Elevated Non-classical ($CD14^{+/low}CD16^{++}$) Monocytes and Urine Fibrotic Biomarkers in HIV Albuminuria

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Conference on Retroviruses & Opportunistic Infections
February 23-26, 2015
Abstract Number: 796

Introduction

Albuminuria is a pathological condition of the kidneys where excess albumin is excreted in the urine. Both in the general and HIV-infected populations, albuminuria has been shown to be a strong and independent risk factor for renal and cardiovascular disease. There is increasing evidence that both pro-fibrotic and pro-inflammatory host responses may be important components driving the pathophysiology of albuminuria. HIV-infected individuals on stable antiretroviral therapy (ART) are observed to have higher rates of albuminuria, along with increased risk for kidney and cardiovascular disease, as compared to the general population. However, it is still unclear how pro-fibrotic and pro-inflammatory responses are associated to the prevalence of albuminuria in HIV-infected individuals on stable ART.

Recently, there has been an increased interest in monocytes and the role they may have in the development of HIV-associated cardiometabolic complications. Monocytes are mononuclear leukocytes that originate in the bone marrow and circulate in the bloodstream before migrating into tissue to terminally differentiate into macrophages or dendritic cells. Through the advancement of flow cytometry, various monocyte subsets have been phenotyped and are traditionally characterized into classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and non-classical ($CD14^{+/low}CD16^{++}$) subsets. Monocytes are described to contribute to both the production of pro-inflammatory and pro-fibrotic cytokines and may be major mediators of HIV-associated inflammation and fibrosis.

In this study, we investigated the relationship of monocyte subsets and urine inflammatory and fibrotic biomarkers to albuminuria in HIV-infected individuals on stable ART.

Study Design & Results

A retrospective study was conducted to examine 96 chronic HIV-infected adults on stable ART from the Hawaii Aging with HIV Cohort-Cardiovascular Disease (HAHC-CVD) study who had documented urine albumin/creatinine ratios (UACR). Criteria for the HAHC-CVD study required patients to be > 40 years old and to be on stable ART > 3 months upon entry. Spot urine samples were obtained from these patients at entry and were assessed by a commercial laboratory (Diagnostic Laboratory Services, Inc.) to determine UACR. Albuminuria is defined as having a UACR value of ≥ 30 mg/g and was used to separate patients into two groups: Patients with albuminuria and patients without albuminuria. Other baseline parameters were assessed in patients upon entry and are described in Table 1.

Of the 96 patients included in this study, banked urine samples from 19 randomly selected patients with a UACR < 30 mg/g and 18 patients with a UACR ≥ 30 mg/g (total n=37) were tested for urinary inflammatory and fibrotic biomarkers using Luminex technology.

Table 1. Baseline characteristics of study patients[(1)] (n=96)

