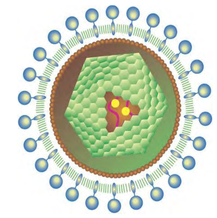
#0214

HIV INFECTION AND SMOKING DIFFERENTIALLY REGULATE ALVEOLAR MACROPHAGES

Charles Preston Neff, Tom Campbell, Andrew Fontenot, and Brent E. Palmer
Division of Allergy and Clinical Immunology, Department of Medicine, University of Colorado, Aurora, CO



Abstract

Background: HIV infection impacts immune cells in the lung leading to pulmonary complications which persist with antiretroviral therapy (ART). Alveolar macrophages (AM) are principle immune cells in the bronchoalveolar compartment and as such play a pivotal role in host defense against pathogenic microorganisms and tissue remodeling. Examination of the effect of HIV on AM is complicated by the high prevalence of smoking in HIV-infected subjects from the United States. Smoking increases auto-fluorescence of AMs, inhibiting the reliability and resolution of traditional flow cytometry. Cytometry by Time of Flight (CyTOF) utilizes pure metal conjugated antibodies and detection by mass cytometry, which effectively bypasses auto-fluorescence. Here, we utilize CyTOF to comprehensively evaluate the effects of HIV infection and smoking on AM.

Methods: Bronchoalveolar lavage (BAL) cells from 10 untreated HIV-infected non-smokers, 9 untreated HIV-infected smokers, 10 HIV-seronegative non-smokers and 9-HIV seronegative smokers was subjected to traditional flow cytometry and CyTOF. Our CyTOF panel consisted of 32 unique markers. Phenotypic analysis using a smaller monoclonal antibody panel was also performed on all samples using traditional flow cytometry.

Results: Compared to those without HIV we found a decrease in CD206 (p=0.0002), and CD163 (p=0.002) positive cells, indicating a loss of alternatively activated AMs (M2) caused by HIV infection. The loss of M2 macrophages indicates an increased inflammatory environment. Smoking increased AM expression of CCR2 (p=0.007) which is a marker of inflammatory macrophages. Together, compared to healthy non-smokers, smoking and HIV increased CXCR4 expression on AM (p=0.004) demonstrating increased susceptibility to X4 tropic HIV infection.

Conclusions: While the aim of characterizing alveolar macrophages during HIV infection and smoking was our primary goal, this study also demonstrates the sensitivity of mass cytometry, and its ability to detect significant differences between patient groups which would have otherwise been masked by auto-fluorescence. Overall, these findings indicate that HIV and smoking drive alveolar macrophages toward an inflammatory state, leading to an overall more inflammatory environment in the lung.

Background

- We and others have shown that HIV infection profoundly impacts immune cells in the lung[1,2].
- Alveolar macrophages (AM) are principle immune cells in the bronchoalveolar compartment and play a pivotal role in host defense against pathogenic microorganisms and tissue remodeling.
- Differential expression of surface markers classifies macrophages to either classically (M1) or alternatively activated (M2).
- M1 macrophages are pro-inflammatory and mediate resistance to pathogens.
- M2 macrophages are anti-inflammatory and promote tissue remodeling.
- High prevalence of cigarette smoking in the HIV-infected population in the United States.
- Smoking increases auto-fluorescence of AM, inhibiting the reliability and resolution of traditional flow cytometry.
- Cytometry by Time of Flight (CyTOF) utilizes pure metal conjugated antibodies and detection by mass cytometry, which effectively eliminates auto-fluorescence.

Methodology

- Bronchoalveolar lavage (BAL) cells and peripheral blood mononuclear cells (PBMC) from 10 untreated HIV-infected non-smokers, 9 untreated HIV-infected smokers, 10 HIV-seronegative non-smokers and 9-HIV seronegative smokers was subjected to traditional flow cytometry and CyTOF.
- To bypass batch effect, 4 batches of 19 samples (2 BAL and 2 PBMC) plus a PBMC staining control were barcoded, pooled and ran per batch simultaneously.
- The CyTOF BAL panel consisted of 32 markers focused on alveolar macrophages (Fig. 1) while the CyTOF PBMC consisted of 36 markers focused on lymphocytes.
- Phenotypic analysis was performed using traditional flow cytometry methods as well as novel clustering algorithms.
- To evaluate gene expression of AMs, a subset of samples were subjected to RNA-seq. Briefly, AMs were purified using anti-CD71 magnetic microbeads, lysed in TRIzol and RNA was extracted using the RNeasy column. Library construction was made using the Illumina TruSeq mRNA library construction kit. Single-end sequencing was performed using an Illumina HiSeq 4000 for 125 cycles.
- Statistical significance was determined using Mann-Whitney T tests or ANOVA Dunnett's multiple comparisons tests.

