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Characteristics of Activated Monocyte Phenotype Support R5-Tropic Human Immunodeficiency Virus

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ABSTRACT

Background: Microbial translocation has been recognized as an important factor in monocyte activation and contributing to AIDS pathogenesis with elevated plasma lipopolysaccharide (LPS) levels, as a marker for microbial translocation, seen in advanced HIV disease. Therefore, the current study was undertaken to assess monocyte activation in vitro by LPS and to determine its impact on monocyte phenotype. **Methods:** Monocytes from non-HIV-infected donors were analyzed for CD14, CD16, CD69, TNFα, and CCR5 by flow cytometry pre- and post-stimulation with LPS. In-vitro cultures were then set up to expose non-activated and activated monocytes to R5-, X4-, and dual (R5/X4)-tropic viruses; and the amount of HIV present on the cells was assayed. **Results:** Non-HIV-infected monocytes, after LPS stimulation, were confirmed to have an activated phenotype with increase in CD16 and CD69 surface expressions ($p<0.05$). The activation phenotype was supported by increase in TNFα production, $p<0.05$. The activated monocytes had increased surface CCR5 (from 21% to 98%; $p=0.05$); and were found to have more R5-tropic virus than non-activated monocytes ($p<0.05$). **Conclusions:** Following activation by LPS, non-HIV-infected monocytes were found to have increase in surface CCR5. These activated monocytes, when exposed to R5-tropic virus, were found to have more virus compared to non-activated monocytes. The significance of the findings could lie in explaining how microbial translocation plays a role in HIV progression; and possibly promoting CCR5-directed strategies in treating HIV.

BACKGROUND

Systemic immune activation in chronic human immunodeficiency virus type 1 (HIV) infection is thought be due to persistent elevated plasma lipopolysaccharide (LPS) levels due to microbial translocation from the gut [1]. The elevated LPS levels lead to monocyte activation and these activated monocyte are thought to play an important role in HIV disease pathogenesis [2, 3]. Studies by Ancuta, et al suggest a role for elevated LPS levels in driving monocyte activation in HIV disease progression. Because LPS can lead to monocyte activation, the current study was design to characterize if the monocyte phenotype supports the clinical observation by assessing its affect on CCR5, the co-receptor for macrophage-tropic virus. Other studies have shown that CCR5 expression is upregulated on HIV-infected monocytes. We hypothesize that the initial activation of monocytes by LPS is an important mechanism leading to increase CCR5 expression on the cells. While the variability in CCR5 expression on cells is dependent on multiple factors, the initial activation by LPS could promote increase in CCR5, thus leading to an increase of R5 virus on monocytes [4, 5]. If shown that monocytes, after activation with LPS, have increase CCR5 and more R5-virus can be recovered from activated cells, then the paradigm for CCR5-adapted treatment or prevention strategies for HIV-infection can be reexamined.

METHODS AND MATERIALS

The study was designed to characterize monocytes, which were recovered from stimulated peripheral blood mononuclear cells (PBMC) from non-HIV-infected volunteers. In separate experiments, activated and non-activated monocytes were then placed in culture and exposed to virus.

Cells and Cell Cultures

The study was reviewed and approved by the University of Hawaii Institutional Review Board. Heparinized blood from two non-HIV-infected donors were processed as previously described with Dubeco's phosphate buffered saline (PBS) and Ficoll-paque (Amersham Biosciences Inc, Piscataway, NJ) to isolate peripheral blood mononuclear cells (PBMC) [6]. The cells were re-suspended in PBS, 2% fetal bovine serum (FBS) (Mediatech Inc., Herndon, VA); washed and re-suspended in RPMI (Mediatech Inc., Herndon, VA) supplemented with antibiotics (100IU/mL Penicillin, 100ng/mL Streptomycin) and 0.2% normal human serum. Priming of cells with IL-10 (10ng/mL rhIL-10) at 37°C, 5% CO_2 for 20 hours was performed as per a previously-established protocol because plasma IL-10 levels are elevated in HIV-infected patients [7-10]. The protocol was established from published work and in our laboratory provided consistent results with recovery of cells [10]. The primed cells were resuspended in RPMI supplemented with antibiotics, 0.2% normal human serum and 1μg/mL indomethacin (Sigma Aldrich, St. Louis, Missouri), at 6 x 10^6 cells/mL; and incubated in Teflon flasks at 37°C, 5% CO_2 for 20 hours by stimulating with 20ng/mL *Escherichia coli* LPS O111:B4 (Sigma Aldrich, St. Louis, Missouri) [10].