Demographic and Clinical Parameters			
Characteristic	Patients with albuminuria	Patients without albuminuria	p-value
n=96	n=18 (19%)	n=78 (81%)	
Age, years	57 [49, 62.5]	50 [45, 55]	0.013*
Male, n (%)	16 (88.9%)	67 (86.0%)	1.000
Race, n (%)			
Caucasian	12 (66.7%)	45 (57.7%)	0.665
Other races	6 (33.3%)	33 (42.3%)	0.665
Smoking history, n (%)	12 (66.7%)	49 (63.6%)	1.000
BMI, kg/m^2	25.9 [21.8, 28.1]	25.7 [23.8, 27.9]	0.789
Systolic blood pressure, mmHg	124.5 [119.8, 141.3]	120.0 [111.3, 129.0]	0.043*
Diastolic blood pressure, mmHg	80.5 [75.0, 84.3]	74.0 [68.0, 80.0]	0.012*
Hypertension, n (%)	12 (66.7%)	24 (30.8%)	0.010*
Current use of ACE inhibitors and/or ARB [(2)], n (%)	9 (50%)	12 (15%)	0.004**
Fasting glucose, mg/dL	91.5 [80.5, 96.75]	87.0 [81.0, 94.0]	0.366
Fasting insulin, µIU/mL	9.4 [4.7, 13.5]	5.9 [3.65, 10.1]	0.060
OGTT [(3)] blood glucose @ 120 min, mg/dL	113.0 [96.0, 138.0]	97.5 [73.3, 115.5]	0.031*
Metabolic syndrome [(4)], n (%)	5 (27.8%)	15 (19.2%)	0.629
Diabetes, n (%)	3 (16.7%)	7 (9.0%)	0.593
HOMA-IR [(5)]	2.6 [1.2, 3.4]	1.2 [0.8, 2.2]	0.017*
eC_{Cr}, Cockcroft-Gault [(6)], mL/min	76.6 [65.8, 104.9]	96.1 [82.0, 111.8]	0.072
eGFR CKD-EPI [(7)], mL/min/1.73 m^2	72.8 [63.8, 101.1]	86.6 [76.2, 99.3]	0.071
UACR [(8)], mg/g	63.35 [44.40, 156.15]	4.95 [3.38, 8.92]	<0.001**
Blood Urea Nitrogen , mg/dL	13.5 [11.0, 18.0]	13.0 [10.0, 16.3]	0.327
Serum creatinine, mg/dL	1.1 [0.9, 1.3]	1.0 [0.9, 1.1]	0.168
Total cholesterol, mg/dL	180.0 [139.8, 267.0]	174.5 [150.8, 192.3]	0.383
LDL cholesterol , mg/dL	119.0 [75.3, 180.5]	104.5 [83.8, 124.3]	0.315
HDL cholesterol, mg/dL	36.0 [31.0, 55.3]	41.0 [32.5, 49.0]	0.683
Triglycerides, mg/dL	116.5 [92.8, 171.8]	109.5 [75.8, 161.5]	0.406
Total cholesterol/HDL ratio	4.14 [3.48, 6.58]	4.09 [3.46, 5.29]	0.546
HIV RNA < 50 copies/mL, n (%)	16 (88.9%)	68 (87.2%)	1.000
Current use of Tenofovir, n (%)	16 (88.9%)	58 (74.3%)	0.312
Current use of Ritonavir, n (%)	7 (38.9%)	29 (37.2%)	1.000
Immunologic Parameters			
Nadir $CD4^+$ T cells, cells/µL	92.0 [33.0, 198.25]	170.0 [39.5, 249.5]	0.277
$CD4^+$ T cells, cells/µL	496.50 [374.5, 554.5]	495.50 [329.75, 635.75]	0.767
$CD8^+$ T cells, cells/µL	885.5 [533.0, 1160.0]	693.0 [552.0, 946.5]	0.177
Activated $CD8^+$ T cells ($CD38^+$), cells/µL	73.2 [53.7, 127.7]	77.8 [46.6, 123.8]	0.962
Total monocytes, cells/L	$4.5x10^8$ [$3.7x10^8$, $6.1x10^8$]	$3.9x10^8$ [$3.2x10^8$, $5.0x10^8$]	0.070
Classical ($CD14^{++}CD16^{-}$) monocytes, cells/L	$3.4x10^8$ [$2.6x10^8$, $4.8x10^8$]	$3.0x10^8$ [$2.2x10^8$, $4.0x10^8$]	0.110
Intermediate ($CD14^{++}CD16^{+}$) monocytes, cells/L	$8.2x10^6$ [$2.3x10^6$, $1.5x10^7$]	$5.0x10^6$ [$2.3x10^6$, $1.2x10^7$]	0.344
Non-classical ($CD14^{+/low}CD16^{++}$) monocytes, cells/L	$2.7x10^7$ [$2.0x10^7$, $5.5x10^7$]	$2.0x10^7$ [$1.3x10^7$, $3.0x10^7$]	0.033*
Transitional ($CD14^{+}CD16^{-}$) monocytes, cells/L	$6.8x10^7$ [$5.2x10^7$, $1.1x10^8$]	$5.8x10^7$ [$4.5x10^7$, $8.2x10^7$]	0.116

