Monocyte and NK Cell Dysfunctional Responses to Mycobacteria During Chronic HIV-1 Infection

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ABSTRACT & BACKGROUND

Background: Coinfection with *M. tuberculosis* (Mtb) and other *Mycobacteria* is a leading cause of morbidity and mortality in HIV+ patients. While neither monocytes nor NK cells are directly infected with HIV, several of their phenotypic and functional properties become altered during chronic infection, and both cell types are important to the control of Mtb. *We hypothesized that HIV-related NK cell dysfunction may result in impaired monocyte-NK cell crosstalk, which we will evaluate in the context of Mycobacteria infection.*

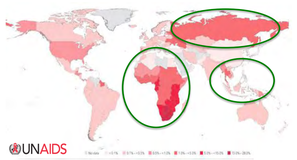
Methods: To assess monocyte cytokine production, whole blood from HIV+ ART-naïve patients and healthy donors was stimulated with *Mycobacteria* for 6h (in the presence of Brefeldin-A) and followed by intracellular cytokine staining. For cellular functional assays, cells were isolated from PBMC via maganetic bead isolations. Monocytes were infected with fluorescent (mCherry) BCG (opportunistic pathogen) or Mtb at MOI10 for 6h to assess phagocytosis. Autologous NK cells were added to assess cell killing.

Results: We observed a decrease in IL-12 (p=0.017), but similar TNF-alpha production, by blood monocytes from HIV+ individuals following a 6h stimulation with *M. Bovis* BCG (opportunistic pathogen), compared to uninfected donors. As IL-12 is known to regulate NK cell functionality, we were also interested in evaluating NK cell function in our cohort. While developing a flow-based functional assay to measure NK cell killing of autologous Mtb-infected monocytes, we found that NK cells from HIV+ donors exhibited decreased killing of target K562 cells compared to controls (p=0.04). Further evaluation established that monocytes from the same HIV+ patients in which we identified lower NK cell killing also exhibited decreased phagocytosis of fluorescently-labeled BCG (p=0.016) but similar phagocytosis of Mtb compared to uninfected donors.

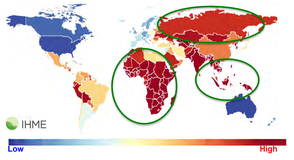
Conclusions: Our data demonstrate a reduction in both monocyte and NK cell functionality in the same HIV+ patients and is suggestive of synergism in the dysfunction of two innate cell types. The assays described have the potential to result in novel therapeutic targets to enhance innate cellular function in HIV+ patients.

HIV and Tuberculosis: A Synergistic Pandemic:

Worldwide HIV Prevalence - 2009



Deaths by Tuberculosis (Cause Rank) - 2010



- Tuberculosis is a leading cause of death in HIV+ patients *(Mayer and Hamilton CID 2010)*
- HIVneg individuals: 5-10% **lifetime** risk of developing TB; HIV+ individuals: 10% **annual** risk *(Havlir DV 1999 NEJM; Jones BE 1993 Am Rev Respir Dis)*
- BCG is a vaccine administered against *M. tuberculosis*; causes disseminated disease in severely immunocompromised patients

SPECIFIC AIMS

Overall Goal: Elucidate differences in innate response to Mycobacteria between HIV+ and HIV-negative humans that reveal novel, key elements in HIV pathogenesis and increased susceptibility to opportunistic infections.

Aim 1. Characterize innate immune gene expression during pathogenic and non-progressive HIV/SIV infections following stimulation with *Mycobacteria.*

Aim 2. Assess monocyte functional responses to *Mycobacteria,* including cytokine production and phagocytosis during pathogenic HIV and non-progressive SIV infection.

Aim 3. Functionally assess the ability of NK cells to kill infected targets during HIV infection.

STUDY POPULATION(S)

Human Cohort Inclusion Criteria

- HIV+ blood from Madison Clinic; healthy blood from Seattle BioMed
- Antiretroviral (ART) naïve or off of ART treatment for >1 year
- PPD-negative and not presenting with TB symptoms
- Recent HIV viral load and CD4+ T cell count available

Median CD4+ T cell count:	Median Viral Load:
585 cells/ul	**4062 copies/mL**



Sooty mangabey (model of non-progressive SIV infection)

- Whole blood from SIVnegative and SIV+ **sooty mangabeys** (model of nonpathogenic infection) was obtained from the Yerkes National Primate Research Center

METHODS

Mycobacteria Culture

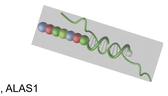
- *Grow to log growth phase*
- *Wash from growth media*
- *Calculate MOI based on OD*



Species	Pathogenicity	Biosafety	Replication time (h)
M. smegmatis	non-pathogenic	BSL-1	3
M. bovis BCG	opportunistic	BSL-2	16-18
M. tuberculosis	pathogenic	(TB)BSL-3	20-24

