Monocytes from HIV-infected individuals show impaired cholesterol efflux and increased foam cell formation after transendothelial migration

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\textbf{Design:} HIV-infected (HIV+) individuals have an increased risk of atherosclerosis and cardiovascular disease which is independent of antiretroviral therapy and traditional risk factors. Monocytes play a central role in the development of atherosclerosis, and HIV-related chronic inflammation and monocyte activation may contribute to increased atherosclerosis, but the mechanisms are unknown.

\textbf{Methods:} Using an in-vitro model of atherosclerotic plaque formation, we measured the transendothelial migration of purified monocytes from age-matched HIV+ and uninfected donors and examined their differentiation into foam cells. Cholesterol efflux and the expression of cholesterol metabolism genes were also assessed.

\textbf{Results:} Monocytes from HIV+ individuals showed increased foam cell formation compared with controls (18.9 vs. 0\%, respectively, \(P = 0.004\)) and serum from virologically suppressed HIV+ individuals potentiated foam cell formation by monocytes from both uninfected and HIV+ donors. Plasma tumour necrosis factor (TNF) levels were increased in HIV+ vs. control donors (5.9 vs. 3.5 pg/ml, \(P = 0.02\)) and foam cell formation was inhibited by blocking antibodies to TNF receptors, suggesting a direct effect on monocyte differentiation to foam cells. Monocytes from virologically suppressed HIV+ donors showed impaired cholesterol efflux and decreased expression of key genes regulating cholesterol metabolism, including the cholesterol transporter \textit{ABCA1} \((P = 0.02)\).

\textbf{Conclusion:} Monocytes from HIV+ individuals show impaired cholesterol efflux and are primed for foam cell formation following transendothelial migration. Factors present in HIV+ serum, including elevated TNF levels, further enhance foam cell formation. The proatherogenic phenotype of monocytes persists in virologically suppressed HIV+ individuals and may contribute mechanistically to increased atherosclerosis in this population.

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Introduction

Cardiovascular disease (CVD) is an increasing cause of morbidity and mortality in HIV+ individuals receiving combination antiretroviral therapy (cART) [1,2]. HIV infection is associated with an increased risk of cardiovascular conditions, including atherosclerosis [3,4] and coronary heart disease [5,6] and an approximately two-fold increased risk of myocardial infarction [7,8]. Importantly, increased CVD risk in HIV+ individuals is independent of traditional risk factors [5–10] and risk prediction algorithms based on these factors alone used in the general community setting underestimate atherosclerosis in HIV+ individuals [11]. Although cART and specific antiretroviral agents may potentiate CVD [12–14], the increased CVD risk observed in HIV+ individuals who are treatment naive [13], undergoing treatment interruption [15], and in elite controllers [16] suggests the involvement of cART-independent factors. Taken together, these observations suggest that unique mechanisms exist in HIV+ individuals which act in addition to traditional factors to increase CVD risk, and these effects persist despite viral suppression.

In HIV+ individuals, CVD is associated with markers of inflammation [17–19] and is increasingly reported in association with markers of monocyte/macrophage activation. We have shown that monocyte and innate immune activation persist in HIV+ individuals despite viral suppression [20,21], and markers of monocyte/macrophage activation, including soluble (s)CD163 [22,23], sCD14 [24] and cellular monocyte activation markers [25–27], are associated with atherosclerosis and CVD in HIV infection. Despite the significant links between chronic inflammation/monocyte activation and increased CVD risk in HIV infection, the mechanism remains unclear.

Proinflammatory cytokines such as tumour necrosis factor (TNF) potentiate atherosclerosis; they activate endothelial cells and monocytes, resulting in increased expression of adhesion molecules, release of chemotactants by endothelial cells, lipid transcytosis and oxidation of low-density lipoprotein (LDL) [28]. Activated monocytes are recruited to inflamed blood vessels and migrate into the vascular neointima, wherein they endocytose lipids and either exit via reverse transendothelial migration or develop into foam cells by phagocytosing highly inflammatory oxidized low-density lipoprotein (oxLDL; via scavenger receptors SR-A and CD36) [29]. Foam cells have reduced migratory capacity [30] and accumulate in the neointima wherein they release proinflammatory molecules contributing to further recruitment of monocytes and progression of atherosclerosis [31]. The ability of macrophages to exit the neointima and remove cholesterol via reverse transendothelial migration is critical to retarding the development of atherosclerotic plaques and promoting plaque regression [32].

