HIV Vasculopathy: Role of Mononuclear cell-associated Krüppel-like Factors 2 and 4

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Abstract

Objective—To determine the relationships between Krüppel-like Factors (KLF) 2 and 4, immune-activation, and sub-clinical vascular disease in HIV-infected patients on antiretroviral therapy.

Design—Double blind, randomized, placebo-controlled trial

Methods—We studied 74 HIV-infected adults on antiretroviral therapy enrolled in a randomized clinical trial of statin therapy. KLF2 and KLF4 gene expression was measured by quantitative polymerase chain reaction from peripheral blood mononuclear cells (PBMCs) at baseline and after 24 weeks of 10mg daily rosuvastatin or placebo. At the same time points, T-cell and monocyte activation were assessed by flow cytometry and vascular health was assessed by cardiac computed tomography and carotid ultrasound.

Results—KLF4 expression was negatively correlated with duration of antiretroviral therapy (r= −0.351, p=0.004) and positively correlated with measures of immune activation: pro-inflammatory monocytes [$CD14^+CD16^+$ (r=0.343, p=0.003)], patrolling monocytes [$CD14^{dim}CD16^+$ (r=0.276, p=0.017] and activated CD8+ T-lymphocytes [$CD8^+DR^+CD38^+$ (r=0.264, p=0.023)]. KLF2 expression was negatively correlated with subclinical atherosclerosis: mean-mean common carotid artery intima media thickness (CIMT) (r= −0.231, p=0.048), mean-max CIMT (r= −0.271, p=0.020) and coronary artery calcium score (r=−0.254, p=0.029). There were no statistically significant changes in KLF2/4 expression in PBMCs after 24 weeks of rosuvastatin.

Conclusions—Expression of KLF4 in PBMCs positively correlates with cellular markers of immune activation, while KLF2 expression negatively correlates with markers of subclinical

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atherosclerosis in this HIV-infected population on antiretroviral therapy. Additional studies are needed to determine if targeted interventions might alter KLF2/4 expression to reduce inflammation and vascular risk in humans.

Keywords

HIV; Cardiovascular Disease; Krüppel-like Factor; Inflammation; Immune Activation

Introduction

There is increasing evidence that immune activation and inflammation contribute to cardiovascular disease (CVD) in the general population[1, 2]. Patients infected with human immunodeficiency virus (HIV) have an increased risk for CVD compared to HIV-uninfected controls despite effective antiretroviral therapy, possibly due to residual immune dysregulation[3, 4]. The mechanisms of immune-mediated vascular disease in controlled HIV infection are complex and include alterations in both innate and adaptive immune cell function in the myeloid[5–7] and lymphocyte[8–10] compartments, respectively. In addition, markers of coagulation, immune activation and inflammation are associated with HIV disease progression and mortality[11–13]. Yet, the processes that regulate inflammation and CVD in HIV are incompletely understood.

Activated monocytes are critical to the initiation and progression of atherosclerotic CVD[14]. There exist three distinct subsets of monocytes based on CD14 and CD16 expression and each have distinct immunophenotypes[15–17]. Canonical monocytes, $CD14^+CD16^-$, are phagocytic and antigen presenting; patrolling monocytes, $CD14^{dim}CD16^+$, express pathogen-recognition receptors and home to the endothelium; pro-inflammatory monocytes, $CD14^+CD16^+$, produce inflammatory cytokines in response to antigen stimulation. HIV-infected patients without known coronary artery disease and HIV-uninfected patients that have had an acute coronary syndrome have similarly high levels of inflammatory and patrolling monocytes suggesting a shared pro-atherothrombotic phenotype[18].

The role of T-cells in the progression of atherosclerosis is well described[1]. HIV infection causes heightened T-cell activation—often defined as co-expression of CD38 and/or HLA-DR—that is only partially reversed by antiretroviral therapy[19] and is an important predictor of mortality[20]. Further, cross-sectional correlations between T-cell activation and subclinical vascular disease have been described[8–10], but the mechanisms linking T-cell activation and vascular disease in HIV+ patients remain elusive.