Stimulated PBMC recovered from Ficoll centrifugation were suspended in 100µL PBS, 2% FBS, 1mM EDTA. Monocyte isolation was achieved using the Human Monocyte Enrichment Kit 'without CD16 depletion' (Stem Cell Technologies, Vancouver, Canada). Briefly 10µL of αCD32 blocking antibody was added to prevent non-specific binding to monocytes by blocking monocyte Fc receptors. The cells were kept at 4°C for 5 minutes; followed by the addition of 5µL of the antibody cocktail (CD2, CD3,CD19, CD20, CD56, CD66b, CD123, glycophorin A) and dextran; and kept at 4°C for 5 minutes. Magnetic beads were then added to the cells followed by PBS, 2% FBS, 1mM EDTA buffer; incubated at 4°C; and the cells were magnetically separated. The unbound monocytes were poured off; and the cells re-suspended in 2.5mL PBS, 2% FBS, 1mM EDTA. The recovered activated monocytes ($CD14^+/CD16^+$ phenotype) were washed twice with 2% FBS/PBS. An aliquot of the activated monocytes was analyzed by flow cytometry. The remaining cells were used for the virus culture experiments described below. Non-activated monocytes were obtained from PBMC isolated from fresh whole blood from the same donors by Ficoll centrifugation followed by Easysep Monocyte Enrichment Kit 'with CD16 depletion' as described above (Stem Cell Technologies, Vancouver, Canada). The antibody cocktail consisted of CD2, CD3, CD19, CD20, CD56, CD66b, CD123, glycophorin A; and anti-CD16 to deplete $CD16^+$ monocytes. An aliquot of the non-activated monocytes were stained and analyzed by flow cytometry; with the remainder of the non-activated monocytes placed in culture for the virus experiments. As described by Adib-Conquy et al, similar experiments were set up by stimulating whole blood and monocytes to determine if differences in stimulating in the presence of other cells is noted [10].

Cell Staining and Phenotypic Characterization

Monocytes, both activated and non-activated, were washed twice and re-suspended in 100μL PBS, 2% FBS. Fluorochrome-conjugated antibodies (anti-CD14-FITC, anti-CD16-Alexa 674, anti-CCR5-APC Cys7, and anti-CD69-Cy7; BD Biosciences Pharmingen, San Diego, CA.) were added as per manufacturer's specifications. The cells were incubated for 20 minutes at room temperature; washed with PBS 2% FBS twice and then fixed in 200μL 1% Para formaldehyde, 2% FBS; and analyzed using FACSAria (BD Biosciences, San Jose, CA) and FACSDiva software (BD Biosciences, San Jose, CA).

Intracellular staining for TNF-α was carried out following LPS stimulation by adding brefeldin A (Sigma Aldrich, St. Louis, Missouri) to the cells three hours before harvesting. The cells were washed twice in PBS, 2% FBS and stained for cell surface markers as above. The cells were then permeabilized with 200μL fixation and permeabilization buffer (BD Biosciences, San Jose, CA); and incubated for 20 minutes. After permeabilization, the cells were washed with the wash buffer; centrifuged; re-suspended in 100μL PBS, 2% FBS with anti-TNF-α-PE; and incubated for 20 minutes at room temperature. The cells were washed with PBS, 2% FBS ; fixed in PBS, 1% Para formaldehyde, 2% FBS; and analyzed as noted above.