We hypothesized that monocyte activation in the setting of chronic HIV infection [20] promotes atherosclerosis. We have previously developed a novel in-vitro model of the initiation of atherosclerotic plaque formation that couples transendothelial migration of primary human monocytes across an activated endothelium with foam cell formation [33–35]. Here, we adapted this model to investigate the atherogeneic potential of monocytes isolated from HIV+ individuals and determine whether inflammatory factors elevated in HIV infection influence early atherosclerotic events mediated by monocytes.

Methods

Recruitment and blood processing
Blood was obtained from HIV+ donors recruited from the Department of Infectious Diseases, The Alfred Hospital, Melbourne, Australia, and healthy control donors of a similar age following written, informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated within 2h of sample collection and were either used immediately (for migration assays) or stored in liquid nitrogen for later mRNA and cholesterol efflux analysis. Monocytes were further purified from PBMC via negative selection using magnetic beads (Miltenyi Biotec, Cologne, Germany) as per the manufacturer’s protocol, which yields monocytes with a purity of 80–85% as determined by flow cytometry (not shown). This study received ethical approval from The Alfred Research and Ethics Committee and from the Royal Women’s Hospital Ethics Committee, Melbourne, Australia.

Cell migration assay and analysis
Hydrated collagen gels were prepared in a 96-well format as described previously [33,36]. Gels were incubated at 37°C for 4–6 days until use. Primary human umbilical vein endothelial cells (HUVEC) were prepared as described [36] and used without further passage. 2 × 10⁴ HUVEC were added to each collagen gel and incubated in Medium 199 (Life Technologies, Carlsbad, California, USA) containing 20% human serum for 3 days to allow confluent monolayers to form. Media were prepared using a single batch of pooled human serum (pHS) prepared from six HIV-seronegative blood donors (Australian Red Cross Blood Service, Sydney, Australia) or autologous serum (from the same donor as the monocytes) as indicated; all sera were heat inactivated at 56°C for 30 min before use. Silver staining was performed on selected wells, in addition to routine phase-contrast microscopy, to verify HUVEC monolayer integrity (Supplementary Fig. 1A) [33]. HUVEC were activated with 10 ng/ml TNF for 4 h [35] or left unactivated, then freshly isolated PBMCs (2 × 10⁷/well) or purified monocytes (5 × 10⁷/well) added for 1 h at
37 °C. Nonmigrated cells were removed by washing and cultures incubated for a further 48 h as described [33]. For TNF blocking, 10 or 20 µg/ml anti-tumour necrosis factor receptor (TNFR) I and anti-TNFRII (R&D Systems, Minneapolis, Minnesota, USA), or respective isotope controls, were added immediately following monocyte migration. Forty-eight hours after monocyte migration, reverse-migrated cells were removed and collagen gels stained with Oil Red O as described [35]. Gels were excised from wells, mounted on glass slides and foam cells counted by bright field microscopy (x40). Foam cells were defined as cells containing Oil Red O stained vesicles within the cytoplasm and determined as a proportion of total migrated cells within the counted area of the gel (Supplementary Fig. 1B). To investigate the phenotype of migrated cells within the collagen matrix, cells were extracted from the collagen by washing the gels without fixation and incubating in 1 mg/ml collagenase D for 20 min at 37 °C, after which they were macerated manually and incubated further for 20 min at 37 °C. Resulting cell suspensions were filtered through 100 µm mesh prior to staining for flow cytometric analysis. Cells extracted from collagen gels were stained with 0.2 µg/ml BODIPY 493/503 (Life Technologies, Carlsbad, California, USA) in 150 mM NaCl for 20 min at room temperature and anti–TNF-PE, CD36–FITC, CD14–APC and CD11b–PE (all from BD Biosciences, San Jose, California, USA) as described [20]. Cells stained with anti–CD14 APC and BODIPY were sorted using a BD influx cell sorter. The specificity of phenotyping antibodies was verified via the use of isotype control antibodies on selected samples (Supplementary Figure 2).

Live cell imaging
Purified monocytes were stained with PKH26 (Sigma-Aldrich, St Louis, Missouri, USA) according to manufacturer’s instructions and added to HUVEC monolayers grown on collagen gels in 96-well optical plates (Greiner Bio-One, Frickenhausen, Germany). Cells were immediately incubated at 37 °C in a humidified temperature-controlled chamber coupled to a DeltaVision deconvolution microscope (Applied Precision-GE Healthcare, Issaquah, Washington, USA) and images were captured in z-series on a charge-coupled device camera (AxioCamMRm Rev. 3, Carl Zeiss Microscopy, Jena, Germany) through a 20 ×, 0.45 NA lens. Detection and tracking of cell migration was performed using the tracking module of the Imaris software (Bitplane, Zurich, Switzerland).