Coronary artery calcification (CAC), as detected by ECG-gated non-contrast computed tomography (CT), is a marker of subclinical coronary atherosclerosis that predicts coronary events better than other surrogate measures of vascular risk[21]. Compared to HIV-uninfected controls, HIV-infected patients on stable antiretroviral therapy (ART) have a higher CAC burden[22, 23]. Similarly, high-resolution ultrasound measures of carotid intima media thickness (CIMT) also predict CVD risk in the general population, particularly stroke[24, 25]. Traditional and non-traditional risk factors are associated with elevated CAC

and CIMT in patients with HIV[8, 9, 22, 23, 26–29]. To date, transcriptional modulators that may explain these observations have not been identified.

The Krüppel-like Factor (KLF) family of transcription factors has regulatory roles in cellular differentiation, metabolism, homeostasis and cellular differentiation. Hemovascular expression of two members of the KLF family, KLF2 and KLF4, has been shown to modulate thrombosis, atherosclerosis, inflammation, barrier function, and vasomotor tone in cell and animal models of disease[30–38]. In addition, statins (HMG-CoA reductase inhibitors) significantly induce levels of KLF2 in mouse and human T-cells[39] and KLF2 and KLF4 in human umbilical vein endothelial cell cultures[36]. The *in vivo* effect of statins on KLF expression in humans is, however, as yet unknown. Given the important roles of KLF2 and KLF4 in modulating CVD progression and inflammation in cell and animal models of CVD, we hypothesized that KLF2 and KLF4 may play a role in mediating the inflammatory CVD phenotype in HIV+ patients.

To address this question, we designed the current study to evaluate the associations between immune activation, subclinical vascular disease, and KLF2/4 expression in PBMCs of a population of HIV infected subjects treated with antiretroviral therapy. We further aimed to compare changes in KLF2/4 expression in PBMCs after 24 weeks of daily rosuvastatin versus matching placebo.

Methods

Study population

This is a sub-study of the Stopping Atherosclerosis and Treating Unhealthy Bone with Rosuvastatin in HIV (SATURN-HIV) trial. SATURN-HIV is a randomized, double-blind, placebo-controlled trial aimed at elucidating the physiological effects of 10mg/daily rosuvastatin on markers of subclinical cardiovascular disease, inflammation and skeletal health in subjects with HIV on stable antiretroviral therapy (clinicaltrials.gov identifier: NCT01218802). The institutional review board at University Hospitals Case Medical Center approved the study and written informed consent was obtained from each participant.

Participants in SATURN-HIV were HIV-infected adults 18 years of age, with HIV-1 RNA < 1,000 copies/mL, on stable antiretroviral therapy (ART) for at least 3 months and cumulative ART for at least 6 months, fasting LDL cholesterol (LDL-C) of 130mg/dL and fasting triglyceride level of 500mg/dL. Additional inclusion criteria included evidence of heighted T-cell activation (CD8+ T cells expressing CD38+HLA-DR+ 19%) and/or screening high-sensitivity C-reactive protein (hs-CRP) 2mg/L. Exclusion criteria included known history of coronary artery disease or diabetes, pregnant or lactating, or uncontrolled inflammatory condition.

A medical history, demographic characteristics and physical exam including height, weight, waist and hip measurements were obtained at the initial screening visit. Metabolic syndrome was defined according to Adult Treatment Panel III guidelines[40] as having 3 of the following 5 criteria: (1) waist circumference >102 cm (men), >88 cm (women); (2) triglycerides 150 mg/dL; (3) high density lipoprotein <40 mg/dL (men), <50 mg/dL

(women); (4) blood pressure 130/ 85 mmHg or on anti-hypertensive treatment; and (5) fasting glucose 110 mg/dL. Twelve-hour fasting venous blood draw was obtained at study entry and at 24 weeks. Participants' HIV-1 RNA levels and CD4+ counts were obtained as part of routine clinical care.

Blood sample preparation

PBMCs were separated from whole blood by centrifugation through a Ficoll-Hypaque gradient. All SATURN-HIV participants had PBMCs collected for T-cell and monocyte analysis. PBMCs not used for these analyses were stored for future studies. This KLF sub-study was performed using only those participants (n=74) who had sufficient quantities of PBMCs available for further analyses at both baseline and at week 24.