HIV-1 Exposure on Activated Monocytes and Non-Activated Monocytes

Monocytes (activated and non-activated monocytes) were isolated and purified from fresh whole blood from the same donors noted above using Ficoll and magnetic bead selection as outlined above. To expose cells to virus, activated and non-activated monocytes were placed separately in 96-well plates ($5x10^5$ cells/well) with the addition of 2ng p24 units of LAI (X4-

tropic strain; NIH AIDS Research and Reference Reagent Program, Bethesda, MD), BaL (R5-tropic strain; NIH AIDS Research and Reference Reagent Program, Bethesda, MD); or p89.6 (dual-X4/R5-tropic strain) HIV-1 strains for 1 hour in triplicate. Following three washes, the cells and virus were lysed; and RNA isolated. The final supernatants from the third washes were recovered to verify that no virus was present in the last wash. The supernatants were also assessed for HIV p24 (XpressBio, Thurmont, MD) to verify that no active virus was present that could suggest that the cells were infected.

The amount of virus that was recovered from the remaining cells was assayed from the isolated RNA by RT-PCR using HIV Gag and β-actin primers with appropriate positive and negative control RNA. Amplified fragments were resolved on 2.5% agarose gels and analyzed by densitometry. From the scanned gels, the ratio of HIV gag light units/β-actin light units was compared between the activated and non-activated monocytes and between the viruses.

Statistical Analyses

Results from the experiments carried out in triplicate were expressed as percentage of cells stained for the selected marker before and after stimulation; and the data were analyzed for statistical significance using the Student's t test for paired values. Differences were considered significant at $p < 0.05$. Experiments, carried out in triplicate, showing differences in the amount of virus detected between cell groups were considered significant by Student's t-test if $p<0.05$.

RESULTS

Monocytes from non-HIV-infected donors were characterized before and after activation to determine the phenotypic change due to LPS-stimulation. The monocytes, before and after stimulation, maintained their CD14-expression with no significant change in surface expression of CD14 (87% versus 92%, respectively), $p>0.05$, Figure 1A, 1B. To confirm the activation status, monocytes were stained for CD16 expression, which showed significant increase in percentages of monocytes with CD16 from 9.5% to 89%, $p<0.05$, Figure 1E. Other markers of monocyte activation confirmed the phenotype after LPS-stimulation with CD69 expression and assessment TNF-α production. Following LPS-activation, expression of surface CD69 on activated monocytes increased from 18% to 91%, Figure 1D. Similarly, production of intracellular TNF-α increased from 0% to 33% post-LPS stimulation, $p<0.05$, Figure 1F. Surface CCR5 expression was assessed on activated monocytes, which increased from 21% to 99% ($p=0.06$), Figure 1C. The parallel experiments that were set up by stimulating whole blood and monocytes, showed similar results in activation phenotype as previously suggested by Adib-Conquy, et al [10]. We assessed the effect of IL-10, by itself, on monocyte phenotype because plasma IL-10 is increased in HIV-infected individuals [7]. When IL-10 was used to prime cells prior to LPS stimulation, more consistency in the amount of activation was demonstrated as others have shown [10].

To assess the impact of increase in CCR5 expression on activated monocytes compared to non-activated monocytes, the experiments with the three different types of HIV-strains (LAI, BaL, and p89.6) confirmed that activated monocytes had more BaL (X5-tropic) compared to non-activated monocytes exposed to the same virus, $p<0.05$, Figure 2. For both LAI (X4-tropic) and p89.6 (R5/X4-tropic), the amount of virus recovered from activated monocytes did not differ

from non-activated monocytes, $p>0.05$, Figure 2. The effect of IL-10, alone, on CCR5 expression, showed a slight increase in CCR5 expression on monocytes, as previously shown [11]. In comparison to IL-10-priming prior to LPS stimulation, however, the level of activation and CCR5 increase was more consistent and greater in magnitude with the IL-10 priming.

The supernatants recovered from the washing steps after an hour-exposure of the cells to the virus showed decreasing amounts of HIV with the last wash having no detectable HIV. This confirmed that the cells left in the culture wells had no free virus. The last wash was negative for p24, consistent with undetectable virus replication.