Gene Expression Analysis

- **Sample preparation**
  - Whole **PBMC** stimulated in triplicate with BCG (Russia) at a ratio of 1:1 in 96-well plate for 4,18h.
  - N=22 (7 HIVneg, 7 HIV+, 4 SIVneg, 4 SIV+)
  - Triplicates pooled for RNA isolation (Qiagen)
- **Nanostring platform**
  - Color coded hybridization and capture probes
  - Absolute transcript abundance
  - Normalized to housekeeping controls GAPDH, ALAS1
  - Background cutoff for detection: average copy # neg controls +/- 2SD



NK Cell Functional Analyses

- Purified NK cells from PBMC using CD56 enrichment magnetic bead kit
- Rested O/N
- Exposed to claret-labeled MHCI-deficient K562 cells for 4h at E:T 1:1 or 4:1
- Visualized NK cell killing via flow cytometry (uptake of amine aqua)

Whole Blood Monocyte Functional Analyses

- 6h stimulation in the presence of Brefeldin-A
- Intracellular cytokine staining for TNFα and IL-12



HIV-infected, healthy human donors

Non-pathogenic — *M. smegmatis* (non-pathogenic) — BCG (opportunistic) — *M. tuberculosis* (pathogenic) — Pathogenic



monocytes

- TNFα: pleiotropic, critical for containment of *M. tuberculosis* *(Bean et al. 1999 JI; Gardam 2003 Lancet Inf Dis)*
- IL-12: pleitropic, important for NK cell activation, drives Th1 response *(Stoffer et al. 1993 Blood; Hsieh et al Science 1993)*

Data Analysis

- Flow cytometry data was analyzed using Flowjo v. 8.8.6
- Statistics were generated in GraphPad Prism using a Mann-Whitney test for nonparametric data

RESULTS (GENE EXPRESSION ANALYSIS OF WHOLE PBMC)

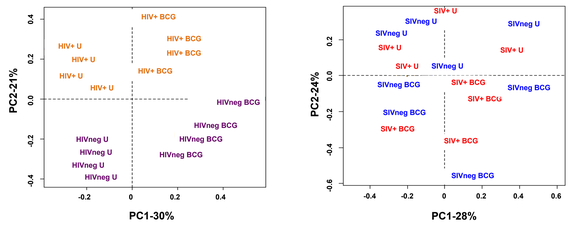


Figure 1. Principal Component Analysis (PCA) reveals hierarchical clustering pattern unique to pathogenic HIV infection. Semi-supervised clustering via PCA demonstrates that BCG stimulation is a key component to differential gene regulation (PCA-1 for humans, A; PCA 2 for mangabeys, B.). Pathogenic HIV status also defines response patterns (PCA-2 for humans); Nonpathogenic SIV status does not define BCG response patterns for mangabeys.

Figure 2. Analysis of differentially-expressed genes at baseline in HIV+ donors. Of 224 targets (A), 30 genes were found to be differentially-expressed during HIV infection at baseline; those genes were submitted for KEGG analysis and found to be significantly enriched for two pathways: antigen processing and cell-mediated toxicity (B). Individual dot plots display differences in NK cell gene expression before (C.) or after (D.) BCG stimulation.

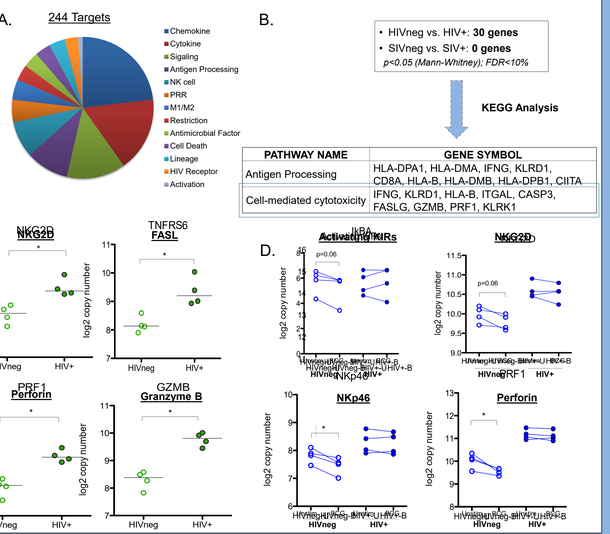
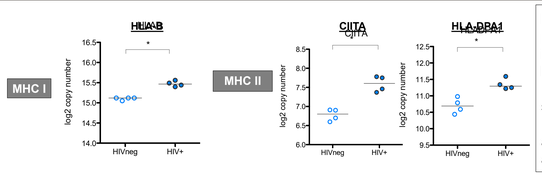


Figure 3. Genes involved in antigen processing pathways have higher baseline expression levels in HIV+ donors. Pathways of HLA class I and class II antigen processing molecules showed significantly higher gene expression in HIV-infected donors. This supports our hypothesis that we would find increased basal immune activation in these donors, and may contribute to increased NK cell activation and subsequent lack of NK cell response to BCG.