Quantitative polymerase chain reaction

RNA was isolated from PBMCs using Trizol. Reverse transcription was then performed using a BioRad iScript kit and quantitative polymerase chain reaction (qPCR) was performed using the probe-based Lightcycler 480 system. KLF2, KLF4, HPRT and GAPDH expression levels were measured and the data herein represents an average copy number of KLF2 and KLF4 expression normalized to both housekeeping genes- HPRT and GAPDH. To determine absolute quantification of gene transcripts, we created a standard curve (copy number vs. Cp) using purified amplicon from each gene above, as described previously[41]. We subsequently derived copy number from a line of best fit for the standard curve using the Cp of each gene. The number of copies of KLF2/4 mRNA reported is that present in 20 picograms of total cellular mRNA. Each subject's PBMC sample was run at least three times in triplicate.

Monocyte subsets

Whole blood was incubated for 15 minutes on ice in FACS lysis buffer (BD Biosciences) and washed in phosphate-buffered saline containing 1% bovine serum albumin and 0.1% sodium azide. The cells were then stained for 30 minutes in the dark on ice, washed and fixed in 1% paraformaldehyde. Monocyte subsets were defined by expression of CD14, CD16, size and granularity[18]. In order to quantify expression of surface markers, we utilized isotype gating. The following fluorescent antibodies were used to identify monocytes: anti-CD14 (Pacific Blue, BD Pharmingen) and anti-CD16 (PE-conjugated, BD Pharmingen). Monocytes were analyzed for differential expression of CD14 and CD16 using a Miltenyi MACS quant flow cytometer and MACS Quantify software. Monocyte subsets were defined using relative expression of CD14 and CD16.

T-cell activation

T cells were quantified by size, granularity and positive expression of CD3 and either CD4 or CD8. T-cell activation was determined using PE-conjugated anti-CD38, FITC-conjugated anti-HLA-DR, peridinin-chlorophyll-protein-complex-conjugated anti-CD3, allophycocyanin-cy7-conjugated anti-CD8 and allophycocyanin-conjugated anti-CD4 antibodies all from BD Biosciences. T-cell activation was defined as expression of co-

expression of CD38 and HLA-DR. Measurements were performed using a LSR II flow cytometer and analyzed using FACSDiva software.

Coronary artery calcium score

Coronary artery calcium (CAC) score was performed as previously described[42]. Briefly, all patients underwent a ECG-gated non-contrast computed tomography (CT) scan of the chest. Calcified lesions were quantified as areas of 6 pixels with density > 130 Hounsfield units. The Agatston method was used to quantify CAC. Subjects were categorized dichotomously as a score of 0 or >0.

Carotid intima media thickness

Vascular ultrasound to assess carotid intima media thickness (CIMT) was performed as described previously[42]. CIMT was measured over the distal one-centimeter segment of the common carotid artery at three angles bilaterally (i.e. 6 segments). Each of these 6 segments has a max value and a mean value over that 1 cm segment. Two measures were used for analysis: average of the 6 mean values (mean-mean IMT) and the average of the 6 max values (mean-max IMT).

Statistical Analysis

Characteristics of study participants were described as median (inter-quartile range) for continuous variables and frequency (%) for categorical variables. Characteristics of those participants included in the KLF analysis were compared to the rest of the study population using an unpaired t-test or Wilcoxon Rank Sum test as distributionally appropriate for continuous variables and Fisher's exact test for categorical variables. Spearman correlation was used to examine the relationships between KLF expression and markers of immune activation, subclinical vascular disease, and HIV-specific factors. Changes in KLF2/4 copy number from 0 to 24 weeks were expressed as percentage change from baseline and were compared between statin and placebo groups using paired t-tests. All statistical tests were two-sided and considered significant at a level of $p<0.05$. Analyses were performed using SAS v. 9.2 (The SAS Institute, Carey, North Carolina, USA).