1. Median values shown with [median Q1, median Q3] or frequency, n with (%). *p <0.05, **p <0.01
2. Angiotensin-Converting Enzyme Inhibitor and Angiotensin II Receptor Blocker
3. Oral Glucose Tolerance Test
4. Metabolic syndrome was defined as having 3 or more of the following: Abnormal obesity, high triglycerides, low HDL cholesterol, high blood pressure, or high fasting glucose.
5. Homeostatic Model Assessment of Insulin Resistance was calculated by: HOMA-IR = [(fasting glucose/18) × fasting insulin]/22.5
6. Estimated Creatinine Clearance was calculated using the Cockcroft-Gault formula: eC_{Cr} = [(140-age (years)) × weight (kg)]/(72 x serum creatinine (mg/dL))
7. Estimated Glomerular Filtration Rate was calculated using the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) formula: eGFR (if serum creatinine ≤ 0.9 mg/dL) = 141 x (Serum Creatinine (mg/dL)/0.9)$^{-0.411}$ x 0.993^{Age} ; eGFR (if serum creatinine > 0.9 mg/dL) = 141 x (Serum Creatinine (mg/dL)/0.9)$^{-1.209}$ x 0.993^{Age}
8. Urine Albumin/Creatinine Ratio was calculated by: UACR (mg/g) = Urine Albumin (mg/dL)/Urine Creatinine (g/dL) = Albumin excretion in mg/day

Figure 1. Flow cytometry gating strategy

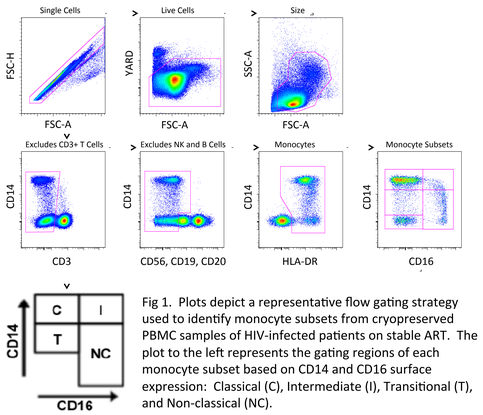

Fig 1. Plots depict a representative flow gating strategy used to identify monocyte subsets from cryopreserved PBMC samples of HIV-infected patients on stable ART. The plot to the left represents the gating regions of each monocyte subset based on CD14 and CD16 surface expression: Classical (C), Intermediate (I), Transitional (T), and Non-classical (NC).

Table 2. Pearson product-moment correlations among UACR and cellular immune parameters[(1)] (n=96)

	CD4 T cells	CD8 T cells	Activated CD8 T cells	Classical Monocytes	Intermediate Monocytes	Transitional Monocytes	Non-classical Monocytes
UACR	r = 0.070	r = 0.150	r = -0.039	r = 0.112	r = 0.067	r = 0.127	**r = 0.238***
CD4 T cells		r = 0.323**	r = -0.008	r = -0.014	r = -0.093	r = 0.110	r = -0.093
CD8 T cells			r = 0.661**	r = 0.368**	r = -0.047	r = 0.312**	r = 0.067
Activated CD8 T cells				r = 0.364**	r = -0.020	r = 0.278**	r = 0.089
Classical Monocytes					r = 0.032	r = 0.306**	r = 0.225*
Intermediate Monocytes						r = 0.016	r = 0.256*
Transitional Monocytes							r = 0.262**

1. UACR and absolute counts of cellular immune parameters were log-transformed prior to analysis
*. Correlation is significant at p <0.05 (2-tailed).
**. Correlation is significant at p <0.01 (2-tailed).

Table 3. Multivariable linear regression model[(1)] using the non-classical ($CD14^{+/low}CD16^{++}$) monocyte subset to predict albuminuria while adjusting for risk factors[(2)] in HIV-infected patients on stable ART (n=96)

	Unstandardized Coefficients		β-value	*p*-value	95% C.I. for B-value	
	B-value	Std. Error			Lower	Upper
Non-classical Monocytes	0.539	0.197	0.259	0.007**	0.148	0.930
Age	0.017	0.008	0.212	0.038*	0.001	0.034
Hypertension	0.260	0.123	0.213	0.037*	0.017	0.504
HOMA-IR	0.026	0.028	0.234	0.017*	0.012	0.125
Total Cholesterol/HDL ratio	0.027	0.030	0.084	0.366	-0.032	0.087
Current use of Tenofovir	0.357	0.132	0.259	0.008**	0.094	0.621
Current use of Ritonavir	0.024	0.113	0.020	0.834	-0.201	0.248

