub>III</sub>B-Infected CD4 T Cells:** A, pDC were co-cultured for 4 hours with HTLV<sub>III</sub>B-infected CD4 T cells and treated with T-20 or AMD-3100 to block fusion. (mean and SD for n=5 (HTLV<sub>III</sub>B), n=6 (T20,AMD), Anova with Turkey Post Hoc Test, p<.05) B, Percent nibbling of HTLV<sub>III</sub>B-infected CD4 T cells increases over time.



**Figure 8. Fusion and nibbling of BaL-Infected CD4 T cells:** pDC were co-cultured for 4 hours with CD4 T cells uninfected or acutely infected with HIV<sub>BaL</sub> and treated with T20, or CXCR4 antagonist 779. Samples were then analyzed on the Amnis ImageStream<sup>®</sup> for cell-cell fusion (A) and pDC nibbling (B) of infected cells. (Anova with Turkey Post Hoc Test, p<.01)



**Figure 9. Differential pDC IFN- $\alpha$  production in response to chronically or acutely infected CD4 T cells:** A, PBMC were mock or directly stimulated with HSV or co-incubated with H9 or HTLV<sub>III</sub>B-infected H9 cells for 18 hrs. B, PBMC were mock or directly stimulated with HSV or co-incubated with CD4 T cells or CD4 T cells acutely infected with HIV<sub>III</sub>B or HIV<sub>BaL</sub> for 18 hrs. Supernatants were analyzed by ELISA for the presence of IFN- $\alpha$ .



**Figure 1. pDC produce IFN- $\alpha$  in response to both active and AT-2-inactivated HIV-1:** PBMC were stimulated with active or AT-2-inactivated HIV<sub>III</sub>B or HIV<sub>BaL</sub> at varying concentrations for 6 hr. Following incubation, cells were stained for DC, fixed overnight and stained intracellularly for IFN- $\alpha$ . HIV<sub>BaL</sub> is a better inducer of IFN- $\alpha$  than HIV<sub>III</sub>B. In both cases, the AT-2-inactivated forms of HIV induces more IFN- $\alpha$  with lower concentrations of virus.

## CONCLUSIONS

- pDC produce IFN- $\alpha$  in response to free HIV virions; however, large amounts of virus are required.
- HIV<sub>BaL</sub> is a better inducer of IFN- $\alpha$  than HIV<sub>III</sub>B however, in both cases, the AT-2 inactivated forms induced higher amounts of IFN- $\alpha$  than the live active forms.
- pDC attempt to interact with HTLV<sub>III</sub>B-infected H9 cells in a similar fashion to HSV infected Raji. Low levels of internalized material can be observed in pDC that have been co-cultured with H9.
- HTLV<sub>III</sub>B-infected H9 cells induce cell-cell fusion with pDC. These fusions may lead to pDC death, but it is evident that they are no longer able to function in a capacity to produce and secrete IFN- $\alpha$  in response to chronically infected T cells
- Similarly, pDC fuse with acutely HTLV<sub>III</sub>B or HIV<sub>BaL</sub> infected CD4 T cells. However, nibbling of these cells is still observed and pDC can produce IFN- $\alpha$  albeit at lower levels than direct stimulation with HIV.
- pDC populations are known to be depleted in chronic HIV infection, similar to CD4 T-cell populations. pDC may attempt to "nibble" on HIV infected cells, but in the process are fused with and destroyed, a potential mechanism for the depletion of pDC in chronic HIV infection.