Results

Of 147 total participants in SATURN-HIV, 74 had sufficient quantities of PBMCs for inclusion in this KLF sub-study. Baseline characteristics of the 74 study participants, including CVD risk factors and HIV disease status, are displayed in Table 1. Median age was 47 and the majority were male. Participants had well-controlled HIV-infection with high median CD4+ T-cell count and 78% with undetectable HIV-1 RNA. Although median 10-year Framingham risk score was low (5% risk of cardiovascular disease), two-thirds were smokers and nearly one-quarter of participants had metabolic syndrome. Participants in the KLF analysis were similar to those who were not included (n=73), except that included participants were more likely to have undetectable (<48c/ml) HIV-1 RNA levels (78% vs. 63%, p=0.04, included vs. excluded; p>0.05 for all other characteristics shown in Table 1). Total white blood cell count was higher among those who were included in the study compared to those who were not included (6.7 vs. 5.7 $\times 10^9$ cells/L); however, total

lymphocytes and total monocytes were not different (both p>0.2). All participants in this sub-study completed a full 24 weeks of study therapy.

KLF4 positively correlates with monocyte and T-cell activation

We hypothesized that expression levels of KLF2/4 in PBMCs would be related to a study participant's level of immune activation at baseline. Copy number of KLF mRNA was determined using standard qPCR techniques. In a cross-sectional analysis of baseline data, KLF4 was positively correlated with two activated monocyte subsets: $CD14^+CD16^+$ monocytes (r=0.34, p<0.01) and $CD14^{dim}CD16^+$ monocytes (r=0.28, p=0.02) (Fig. 1A). Similarly, we observed that KLF4 was positively correlated with activated CD8+CD38+HLA-DR+ T-cells (r=0.26, p=0.03) (Fig. 1B). Despite a borderline statistically significant positive correlation with total lymphocyte count (r=0.220, p=0.06) and a borderline negative correlation with total monocyte count (r=−0.213, p=0.07), we observed no significant correlations between KLF2 and markers of activated monocytes or T-cells (all p>0.1).

KLF2 negatively correlates with coronary calcium score and carotid intima media thickness

Secondly, we hypothesized that KLF2 and KLF4 would also be related to measures of subclinical vascular disease. Indeed, KLF2 was negatively correlated with mean-mean IMT (Table 2; r= −0.231, p=0.048), mean-max IMT (r= −0.271, p=0.020), and with the presence of coronary calcium (r= −0.254, p=0.029). We found no significant correlation between KLF4 and CAC or IMT (Table 2; all p >0.05).

KLF4 positively correlates with measures of HIV disease duration and ART use

We further explored whether KLF2 and KLF4 would be related to measures of HIV disease severity and duration. KLF4 was negatively correlated with duration of HIV infection (r= −0.297, p=0.01) and duration of ART (r=−0.351, p=0.004), including PI duration (r=−0.322, p=0.02) and nucleoside reverse transcriptase inhibitor (NRTI) duration (r=−0.307, p=0.009), but not non-nucleoside reverse transcriptase inhibitor (NNRTI) duration (r=0.104, p=0.433). We did not observe any relationship between these measures and KLF2. Neither KLF2 nor KLF4 was associated with HIV-1 RNA level, current CD4+ T-cell count, or nadir CD4+ T-cell count (all p>0.6).

Rosuvastatin therapy for 24 weeks does not alter the expression of KLF2 or KLF4 in PBMCs

After 24 weeks of therapy, there were no significant changes in KLF2 or KLF4 expression in PBMCs of those treated with 10mg of daily rosuvastatin compared to those who received placebo [mean (standard deviation) change of +0.4(1.8)% vs. +0.1(0.7)%, p=0.638 for KLF2 and +2.7(6.2)% vs. +3.4(8.8)%, p=0.799 for KLF4, statin vs. placebo, respectively].

Discussion

Studies to elucidate the mechanisms that link immune activation and premature vascular disease in the HIV-infected population are urgently needed in order to develop targeted

therapies that improve outcomes. In this clinical study of HIV infected patients on antiretroviral therapy, we have uncovered important relationships between two transcriptional regulators of atherothrombotic vascular disease—KLF2 and KLF4—and levels of cellular immune activation, subclinical atherosclerosis, duration of HIV infection, and ART use.