1. The dependent variable for the performed multivariable linear regression model was urine albumin/creatinine ratio (UACR) and was log-transformed prior to analysis. The non-classical ($CD14^{+/low}CD16^{++}$) monocyte subset count was log-transformed prior to analysis and was inputted as a predictor variable for albuminuria to assess its significance in a multivariable model.
2. The multivariable model was adjusted for risk factors of albuminuria. Univariable analyses were done on risk factors to observe level of significance in predicting albuminuria and results are as follows: Age, B=0.017, p=0.035, C.I.=0.001-0.033; Hypertension, B=0.463, p<0.001, C.I.=0.235-0.691; HOMA-IR, B=0.077, p=0.010, C.I.=0.019-0.136; Total Cholesterol/HDL ratio, B=0.067, p=0.046, C.I.=0.001-0.132; Current use of Tenofovir, B=0.215, p=0.131, C.I.=-0.066-0.496; Current use of Ritonavir, B=-0.027, p=0.827, C.I.=-0.274-0.220.

*. Significant at p <0.05 (2-tailed).
**. Significant at p <0.01 (2-tailed).

Table 4. Comparison of urine inflammatory and fibrotic biomarkers between HIV-infected patients on ART with and without albuminuria[(1)] (n=37)

Urine Inflammatory Biomarkers			
Biomarker	Patients with albuminuria	Patients without albuminuria	p-value
n=37	n=18	n=19	
MCP-1[(2)]	80.0 [22.3, 178.9]	132.0 [7.3, 214.8]	0.663
IP-10[(3)]	5.3 [0.4, 17.3]	3.0 [0, 14.8]	0.274
IL-18[(4)]	4.8 [0.9, 17.4]	8.5 [0, 19.0]	0.604
Urine Fibrotic Biomarkers			
TGF-β_1[(5)]	14.4 [5.8, 33.8]	3.5 [0, 11.5]	0.039*
TGF-β_2	12.8 [5.1, 20.5]	7.5 [5.0, 21.3]	0.503
TGF-β_3	8.1 [6.9, 22.1]	7.3 [3.3, 17.3]	0.152
Collagen IV	1160.9 [565.9, 1470.1]	702.0 [344.5, 888.5]	0.042*
TIMP-1[(6)]	678.8 [235.3, 1420.2]	516.5 [171.5, 751.3]	0.178

1. Medians of net median fluorescence intensity (MFI) averages are shown with [median Q1, median Q3].
2. Monocyte Chemotactic Protein-1 (CCL2)
3. IFN-γ-Inducible Protein 10 (CXCL10)
4. Interleukin-18
5. Tumor Growth Factor-β
6. Tissue Inhibitor of Metalloproteinase-1

*. Significant at p <0.05 (2-tailed).
**. Significant at p <0.01 (2-tailed).

Table 5. Pearson product-moment correlations among non-classical ($CD14^{+/low}CD16^{++}$) monocytes and urine biomarkers[(1)] (n=37)

	IP-10	MCP-1	IL-18	TGF-β_1	TGF-β_2	TGF-β_3	Collagen IV	TIMP-1
Non-classical Monocytes	r=0.008	r=-0.141	r=-0.037	**r=0.464***	r=0.063	r=-0.189	r=0.032	r=0.029
IP-10		r=0.393	r=0.334	r=0.393	r=0.346	r=0.039	r=0.254	r=0.297
MCP-1			r=0.187	r=0.314	r=0.520**	r=0.362*	r=0.558**	r=0.592**
IL-18				r=0.201	r=0.001	r=-0.198	r=0.221	r=-0.082
TGF-β_1					r=0.752**	r=0.132	r=0.250	r=0.424*
TGF-β_2						r=0.389*	r=0.617**	r=0.730**
TGF-β_3							r=0.317	r=0.520**
Collagen IV								r=0.848**

1. Absolute counts of non-classical ($CD14^{+/low}CD16^{++}$) monocytes and net MFI averages of urine biomarkers were log-transformed prior to analysis

*. Correlation is significant at p <0.05 (2-tailed).
**. Correlation is significant at p <0.01 (2-tailed).