Analysis of plasma lipids and soluble factors
Plasma TNF and CXCL10 levels were measured using commercial ELISA kits (human TNF-α ELISA, Cardinal Bio-research, New Farm, Australia, and Human CXCL10/IP-10 Quantikine ELISA KIT, R&D Systems, Minneapolis, Minnesota, USA). Cholesterol and oxidized LDL levels in plasma were measured using the HDL and LDL/VLDL Cholesterol Assay Kit and the Human Oxidized LDL ELISA Kit (CML-LDL) respectively (both from Cell Biolabs, San Diego, California, USA).

Cholesterol efflux assays
PBMCs (2.5–5 × 10⁶) were incubated for 1 h at 37 °C in RPMI containing 10% FBS (RH-10), 1.5 µmol/l BODIPY-cholesterol (Avanti Polar Lipids, Alabaster, Alabama, USA), 6 µmol/l cholesterol and 300 µmol/l methyl-β-cyclodextrin (both from Sigma-Aldrich). Cells were washed twice with PBS (PBS without Mg²⁺ or Ca²⁺), resuspended in RH-10 and cholesterol was allowed to efflux from the cells for 30 min at 37 °C in either the absence or presence of 1 µmol/l of methyl-β-cyclodextrin. Cells were washed, fixed with 1% formaldehyde, immunostained as above and analyzed by flow cytometry.

Gene expression analysis of cholesterol efflux and metabolism intermediates
The expression of proteins involved in cholesterol metabolism and transport was assessed via quantitative real-time PCR (qPCR). Monocytes were isolated from frozen PBMC, rested for 4 h in RPMI containing 20% FBS at 37 °C and total RNA was extracted. Potential DNA contamination was removed by DNase I treatment (37 °C for 20 min, 75 °C for 10 min, Roche, Basel, Switzerland). cDNA synthesis was performed using a combination (1:2) of oligo(dT) and random hexamer primers, respectively (Transcriptor First Strand cDNA synthesis kit, Roche) and qPCR analysis was performed using FastStart Universal SYBR Green Master Mix (Roche) for GAPDH, adenosine triphosphate (ATP)-binding cassette transporter A1 (ABCA1) and Acyl-coenzyme A: cholesterol acyltransferase (ACAT), and Brilliant SYBR II (Agilent Technologies, Santa Clara, California, USA) for ABCG1 and HMG-CoA reductase on a MX3005P qRT-PCR machine (Agilent Technologies). The primers used were as follows: GAPDH (Fwd 5′-CATGTCACCTCAAGGC-3′, Rev 5′-CCAG-CATCGCCCCACTTG-3′), ABCA1 (Fwd 5′-GACTCTAGAAATGCTGAAA-3′, Rev 5′-AGTTCCCTGGAGGTCTTGTTCAC-3′), ABCG1 (Fwd 5′-CAGGAAGATTAGACACTGGTG-3′, Rev 5′-GAAGGG-GAATGGGAGAGAA-3′), ACAT (Fwd 5′-CAAGGGCTCCTCTTTAGATGAAC-3′, Rev 5′-GAATAAACCTACACTACCTCAG-3′), and HMG-CoA reductase (Fwd 5′-CGCGAGAAGTTGATGATGAGAG-3′, Rev 5′-CGACCTGTGTGTAACATGTCATT-3′) at final concentration of 280 nmol of each primer. PCR conditions were 95 °C for 10 min then 40 cycles of 95 °C for 15 s, 60 °C for 1 min. Absolute quantification of gene expression was determined via comparison with a standard curve generated from serial dilution of a plasmid engineered to contain the amplicon of interest. Expression of all genes was standardized to GAPDH.

Data and statistical analysis
Nonparametric, two-tailed Mann–Whitney U test was used for unpaired data and Wilcoxon rank-sum test for
paired data. Parametric unpaired t-test was used for data passing the D’Agostino–Pearson omnibus normality test. Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, California, USA) where values of $P<0.05$ were considered statistically significant.