KLF2 and KLF4 represent two of the 18 members of the family of transcription factors called Krüppel-like factors. While the KLFs have diverse roles in fundamental physiological processes including cell differentiation and homeostatic balance, immune cell expression of KLF2 and KLF4 in particular have been implicated in regulation of essential aspects of hemovascular biology[43, 44]. In animal models of disease, KLF2 and/or KLF4 expression in immune cells have been shown to protect from development of atherosclerosis[35], insulin resistance and obesity[37], and regulate the immune response in inflammatory arthritis[45], myocarditis[39], and polymicrobial infection[46]. The precise transcriptional and signaling pathways by which KLF2/4 exert their immune effects is a subject of intense investigation. Several studies have shown roles for KLF2 or KLF4 in the regulation of T-cell and monocyte/macrophage activation *in vitro* and *in vivo* using cell culture and animal models[39, 47–50]. Early *in vivo* mouse studies suggested that the anti-inflammatory transcription factor KLF2 maintains T-cells in a quiescent state; however subsequent studies indicate that KLF2 regulates a composite of T cell functions, trafficking in particular, that can give the impression that it has a direct effect on quiescence[47, 48]. As reviewed by Hart et al.[43], constitutive expression of KLF2 in naïve T-cells is extinguished after T-cell stimulation, and cytokines involved in effector differentiation repress re-expression of KLF2. Loss of KLF2 leads, in turn, to transcriptional down-regulation of sphingosine 1-phosphate receptor 1 and thus activated T-cells are retained in lymphoid sites[51]. Furthermore, once CD8+ T-cells are activated, elevated levels of KLF2 are required to maintain the memory cell phenotype[52]. Of note, KLF2 also regulates expression of CCR5 in response to activation, thus making it a potential target to modify susceptibility to R5-tropic HIV-1 infection[53]. Susceptibility of CD4+ T-cells to HIV-1 infection may be further increased by KLF2 regulated expression of CD62L (L-selectin)[54]. Finally, more recent data suggests that in addition to its important role in T-cell biology, KLF2 is also a negative regulator of monocyte activation[49]. Data regarding immune expression of KLF2 in humans is limited. Expression was shown to be decreased in circulating monocytes from patients with coronary artery disease[49] or by RNA analysis of whole blood in patients with sepsis[46]. In our study, we found no correlation between KLF2 copy number in PBMCs and levels of inflammatory monocyte subsets or CD38+HLA-DR+ T-cells. Relationships between KLF2 and markers of T-cell turnover and senescence—which are altered by HIV infection—should be explored in future studies.

The important role of the transcription factor KLF4 in monocyte and tissue macrophage activation has been previously described[50, 55]. Its role in T-cell activation is less clear, though it does appear to be required for T helper 17 differentiation[56, 57] and is expressed in CD8+ T cells where it contributes to T-cell quiescence downstream of Ets transcription factor ELF[58]. Inflammatory cytokines such as interferon and lipopolysaccharide induce tissue macrophages to produce KLF4 and overexpression of KLF4 induces macrophage

activation[50]. As for KLF2, the expression in and role of KLF4 in immune tissue from human samples is limited. One study demonstrated a 50% reduction KLF4 expression in adipose tissue from obese (versus lean) patients[36]. Decreased KLF4 signal was attributed to loss of expression in adipose tissue macrophages and hypothesized to play a role in the metabolic dysregulation seen in obese patients[36]. In this study, we report modest positive correlations between KLF4 copy number in PBMCs and activated CD8+CD38+HLA-DR+ T-cells and pro-inflammatory $CD14^+CD16^+$ monocytes. KLF4 was also positively correlated with $CD14^{dim}CD16^+$ monocytes, so-called "patrolling monocytes." These patrolling monocytes play a homeostatic role in maintaining a healthy endothelium, but can become activated in response to certain viral infections[16]. Higher levels of these monocytes have been associated with CAC progression[59] and carotid stiffness[60] in HIV-infected subjects. Initiation of ART reduces T-cell activation[19] and starting ART earlier in the course of HIV infection is associated with a lower T-cell activation setpoint[61]. The negative correlation between KLF4 levels and duration of ART in our study is, therefore, intriguing; however, whether KLF4 mediates the relationship between ART and immune activation requires further investigation in longitudinal clinical trials of ART.