DISCUSSION

The current study focused on assessing the phenotype of non-HIV-infected monocytes before and after activation; and determined if activated monocytes had more R5-tropic virus. Following activation by LPS, monocytes in culture consistently had higher CD16 expression on the surface of M/MΦ, which was expected since CD16 is a known marker of monocyte activation [12]. Other markers of monocyte activation were also increased: TNFα production and CD69 expression [13]. While CCR5 is a known receptor on monocytes, our results demonstrate that an increase in CCR5 surface expression could be induced on M/MΦ upon stimulation with LPS, with some heterogeneity noted in the staining. While we also demonstrated that IL-10, by itself, up-regulates CCR5 gene expression on human monocytes, as previously shown; using IL-10 to prime the cells prior to LPS stimulation provided a more consistent model for activation, as shown by others [10, 11]. Our results are in contrast to a previous study showing decreased CCR5 expression after LPS-stimulation of monocyte-derived macrophages [14]. However, our data were obtained from primary monocytes, which is a different model than what was used by Franchin et al [14].

We acknowledge that the data generated are limited by the number of donors who provided PBMC and monocytes for the study. Because the baseline phenotype of monocytes varies amongst individuals, and some volunteers we attempted to isolate monocytes had baseline activated phenotype, we limited the current studies to donors with low baseline activated monocytes [15]. Increase in donor pools is currently being planned as resources become available in order to assess the generality of the results. Additional limitations of the study include the number of replicates performed was limited, which will need to be increased in order to assess any trend or significance.

The significance of monocyte activation occurring in parallel with increase surface expression of CCR5 HIV co-receptor lies in the increased potential for CCR5-tropic viral strains to bind and infect the cells. In the setting of microbial translocation with increase plasma LPS levels, non-HIV-infected M/MΦ could be exposed to this chronic inflammatory environment which could increase surface expression of CCR5. This may, in turn, provide a mechanism for these cells to bind more R5-tropic virus and infect the cells. Our results are consistent with recent findings clinically where elevated plasma LPS was suggested to be a likely cause of systemic immune activation in chronic HIV infection, which induces monocyte activation [1]. The implications are that peripheral immunological events may play a key role in the pathogenesis of HIV-1. Therefore, these activated cells could be targets for preventing newly-formed M/MΦ from becoming infected as they enter the circulation. New R5-inihibitor drugs could be identified as targets for future consideration in treatment or preventative strategies [16]. In conclusion, we believe that M/MΦ activation by LPS leads to a phenotype that supports R5-tropic virus. If confirmed through expanded studies, the information could shift the paradigm of how to treat or intervene to prevent M/MΦ from being infected chronically.

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FIGURE LEGENDS

Figure 1. Representative Scatter Plots of M/MΦ Surface Expression Markers Following LPS Stimulation. Cells were stained for CD14, CD16, CD69, TNFα, and CCR5. The scatter plots show examples of the post-LPS stimulation experiments, n=3. **Panel A**: Monocytes gated on forward- and side-scatter plots; **Panel B**: Compared to pre-LPS stimulation, expression of CD14 on isolated monocytes remained stable before and after stimulation; 87% versus 92%, $p>0.05$; **Panel C**: CCR5 expression on CD14 monocytes increased from 21% to 99%, $p=0.06$; **Panel D**: CD69 expression after LPS stimulation on CD14 monocytes increased from to 18% to 91%, $p<0.05$; **Panel E**: CD16 expression increased from 9.5% to 89% after LPS stimulation, $p<0.05$; **Panel F**: TNFa production increased from 0% to 33% after LPS stimulation, $p<0.05$.

Figure 2. Relative Amounts of Virus Recovered from Activated versus Non-Activated Monocytes. **A:** Higher amount of BaL virus (R5-tropic) recovered from activated monocytes compared to non-activated monocytes, $p<0.05$, n=3; **B & C**: For both LAI (X4-tropic) and p89.6 (R5/X4-tropic), the amount of virus recovered from activated monocytes did not differ from non-activated monocytes, $p>0.05$, n=3 for both viruses.