Lineage Markers

label	target	clone	function
89Y	**CD45**	**HI30**	**marker**
146 Nd	CD64	10.1	Mono/Mac marker
149 Sm	(NCAM)	6.2	NK marker
151 Eu	CD14	M5E2	Mono marker
152 Sm	CD66b	80H3	Granulocyte marker
162 Dy	CD11c	Bu15	marker
165 Ho	CD19	HIB19	B cell marker
168 Er	(MMR)	15-2	Mac marker
170 Er	CD3	UCHT1	T cell marker
209 Bi	(Mac-1)	ICRF44	marker
154 Sm	CD163	GHI/61	M2 marker
163 Dy	CD33	WM53	Granulocyte marker
175 Lu	CD71	OKT-9	Transferrin receptor

Costimulation Markers

label	target	clone	function
175 Lu	CD71	OKT-9	Transferrin receptor
142 Nd	CD40	5C3	Costimulation
143 Nd	HLA-DR	L243	MHC
150 Nd	CD86	IT2.2	Costimulation
159 Tb	(PD-L1)	29E.2A3	Costimulation
161 Dy	CD80	2D10.4	Costimulation
172 Yb	(PD-L2)	12	Costimulation
174 Yb	(PD-1)	H7	Costimulation

Chemokine/PAMPs Markers

label	target	clone	function
147 Sm	CD13	WM15	aminopeptidase
153 Eu	CCR2	*	Chemokine receptor
167 Er	(CCR7)	G043H7	Chemokine receptor
156 Gd	(CXCR4)	12G5	HIV receptor
171 Yb	(CCR5)	NP-6G4	HIV receptor
155 Gd	CD36	5-271	Scavenger receptor
148 Nd	CD16	3G8	Fc Receptor
160 Gd	CD23	5*	Fc Receptor
169 Tm	CD32	FUN-2 *	Fc Receptor
166 Er	IL-1RI	al *	Inflammation
158 Gd	TLR4	*	Microbe detection
176 Yb	TLR2	TLR2.1	Microbe detection

Fig 1. BAL CyTOF monoclonal antibody panel. Lineage makers were used to identify immune cell subsets with a focus on AM (left). AMs were then evaluated for the expression of 20 unique markers of costimulation, chemokine receptors or pathogen-associated molecular patterns (PAMP) (center and right).

Results

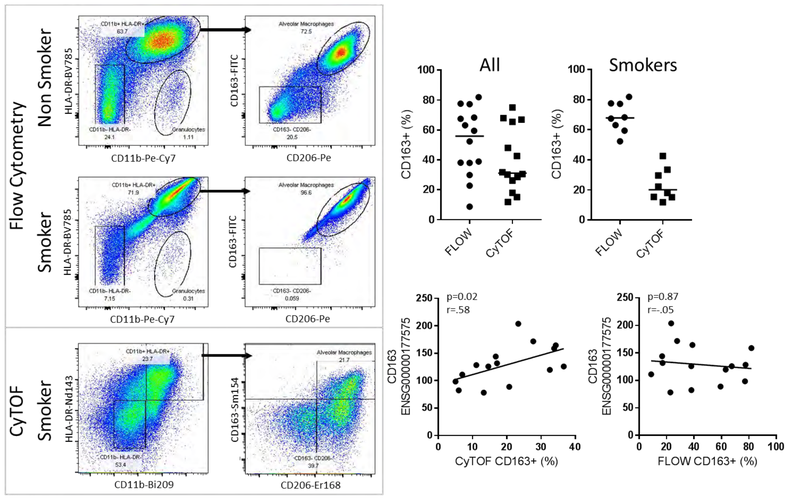


Fig. 2. CyTOF eliminates AM auto-fluorescence. BAL was subjected to traditional flow cytometry and CyTOF for a subset of markers including CD11b, HLA-DR, CD206 and CD163. Representative dot plots using traditional flow cytometry from a non-smoker and smoker compared to the same smoker using CyTOF are shown. Percent of CD163+ cells on all subjects or just smokers is shown on the right. Gene (RNA-seq) and protein expression are correlated with CyTOF but not with flow cytometry.

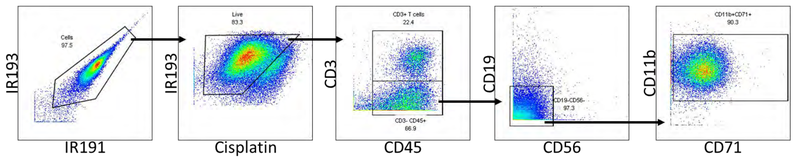


Fig. 3. Representative CyTOF gating strategy of AM. BAL was stained with 33 metal-labeled antibodies (Fig. 1). Viable alveolar macrophages were defined by DNA content, CD45+CD3-CD19-CD56- and CD11b+CD71+ markers.