Results

HIV infection increases foam cell formation by monocytes following transendothelial migration

We have previously shown that HIV infection of monocyte-derived macrophages in vitro impairs their ability to reverse migrate across a HUVEC–collagen gel model of early atherosclerotic events [36], suggesting these cells may have a greater propensity to differentiate into foam cells, and thus be retained in the collagen matrix. To investigate whether HIV infection in vivo is associated with increased propensity for monocytes to develop into foam cells, we utilized a refined version of this model to analyze the atherogenic properties of monocytes isolated by negative selection from fresh whole blood. Ageing is well documented to increase the risk of atherosclerosis, thus we investigated the effect of HIV infection in younger HIV+ individuals and uninfected controls of a similar age. Monocytes were isolated from seven healthy controls [all men, median age (range) 29.0 (25–56) years] and eight HIV+ men [median age 36.5 (26–47) years]. Of the HIV+ individuals, four were receiving cART and had low (80 copies/ml, $n=3$) viral loads and the remaining four were not receiving cART and had a median viral load of 23 400 copies/ml (range: 300–254 800 copies/ml).

When added to unstimulated HUVEC in media containing 20% pHS, a significantly increased proportion of monocytes from HIV+ individuals differentiated into foam cells as compared with monocytes from controls (median foam cells 18.9 vs. 0%, respectively, $P=0.004$, Fig. 1A; all samples tested using the same batch of pHS). To determine whether factors present in HIV+ serum may further influence foam cell formation, we analyzed foam cell formation using culture media containing autologous serum rather than pHS and found that although foam cell formation by control monocytes was not significantly altered in the presence of autologous serum, serum from HIV+ individuals significantly increased foam cell formation by autologous monocytes ($P=0.03$, Fig. 1A).

Consistent with our previous findings, activation of HUVEC with TNF prior to monocyte migration significantly increased foam cell formation by monocytes from control donors ($P=0.016$ for both pHS and autologous serum, Fig. 1A vs. B) and also HIV+ monocytes in the presence of pHS ($P=0.008$). As seen with unactivated HUVEC, autologous serum from HIV+ but not controls, significantly increased foam cell formation when monocytes were added to TNF-activated HUVEC ($P=0.02$ vs. 0.30, respectively, Fig. 1B). The increased rate of foam cell formation by control monocytes following endothelial cell activation meant that differences between control and HIV+ monocytes were only observed in the presence of autologous serum ($P=0.03$,

Fig. 1. Monocytes from HIV+ individuals show increased foam cell formation, which is enhanced by HIV+ serum. Percentage of foam cells present within collagen were measured following Oil Red O staining, as described in Methods, 48h after monocyte migration for seven HIV− controls and eight HIV+ male donors using (a) nonactivated and (b) TNF-activated endothelium (HUVEC) in the presence of either pooled human control serum (pHS – identical batch used for all samples) or autologous serum (AS) from each individual donor. Bars represent median and IQR of data collected from 11 independent experiments. (c) Percentage of foam cells present within collagen was measured using monocytes prepared from six different HIV-negative donors added to TNF-activated HUVEC, and incubated with either pHS or serum from five different HIV+ donors receiving cART and with viral load <20 copies/ml. Plots show median and IQR. Paired two-tailed Wilcoxon rank-sum test was used for within group comparisons and unpaired two-tailed Mann–Whitney U test for between group comparisons. *$P<0.05$, **$P<0.01$. AS, autologous serum; cART, combination antiretroviral therapy; HUVEC, human umbilical vein endothelial cells; IQR, interquartile range; pHS, pooled human serum; TNF, tumour necrosis factor.
Fig. 1B). Similar levels of foam cell were formed by monocytes from young HIV+ individuals receiving cART with low/undetectable viral loads as compared with young untreated HIV+ individuals with viraemia (Supplementary Fig. 3A and B). We also analyzed foam cell formation in an expanded cohort of 17 HIV+ men aged 26–68 years consisting of 13 individuals receiving cART (12 virologically suppressed with plasma viral loads less than 40 copies/ml and one individual with 80 copies/ml) and the four viraemic individuals not receiving cART defined above. This analysis showed a nonsignificant trend towards increased foam cell formation with age; however, there were no differences between virologically suppressed and nonsuppressed HIV+ individuals (Supplementary Fig. 3C). Data from this expanded cohort of largely virologically suppressed individuals also confirmed the proatherogenic effect of autologous HIV+ serum (Supplementary Fig. 3D).