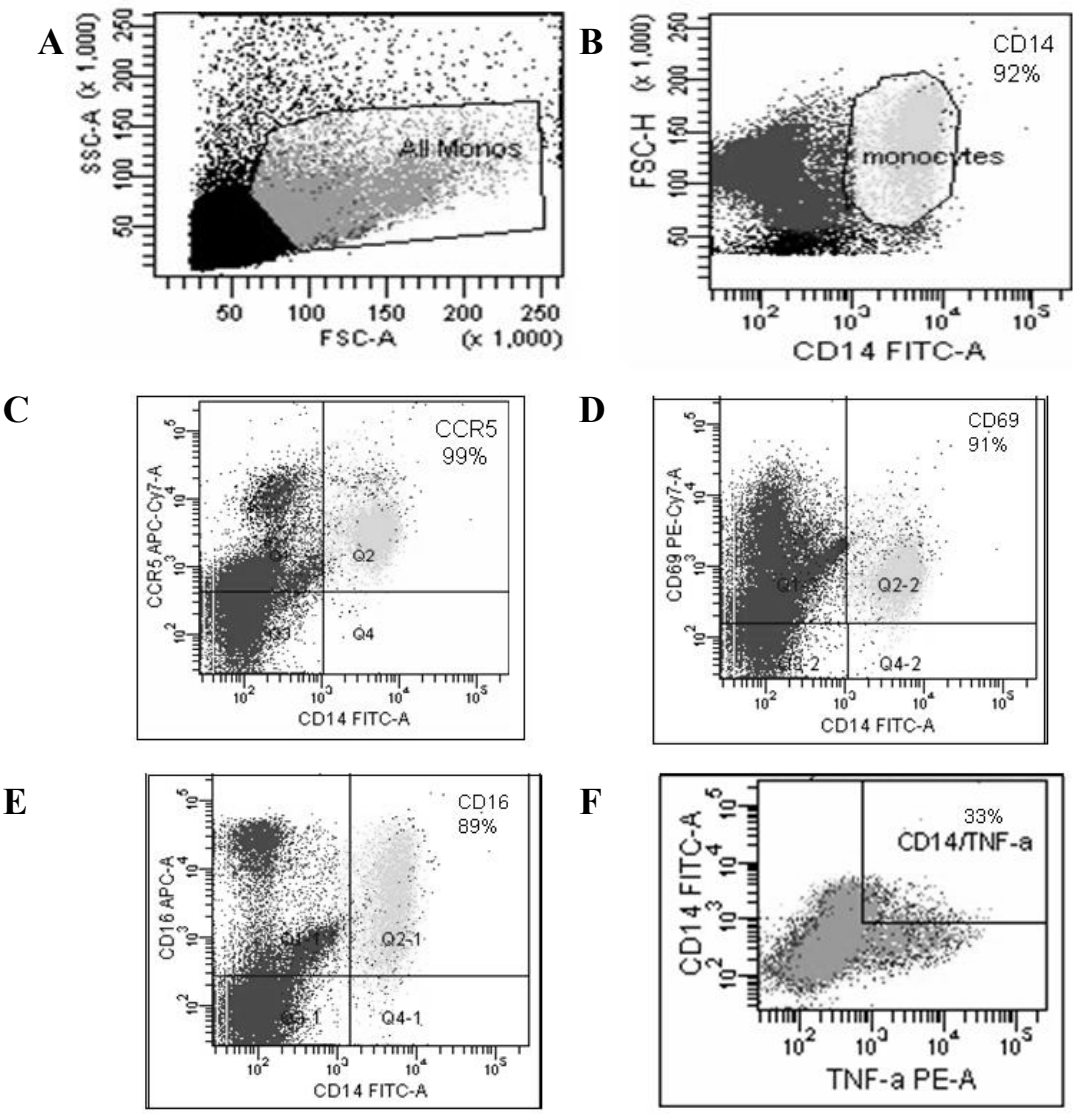
A
SSC-A (x 1,000)
FSC-A (x 1,000)
All Monos
B
FSC-H (x 1,000)
CD14 FITC-A
CD14
92%
monocytes
C
CCR5 APC-Cy7-A
CD14 FITC-A
CCR5
99%
Q2
Q4
D
CD69 PE-Cy7-A
CD14 FITC-A
CD69
91%
Q2-2
Q4-2
E
CD16 APC-A
CD14 FITC-A
CD16
89%
Q2-1
Q4-1
F
CD14 FITC-A
TNF-a PE-A
33%
CD14/TNF-a