Summary

- Among a sample population of 96 HIV-infected patients on ART from the HAHC-CVD study who were predominately Caucasian males, 18 patients (19%) had albuminuria. Patients with albuminuria were older; had higher rates of hypertension; and had higher insulin resistance as measured by HOMA-IR.
- The non-classical ($CD14^{+/low}CD16^{++}$) monocyte subset was significantly elevated in patients with albuminuria. No significant differences were observed in T cells and other monocyte subsets.
- The non-classical ($CD14^{+/low}CD16^{++}$) monocyte subset was significantly correlated to UACR. No significant correlations to UACR were observed in the other measured cellular parameters.
- In a multivariable model adjusted for age, hypertension, HOMA-IR, total cholesterol/HDL ratio, and Tenofovir/Ritonavir use; the non-classical ($CD14^{+/low}CD16^{++}$) monocyte subset remained a significant predictor of albuminuria.
- In a subset of 37 patients assessed for urinary inflammatory and fibrotic biomarkers, urine TGF-β_1 and collagen IV was significantly elevated in treated HIV-infected patients with albuminuria as compared to treated HIV-infected patients without albuminuria.
- Elevated levels of urine TGF-β_1 significantly correlated with the elevation of the non-classical ($CD14^{+/low}CD16^{++}$) monocyte subset.

Conclusions

- Albuminuria in HIV-infected patients on stable ART is associated with elevated counts of the non-classical ($CD14^{+/low}CD16^{++}$) monocyte subset.
- Non-classical ($CD14^{+/low}CD16^{++}$) monocytes are associated with elevated levels of TGF-β_1, a potent pro-fibrotic cytokine, in the urine of HIV-infected patients on ART with albuminuria.
- **Our findings suggest that non-classical ($CD14^{+/low}CD16^{++}$) monocytes may play a role in the pro-fibrotic response, which may contribute to renal dysfunction during treated chronic HIV infection.**
- Interventions targeting non-classical ($CD14^{+/low}CD16^{++}$) monocytes and TGF-β_1 may be suitable courses of treatment for HIV-infected patients on ART with albuminuria.
- A larger study needs to be performed to validate findings and to further elucidate the role monocytes play in renal dysfunction in chronic HIV-infected patients on stable ART.

Methods

PBMC isolation and flow cytometry assay

Whole blood was drawn into EDTA tubes and cells were processed for peripheral blood mononuclear cells (PBMC) isolation within one hour of collection and cryopreserved before use. Cryopreserved PBMC were thawed in serum-free media containing 10 µg/ml of DNAse and were rested overnight at 37° C and 5% CO_2 in a polypropylene 96-well plate. Cells were then surface-stained with CD3, CD14, CD16, CD56, CD19, CD20, HLA-DR antibodies, and with Live/Dead fixable yellow dead cell stain (YARD). Data was acquired on a custom 4-laser BD LSRFortessa Cell Analyzer and all compensation and gating analyses were performed in FlowJo analytical software. Percentage of monocyte (classical, intermediate, non-classical, and transitional) subsets were determined based on CD14 and CD16 staining and absolute numbers of each subset were calculated from WBC and monocyte percent obtained from available clinical CBC performed on each patient.

Spot urine collection and Luminex assay

Spot urine samples were collected at entry and were aliquoted and cryopreserved before use. Cryopreserved urine aliquots were thawed and prepared following manufacturer's guidelines for each kit. Each sample was measured in duplicate. Urine MCP-1 and IL-18 were measured using the Bio-Plex Pro™ RBM Human Kidney Toxicity Panel 1 (Bio-Rad). Urine TGF-β_1, TGF-β_2, and TGF-β_3 were measured using the Bio-Plex Pro™ TGF-β Assay (Bio-RAD). Urine IP-10, Collagen IV, and TIMP-1 were measured using the Milliplex® MAP Human Kidney Injury Magnetic Bead Panel 1 (EMD Millipore). Data was acquired on a Luminex 200™ analyzer (Luminex) and data analysis was done using Bio-Plex Manager™ software (Bio-Rad). Net median fluorescent intensity (MFI) was calculated (MFI value minus background value) and average net MFI of duplicate samples was determined.

Statistical analysis

Statistical analyses (Mann-Whitney U and Chi-squared tests for group comparisons, Pearson product-moment for correlations, and multivariable linear regression) were done in SPSS version 22 software and statistical significance was set at a p-value <0.05.

Acknowledgements

This study was supported by grants R01HL095135, U54RR026136, U54MD007584, and G12MD007601, the Dean's Office, John A. Burns School of Medicine, the Vice Chancellor's Office for Research of the University of Hawaii at Manoa. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.