We then determined whether the proatherogenic effect of HIV+ serum was only evident in combination with autologous HIV+ monocytes or whether it was also capable of enhancing foam cell formation by monocytes from HIV-seronegative individuals. To investigate this, we analyzed foam cell formation in the model described above using monocytes from six HIV-uninfected controls in the presence of either pHS, or serum from five cART-treated, virologically suppressed HIV+ individuals. Foam cell formation by control monocytes was significantly increased by all five HIV+ sera used (Fig. 1C), confirming the presence of soluble factor(s) in HIV+ sera that promote foam cell formation. Significantly, the sera used in this experiment were all from patients with undetectable viral load, indicating the responsible factors remain elevated despite viral suppression. The above data indicate that HIV infection promotes the differentiation of monocytes into foam cells via a mechanism involving both soluble factors present in the serum and an intrinsic alteration to monocyte function.

**Live cell imaging of monocyte migration in the transendothelial migration assay**

To investigate the effect of HIV infection on monocyte movement postmigration, we labelled monocytes with the fluorescent membrane dye PKH26, and recorded individual cell tracks within the collagen layer using live cell imaging over 48 h (Fig. 2 and Fig2mov1 and Fig2mov2). Tracks obtained from monocytes from HIV+ individuals showed a greater number of slowly migrating cells (indicated by yellow foci in Fig. 2B vs. control donor in 2A), consistent with more monocytes developing into nonmotile foam cells. This behaviour was observed for monocytes from virologically suppressed HIV+ individuals receiving cART using either control serum or autologous serum (data not shown). We calculated the distribution of displacement length using tracks combined from at least three independent experiments and found that monocytes from HIV+ individuals migrated shorter distances than those from controls (evidenced by a shift to the left in the distance frequency histograms for HIV+ samples, Fig. 2C) and this effect was heightened in the presence of autologous HIV+ serum (Fig. 2C).

Although the initial migration speed of monocytes from HIV+ individuals was not different from control monocytes in the presence of pHS, the presence of autologous serum was associated with a significant increase in initial migration speed (Fig. 2D), suggesting factors in HIV+ sera accelerate monocyte migration at early time points after transendothelial migration. In contrast, tracks from HIV+ donors during the later stages of observation (i.e., 38–48 h) showed a lower migration speed as compared with control monocytes (Fig. 2E). Interestingly, the average migration speed of monocytes from HIV+ individuals was higher in the presence of autologous serum vs. pHS, although the speed was still significantly lower than that of control monocytes (Fig. 2E). Analysis of monocyte speed over the entire observation window (0–48 h) confirmed the overall average migration speed of monocytes from HIV+ individuals was lower than that of controls (data not shown). These data indicate that monocytes from HIV+ individuals have impaired movement post-transendothelial migration, consistent with their differentiation into foam cells, and that factors present in the serum of HIV+ individuals enhance this effect.

**Plasma tumour necrosis factor and CXCL10 levels are increased in HIV+ individuals**

HIV infection is associated with dyslipidaemia because of the combined effects of HIV and certain antiretroviral drugs which may influence the atherogenic properties of monocytes and sera from HIV+ individuals. To determine whether altered cholesterol levels in autologous serum were responsible for the increase in foam cell formation by monocytes from HIV+ individuals in this study, we measured plasma levels of high-density lipoprotein, LDL and oxLDL by ELISA. No significant differences were observed in plasma levels of any of these forms of cholesterol between HIV+ and control individuals (Supplementary Fig. 4), suggesting that the heightened foam cell formation by HIV+ monocytes observed here is unlikely to be due solely to altered levels of these cholesterol species in the blood.

Inflammatory factors activate monocytes and can also drive foam cell formation independent of dyslipidaemia [37]; therefore, we measured plasma levels of the proinflammatory cytokine TNF and the monocyte activation biomarker CXCL10 in our cohort. Both CXCL10 and TNF levels were significantly elevated in HIV+ as compared with controls (Fig. 3A and B, $P=0.01$ and 0.03, respectively). Taken together, these data suggest that plasma markers of monocyte activation and inflammation, but not plasma cholesterol levels, are significantly increased in these HIV+ individuals who also show increased foam cell formation.
Anti-tumour necrosis factor receptor blocks foam cell formation independent of human umbilical vein endothelial cell activation

We have shown here and previously [35] that TNF activates endothelial cells to increase foam cell formation, and plasma TNF levels have been found to be elevated in HIV patients irrespective of cART [38]. We therefore considered whether TNF promotes foam cell differentiation independently of its effects on HUVEC. Monocytes from control donors were allowed to migrate through TNF-activated HUVEC, then blocking antibodies to TNFR I and II were added to media containing either control pHS or serum from virologically suppressed HIV+ donors and cells incubated for 48 h. Monocytes from each control donor were incubated with serum from three different HIV+ donors, with serum from a total of six HIV+ donors utilized for these experiments. Anti-TNFR antibodies reduced foam cell formation in a dose-dependent manner compared with cells treated with isotype control antibodies (Fig. 3C), and this inhibition was observed when either control serum (pHS) or HIV+ serum was used. These data suggest that endogenous TNF in serum, and/or TNF produced by activated monocytes during foam cell differentiation, enhances foam cell formation independently of its effects on endothelial activation, and that increased levels of TNF in individuals with HIV likely contributes to the proatherogenic effect of HIV+ serum.