RESULTS (NK CELL AND MONOCYTE FUNCTIONAL ASSAYS)

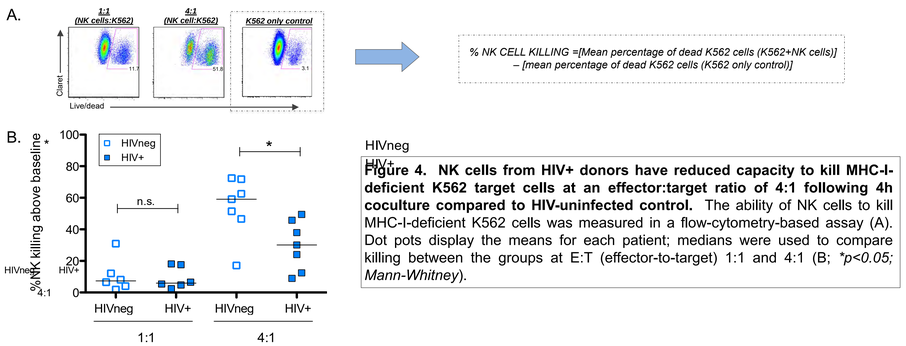


Figure 4. NK cells from HIV+ donors have reduced capacity to kill MHC-I-deficient K562 target cells at an effector:target ratio of 4:1 following 4h coculture compared to HIV-uninfected control. The ability of NK cells to kill MHC-I-deficient K562 cells was measured in a flow-cytometry-based assay (A). Dot pots display the means for each patient; medians were used to compare killing between the groups at E:T (effector-to-target) 1:1 and 4:1 (B; **p<0.05; Mann-Whitney*).

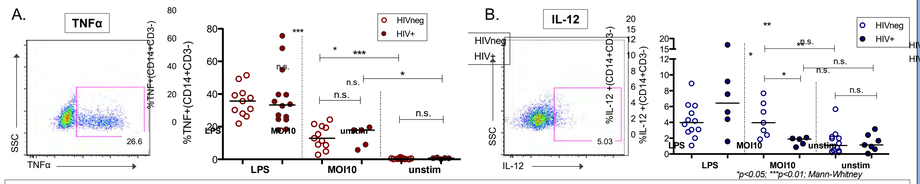
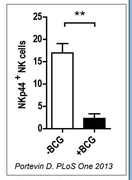


Figure 5. Whole blood monocyte inflammatory cytokine production following 6h stimulation with BCG. A similar proportion of blood monocytes from HIV+ patients produce TNFα (A.) while a lower proportion produce IL-12 (B.) compared to uninfected donors following stimulation with opportunist BCG (**p<0.05; **p<0.01; ***p<0.005; Mann-Whitney*).

CONCLUSIONS AND FUTURE DIRECTIONS

- **Nanostring analysis reveals several genes that become differentially regulated upon PBMC stimulation with opportunist BCG, and that SIV status may not be a key factor in these responses, while HIV is a key factor in response patterns.**
  - At baseline, HIV-infected donors displayed higher levels of antigen processing (indicative of immune activation) and higher levels of NK cells-related gene expression; these pathways were found to be significantly enriched in our gene set; SIV-infected sooty mangabey gene expression was similar to uninfected controls.
  - We found decreased levels of mRNA associated with activated NK cells in healthy donors (activating KIRs, NKG2D, NKp46) following BCG stimulation.

NK cell response to BCG is characterized by apoptosis/depletion of activated, IFNγ-producing NK cells in healthy individuals (Portevin D. PLoS One 2013)

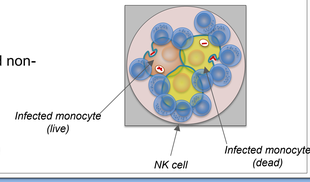


- **Despite having a higher proportion of inflammatory monocytes, HIV+ patients display a lower proportion of IL-12-producing monocytes compared to uninfected donors following stimulation with opportunist BCG.**
  - In contrast to HIV+ patients, monocytes from HIVneg donors upregulate IL-12 in response to BCG (compared to an unstimulated control); responses to LPS were similar.
  - Monocytes from both HIVneg and HIV+ donor produce TNFα in response to both BCG and LPS; this does not differ between the groups.
- **NK cells from HIV+ patients in our Seattle-based cohort have reduced capacity to kill MHC-I-deficient K562 target cells compared to HIV-uninfected controls**
  - This is seen at an effector:target ratio of 4:1 (but not 1:1) following 4h coculture

Together, our data present potential for identifying novel therapeutic targets to enhance innate cellular function in HIV+ patients, which may ultimately stymie the resurgence of HIV-related coinfections.

Future Directions:

- Validate differentially-expressed targets (Nanostring) via real-time PCR
- Examine innate cell interactions in response to *Mycobacteria* in hosts of pathogenic and non-pathogenic infections
  - Monocyte phagocytosis
  - NK cell killing
  - Assess the role of cytokines and the cytokine milieu in responses to *Mycobacteria*



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Patients and healthy donors

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