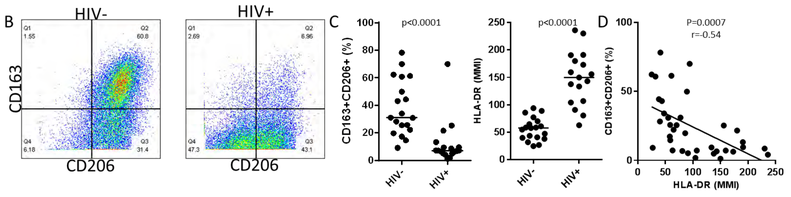


Fig. 4. HIV infection is associated with decreased M2 and increased activated AM. (B) Representative dot plots of AM expression of CD163 and CD206 from an HIV-uninfected and HIV-infected subject. **(C)** Cumulative data of CD163+CD206+ and HLA-DR expression on AM from HIV-uninfected and HIV-infected subjects. **(D)** Correlation of HLA-DR expression and CD163+CD206+ expression on AM. MMI, mean metal intensity

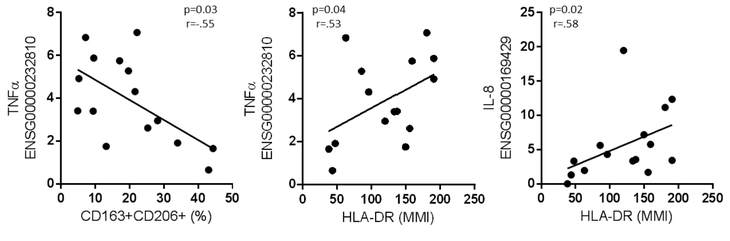


Fig 5. Gene expression of inflammatory cytokines is inversely correlated with the frequency of M2 AMs and positively correlated with activation RNA-seq was used to evaluate gene expression levels of CD71 purified AM. **(Left)** Correlation of gene expression levels and percent CD163+ cells. **(Center and Right)** Gene expression of inflammatory cytokines IL-8 and TNF-α correlated with HLA-DR expression.

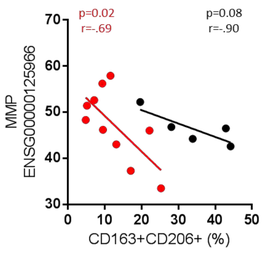


Fig 6. MMP expression is inversely correlated with M2 AM in HIV-infected subjects. Percent of M2 AM correlated with gene expression of matrix metalloproteinases (MMP). Red, HIV-infected; Black, HIV-uninfected

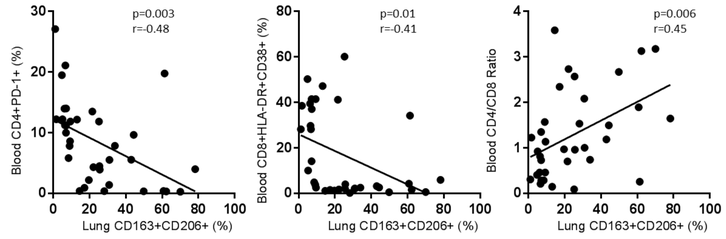


Fig 7. HIV disease progression in the blood correlates with AM depletion in the lung. PD-1 expression on CD4+ T cells, CD8+ T cell activation and CD4/CD8 T cell ratio in the blood were evaluated using CyTOF and correlated to percentage of M2 AM.

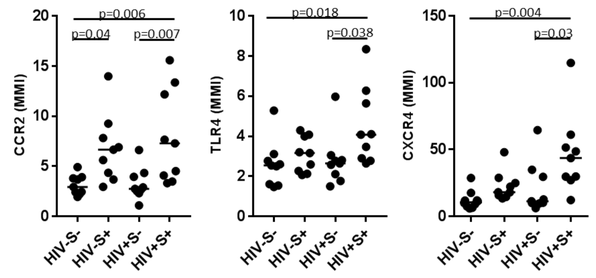


Fig 8. Smoking increases AM expression of chemokine and PAMP receptors. AMs were evaluated for CCR2, TLR4 and CXCR4 expression by CyTOF and subjects were grouped by smoking and HIV status.

Conclusions

- CyTOF effectively eliminates auto-fluorescence associated with AM and smoking.
- Gene and protein levels of CD163 are correlated with CyTOF but not traditional flow cytometry.
- Levels of anti-inflammatory CD163+CD206+ M2 AM are decreased with HIV infection while expression of HLA-DR is increased, highlighting an inflammatory lung environment
- Percent of M2 AM are inversely correlated with TNF-α while activated AM are correlated IL-8 and TNF-α.
- Matrix metalloproteinases (MMP), which cleave CD163, are inversely correlated with percent of M2 AM, suggesting a possible mechanism for induced shedding of CD163 in HIV-infected subjects.
- Disease progression in the blood correlates with loss of M2 AM.
- Smoking impacts AM expression of chemokine and PAMP receptors, enhancing the inflammatory environment of the lung

References

1. Neff, C.P. et al. Lymphocytic alveolitis is associated with the accumulation of functionally impaired HIV-specific T cells in the lung of antiretroviral therapy-naive subjects. Am J Respir Crit Care Med. 2015
2. Logue, E.C. et al. Upregulation of Chitinase 1 in Alveolar Macrophages of HIV-Infected Smokers. J Immunolo. 2019