Foam cells downregulate markers associated with immunity and endothelial attachment

To characterize the foam cells generated in our model following monocyte transendothelial migration, we labelled migrated cells extracted from the collagen with
the fluorescent lipophilic dye BODIPY to enable detection of foam cells and macrophages via flow cytometry. To confirm the ability of BODIPY staining to identify foam cells, we analyzed labelled cells using imaging flow cytometry and found that cells with high BODIPY staining exhibited a morphology consistent with foam cells (Supplementary Fig. 5). By gating BODIPY	extsuperscript{high} cells, the percentage of foam cells was determined based on fluorescence intensity (rather than visually which may be subjective). This gave similar values to those determined by Oil Red O staining and light microscopy analysis assayed in parallel (Supplementary Fig. 5). Taken together, these data confirm that both BODIPY and Oil Red O staining were able to identify similar populations of foam cells and macrophages within our model.

Having validated the use of fluorescent BODIPY staining for identification of foam cells, we labelled cells extracted from the collagen matrix with both anti–CD14 and BODIPY and analyzed them by standard flow cytometry. This revealed two distinct populations of CD14	extsuperscript{+} cells: large, BODIPY	extsuperscript{high}CD14	extsuperscript{low} cells with high granularity, consistent with foam cells, and a population of smaller BODIPY	extsuperscript{low}CD14	extsuperscript{high} cells with relatively low granularity, consistent with monocytes/immature macrophages (Fig. 4A). The characterization of these two novel populations was confirmed by microscopic analysis of cells sorted by fluorescence-activated cell sorting (FACS) and was consistent in experiments using monocytes from 14 independent donors (Fig. 4B and C).

We further analyzed the phenotype of macrophages and foam cells by measuring expression of the β2 integrin subunit CD11b and the oxLDL receptor CD36. Foam cells had lower expression of both receptors compared with macrophages, consistent with a role in lipid storage as opposed to migration and active lipid accumulation (Fig. 4D and E). Moreover, foam cells had significantly higher intracellular levels of TNF compared with macrophages (Fig. 4F), indicating a proinflammatory phenotype. These observations show that foam cell formation is associated with increased cell size, reduced surface expression of CD14, CD11b and CD36 but an increase in the production of the proinflammatory cytokine TNF.

**Monocytes from HIV+ individuals showed impaired cholesterol efflux and reduced expression of cholesterol transporters**

The above data indicate that monocytes from HIV+ individuals have an increased potential to form foam cells, independent of the atherogenic effects of serum, suggesting that HIV is associated with intrinsic changes to monocytes that may alter lipid transport. We therefore assessed the ability of monocytes from virologically suppressed cART-treated individuals to efflux cholesterol using an ex-vivo assay. PBMC from HIV+ individuals and controls of a similar age \([n = 8\) each, all men, median age (range) 43.5 (33–51) and 36.5 (28–52) respectively\] were loaded with BODIPY-labelled cholesterol and the subsequent cholesterol efflux from monocytes determined via flow cytometry. This analysis revealed that monocytes from HIV+ individuals were significantly impaired in their ability to efflux cholesterol from the cell, and this impairment was evident both in the absence and presence of the exogenous cholesterol scavenger methyl-β-cyclodextrin (Fig. 5A and B, respectively).
To investigate the mechanistic basis for this impairment, we analyzed the expression of key genes involved in cholesterol synthesis and transport. RNA was extracted from monocytes purified from virologically suppressed HIV+ individuals on cART and age-matched controls and gene expression analyzed via qPCR. This analysis revealed significantly reduced expression of the key cholesterol efflux transporter ABCA1 in monocytes from HIV+ individuals (Fig. 5C, \( P = 0.02 \)), whereas expression of ABCG1 showed a similar although not statistically significant downregulation (Fig. 5D). Expression of \( \text{HMG-CoA reductase} \), the rate-determining enzyme for cholesterol biosynthesis, was also significantly reduced in monocytes from individuals with HIV (Fig. 5E, \( P = 0.02 \)), whereas monocyte expression of the cholesterol esterase ACAT was not altered by HIV infection (Fig. 5F). These data indicate that HIV infection is associated with a decreased expression of key cholesterol efflux genes in monocytes and an impaired ability to efflux cholesterol from the cell, which likely contributes to the increased potential of monocytes from HIV+ individuals to form foam cells.