Because our study population was extensively characterized by non-invasive vascular imaging tests, we were able to explore the relationship between KLF expression in PBMCs and the extent of subclinical atherosclerosis. We observed that HIV-infected subjects with detectable coronary calcium and higher carotid IMT had lower levels of KLF2. It has long been recognized that KLF2 expression in endothelial cells is induced by shear stress[62] and that carotid IMT is thicker in areas of low shear stress such as the carotid bifurcation[63]; however, to our knowledge, this inverse relationship between peripheral blood KLF2 expression and carotid IMT has not previously been described in humans. It is important to acknowledge that we did not directly measure endothelial KLF expression in this study. Despite correlations with cellular immune activation markers, KLF4 was not associated with any measure of subclinical atherosclerosis in our cross-sectional analysis.

Statins profoundly reduce the risk of cardiovascular events in multiple populations, and part of this risk reduction appears to be related to reductions in inflammation that are independent of LDL-lowering[64, 65]. Prior studies have suggested that KLF2 and KLF4 may be important transcriptional regulators of this anti-inflammatory effect of statins[36, 39, 66, 67]. Surprisingly in the present study, there was no effect of 24 weeks of statin therapy on KLF2/4 levels in PBMCs. This is an important observation that must be confirmed in larger human studies. A potential explanation may be that the concentrations used in prior cell culture and small animal experiments (0.1–10 μM) are much higher than the blood concentration of statins typically achieved in humans (0.001–0.015 μM)[68] or that timing of blood draws and medication administration was mismatched to detect statin-mediated KLF expression. We measured KLF RNA expression in our study as a surrogate marker of protein levels in PBMCs. Many studies have shown strong correlation between RNA and protein[37, 38, 46, 55]; however, there are no data on whether this relationship is altered by HIV infection. In this study, KLF RNA expression was measured in PBMCs. Future studies should assess the effect of statins on KLF expression in vascular endothelial cells. Finally,

whether this lack of statin effect is unique to HIV-infected individuals is unknown and should be explored in HIV-uninfected populations.

This study describes a novel examination of factors that transcriptionally regulate inflammation and vascular health in chronic HIV-infection. These transcription factors may also be altered by duration of ART use. The novel findings of this study are strengthened by the extensive immunophenotyping and subclinical vascular disease characterization of study subjects. Limitations of this study include a relatively small sample size due in part to the time and expense required to measure KLF with qPCR; however it is larger than any previous study using human PBMCs. We cannot exclude the possibility that the observed relationships are explained by unmeasured confounding. Additionally, cross-sectional associations cannot determine causality.

In conclusion, KLF2 and KLF4 may play a role in mediating the inflammatory CVD phenotype in HIV-infected patients. Future studies should examine longitudinal relationships between KLF2/4, immune activation, and subclinical vascular disease. Regulation of KLF2/4 expression may represent a novel therapeutic target to prevent vascular disease in HIV infection and other chronic inflammatory diseases.