Figure 1

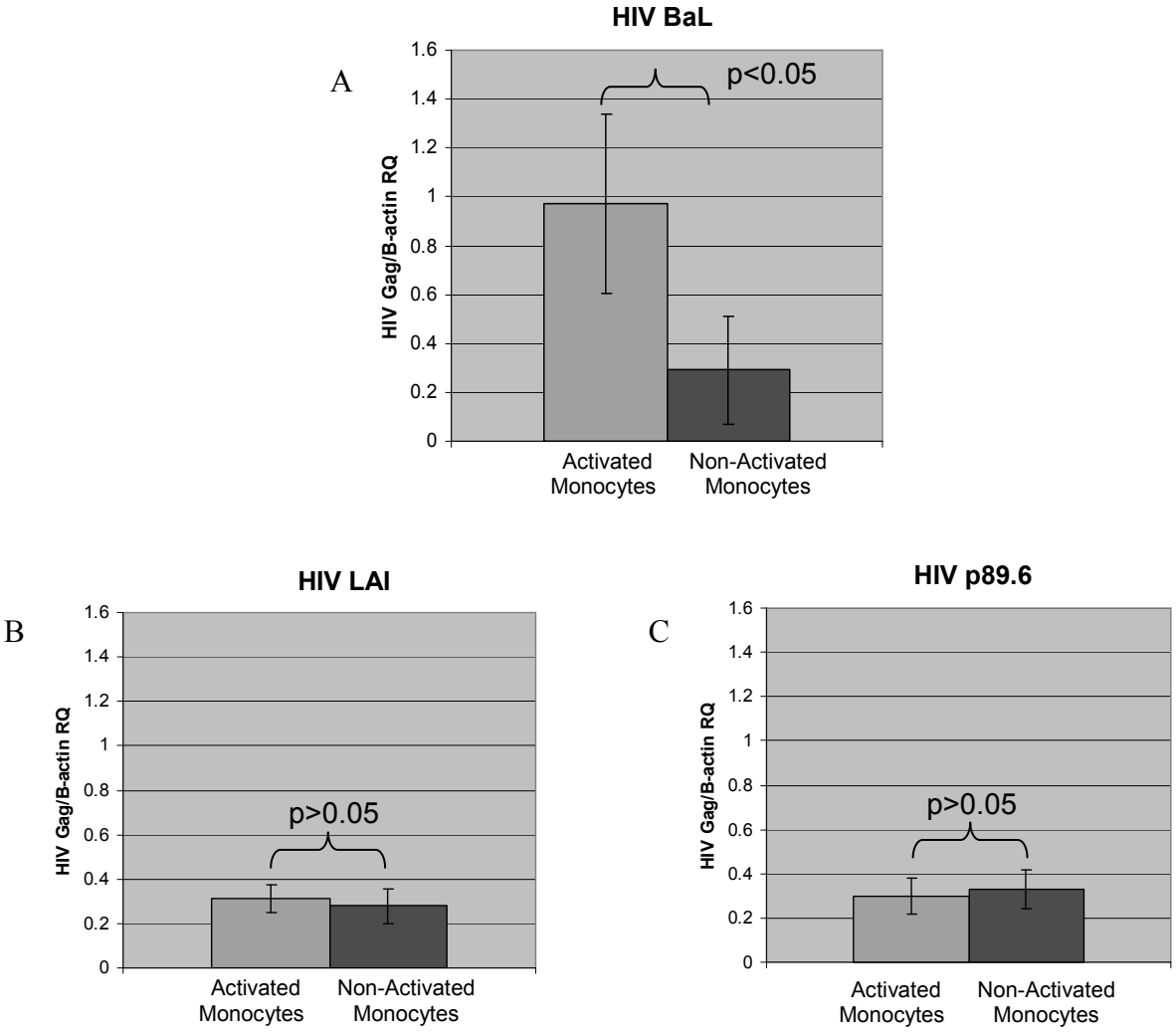
A
HIV BaL
HIV Gag/B-actin RQ
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
p<0.05
Activated Monocytes
Non-Activated Monocytes
B
HIV LAI
HIV Gag/B-actin RQ
p>0.05
Activated Monocytes
Non-Activated Monocytes
C
HIV p89.6
HIV Gag/B-actin RQ
p>0.05
Activated Monocytes
Non-Activated Monocytes

Figure 2