Discussion

Using a model of atherosclerosis that couples primary human monocyte transendothelial migration and foam cell differentiation, we show that monocytes from HIV+ individuals have a higher rate of foam cell formation, which is because of both intrinsic changes to monocyte function and a proatherogenic effect of soluble factors present in HIV+ serum. These findings may provide some of the first mechanistic evidence regarding the pathogenesis of increased atherosclerosis and CVD in the setting of HIV infection.
In this study, we adapted our in-vitro model which couples monocyte transendothelial migration and foam cell formation to determine the functional properties of human monocytes purified from an HIV+ patient cohort. The strengths of this model include the following: monocytes migrate through an activated primary human endothelial monolayer in response to chemokines secreted in situ and into a fibrous collagen matrix that mimics the subendothelial neointima; it measures foam cell formation by migrated monocytes, rather than in vitro differentiated macrophages; and exogenous modified lipoprotein is not added, therefore, foam cells form in response to endogenous lipids in the human-serum containing media and/or generated in response to endothelial activation. This model is therefore a physiologically relevant system for studying the early stages of foam cell formation in vitro by migrating human monocytes.

Although HIV infection and certain antiretroviral drugs including protease inhibitors can cause dyslipidaemia [39], plasma levels of LDL, high-density lipoprotein and oxLDL in the HIV+ individuals who contributed to this study were not different from controls, suggesting that heightened foam cell formation observed here cannot be explained by lipidaemic HIV+ serum alone. Endothelial...
activation and/or monocyte transendothelial migration may affect levels of oxLDL within the gels, however, which could influence foam cell formation. Significantly, serum from HIV+ individuals had elevated levels of TNF and foam cell formation was blocked by anti-TNF antibodies added after monocyte migration, supporting a proatherogenic role for TNF in these individuals via mediating monocyte activation and increased foam cell formation. Our data were qualitatively supported by live cell imaging showing monocytes from HIV+ individuals moved more slowly within the collagen matrix of the model, particularly at later times, consistent with differentiation into foam cells. These novel data regarding the proatherogenic properties of monocytes from HIV+ individuals may provide a mechanistic explanation for increased atherosclerosis in this population.

In addition to the proatherogenic effects of HIV+ serum, our data suggest HIV infection induces changes to monocytes that predispose them to foam cell formation. Indeed, we found an altered expression of critical cholesterol synthesis and transport proteins in monocytes from HIV+ individuals. The reduced gene expression of ABCA1 may translate to an impaired ability to remove cholesterol from the cell, consistent with our observation of impaired cholesterol efflux in monocytes from individuals with HIV. Feeney et al. [40] also examined the expression of cholesterol metabolism genes in monocytes and found reduced levels of ABCA1 mRNA in viremic but not virologically suppressed HIV+ participants, whereas expression of the lipid uptake receptors CD36 and LDL-receptor was reduced in both HIV+ groups. The different result regarding ABCA1 expression may be because of differences in the methodology of monocyte isolation (positive selection in the Feeney et al. study as compared with negative selection used in our study), differences in the cohorts used (50% women and 64% non-White vs. 100% White men in our study) or the smaller sample size in our study. Thus, these findings need to be confirmed in a larger cohort adequately powered to examine the effect of demographic factors including sex and race. The reduced expression of the cholesterol synthesis enzyme HMG-CoA reductase in monocytes shown here is consistent with the previously reported study and may represent a cellular response to increased intracellular cholesterol levels.