Acknowledgements

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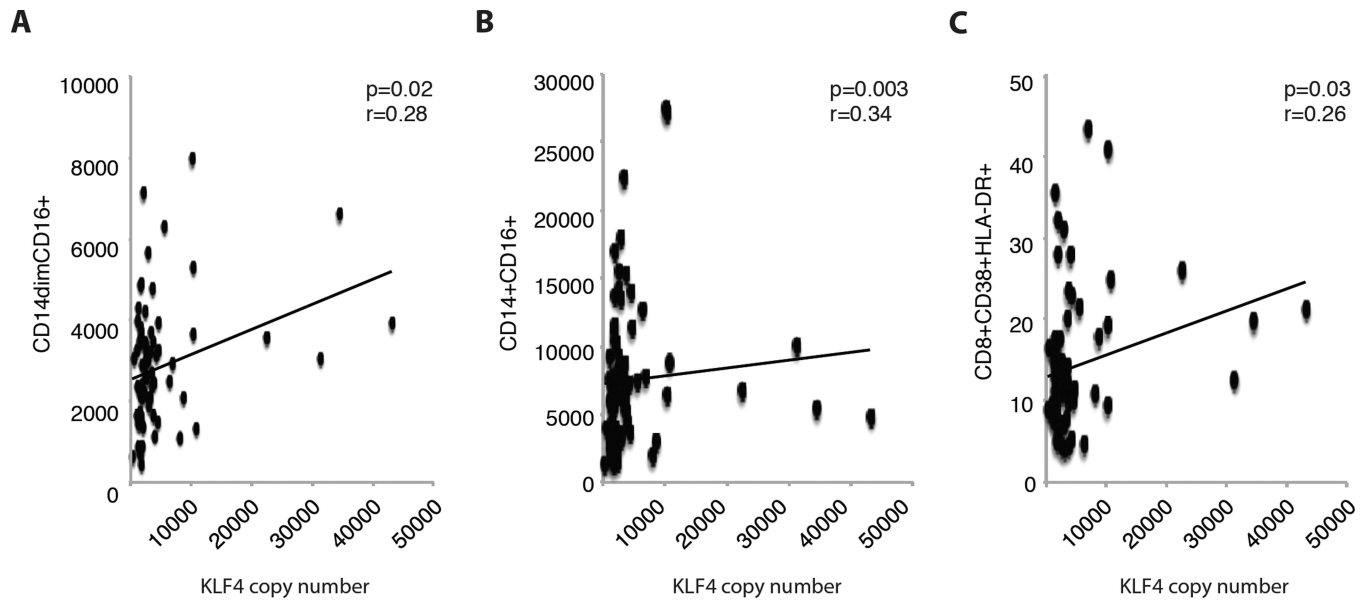
A

B

C

Figure 1.
Baseline expression of KLF4 in peripheral blood mononuclear cells was correlated with the
percentage of (A) CD14dimCD16+ monocytes, (B) CD14+CD16+ monocytes, and (C)
CD8+ T-cell activation.

Table 1

Baseline characteristics of study participants

Demographics	n=74
Age (years)	47 (42–52)
Male	60 (81%)
African-American	49 (66%)
HIV Parameters	
Current CD4+ (cells/mm^3)[†]	628 (473–807)
Nadir CD4+ (cells/mm^3)	231 (114–312)
HIV Duration (years)	14 (7.4–19)
ART Duration (years)	5.2 (2.8–7.1)
Undetectable HIV-1 RNA (<48c/ml)	58 (78%)
CVD Risk Factors	
Systolic Blood Pressure (mmHg)	120 (110–130)
HDL Cholesterol (mg/dL)	45 (35–57)
LDL Cholesterol (mg/dL)	102 (77–114)
Body-Mass Index (kg/m^2)	27 (24–31)
Metabolic Syndrome	16 (22%)
Current Smoker	49 (66%)
Family History of MI	25 (34%)
10-year Framingham Risk Score (%)	5 (1–9)
Current Medication Use	
Anti-hypertensive Medication	15 (20%)
Protease Inhibitor	33 (45%)
AZT or D4T	2 (3%)

Data presented as median (inter-quartile range) or frequency (%).

ART, antiretroviral therapy; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MI, myocardial infarction; AZT, zidovudine; D4T, stavudine

[†] Normal CD4+ T-cell count for HIV-uninfected individuals is 350–2740 cells/

Table 2

Relationship between KLF2 and KLF4 expression and measures of subclinical atherosclerosis

	KLF2		**KLF4**	
	r	**p-value**	**r**	**p-value**
CAC >0	**−0.254**	**0.029**	0.215	0.066
Mean-mean CIMT	**−0.231**	**0.048**	0.177	0.131
Mean-max CIMT	**−0.271**	**0.020**	0.151	0.199

KLF, Krüppel-like Factor; CAC, coronary artery calcium; CIMT, carotid intima-media thickness