The foam cell data presented here were generated from a combination of virologically suppressed and non-suppressed individuals. Although analysis of foam cell formation in an expanded HIV+ cohort aged 26–68 years did not reveal any significant differences associated with detectable levels of viremia, this requires confirmation in a larger sample set. The fact that serum from virologically suppressed individuals enhanced foam cell formation suggests the persistence of proatherogenic factors in serum in individuals receiving effective cART. Furthermore, our findings of impaired cholesterol efflux from monocytes were also made in virologically suppressed individuals, consistent with our findings that increased monocyte and innate immune activation associated with HIV infection are maintained following viral suppression by cART [20,21]. It remains possible that antiretroviral agents may contribute to this phenotype in cART-treated individuals; however, this requires investigation. Given only a very small proportion of monocytes are found to be infected with HIV \textit{in vivo} (<0.1%), the proatherogenic monocyte phenotype observed here is likely secondary to HIV-associated immune activation and inflammation and not a result of direct infection, although it remains possible that nonproductive/latent HIV infection may also alter monocyte phenotype. Our data suggest monocytes from virologically suppressed individuals receiving cART exhibit a proatherogenic activity and that this may be a pathogenic factor underlying increased atherosclerotic risk in HIV+ individuals.

Endothelial activation plays an integral role in atherosclerosis and monocyte transendothelial migration via the expression of adhesion molecules [41], and soluble endothelial activation markers are elevated in HIV+ individuals and independently associated with carotid intima-media thickness, a surrogate measure of atherosclerosis [42]. Zietz et al. [43] examined the aortic endothelium of patients largely with advanced HIV disease and found structural abnormalities which were accompanied by increased expression of adhesion molecules and mononuclear cell infiltration. Thus, the proatherogenic effects of HIV+ monocytes observed here may well be potentiated by heightened endothelial cell activation \textit{in vivo}. Proinflammatory cytokines are well documented to be associated with increased cardiovascular risk factors and outcomes in both HIV+ and seronegative individuals [44]; however, viral factors in the blood may also contribute to increased atherosclerosis in HIV infection. HIV infection and HIV-derived single stranded RNAs increase foam cell formation by monocyte-derived macrophages \textit{in vitro} [45,46]. The HIV accessory protein Nef promotes foam cell formation in these cells by inhibiting cholesterol efflux [45], and high concentrations of Nef persist in plasma of HIV+ individuals receiving cART [47]. Taken together, it is therefore likely that changes to both monocyte activation and gene expression together with inflammatory cytokines and virus-specific factors in serum contribute to the proatherogenic potential of monocytes observed here. Heightened foam cell formation by monocytes/macrophages \textit{in vitro} is consistent with increased prevalence of atherosclerotic plaques in HIV+ as compared with uninfected individuals [4,22,48], and it would be of interest to determine whether this is associated with altered plaque morphology in HIV+ individuals \textit{in vivo}.

We devised a technique to differentiate between monocyte-derived macrophages and monocyte-derived...
foam cells produced in our model based on CD14 and BODIPY staining and determined that foam cells have an inflammatory phenotype, as defined by higher TNF and downregulated CD11b expression similar to that described by others [49]. Higher intracellular TNF levels compared with migrated macrophages suggests foam cells may have a positive feedback role in activating the endothelium and promoting oxidation of LDL during the development of atherosclerotic plaques, as well as acting on nearby foam cells to enhance cellular activation. We also found foam cells downregulated expression of CD14, CD11b and CD36, the latter two being critical for monocyte attachment and lipid uptake, respectively. Downregulation of CD36 expression was unexpected as CD36 contributes to foam cell formation [35]. Although reduced CD36 expression in response to uptake of modified LDL particles has been reported by others [50], the majority of studies suggest oxLDL particles increase CD36 expression [51,52] and CD36 is highly expressed in foam cells obtained from mature human plaques [53]. The kinetics of CD36 expression during the process of foam cell formation is poorly understood, and the decreased CD36 expression we observed may be the result of receptor internalization due to endocytosis of lipid, or may be a transient response to lipid uptake immediately after migration. Indeed, our model analyses foam cells formed within 48 h of migration, whereas foam cells extracted from human plaques likely developed over longer periods. This, combined with findings from parallel studies indicating that foam cells extracted from our model are smaller and contain less cholesterol ester than mature foam cells extracted from atherosclerotic plaques (unpublished findings), suggests they likely represent immature foam cells or foam cell precursors. Studying the behaviour of these cells is therefore highly relevant for strategies aimed at preventing or reversing the early stages of foam cell formation.

Our study reports novel data indicating that monocytes from HIV+ individuals, a population characterized by chronic inflammation and immune activation, have a greater propensity to differentiate into foam cells and that elevated levels of TNF in plasma of these individuals further enhances this effect. This may explain the increased risk of atherosclerosis in these individuals after adjusting for viral and traditional risk factors and warrants further clinical investigation of the relationship between monocyte activation, atherogenic behaviour and the development of atherosclerosis in HIV+ individuals receiving cART.

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Conflicts of interest

There are no conflicts of interest.

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