Platelet Transcriptome Profiling in HIV and ATP-Binding Cassette Subfamily C Member 4 (ABCC4) as a Mediator of Platelet Activity

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VISUAL ABSTRACT

Healthy subjects

Subjects with HIV

HIV

Platelet RNA Sequencing

Platelet-Endothelial Cell Adhesion in HIV

Platelet Activation

Platelet-mediated inflammatory response

Platelet activation

Platelet-mediated inflammatory response

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HIGHLIGHTS

- Platelet activity and its effector cell properties are increased in persons with virologically suppressed HIV on antiretroviral therapy.
- The platelet transcriptome is differentially expressed in participants with HIV compared with healthy individuals.
- ABCC4 expression and translation was enhanced in HIV-infected subjects compared with healthy individuals.
- ABCC4 is a membrane transporter that plays an important role in regulating several cardiovascular processes, including platelet activation and aggregation.
- Platelet ABCC4 inhibition in HIV attenuated platelet activation and platelet effector cell function by regulating cyclic nucleotide homeostasis and the extrusion of platelet proinflammatory mediators.
ABBREVIATIONS AND ACRONYMS

ABCC4 = ATP binding cassette subfamily C member 4
ART = antiretroviral therapy
BSA = bovine serum albumin
cAMP = cyclic adenosine monophosphate
CVD = cardiovascular disease
HIV = human immunodeficiency virus
HUVEC = human umbilical vein endothelial cell(s)
IL = interleukin
NSAID = nonsteroidal anti-inflammatory drug
PAH = pulmonary artery hypertension
PBS = phosphate-buffered saline
qPCR = quantitative polymerase chain reaction
RNA-Seq = RNA sequencing
RT = room temperature
SIP = sphingosine-1-phosphate
VASP = vasodilator-stimulated phosphoprotein

SUMMARY

An unbiased platelet transcriptome profile identified ATP binding cassette subfamily C member 4 (ABCC4) as a novel mediator of platelet activity in virologically suppressed human immunodeficiency virus (HIV)-infected subjects on antiretroviral therapy. Using ex vivo and in vitro cellular and molecular assays we demonstrated that ABCC4 regulated platelet activation by altering granule release and cyclic nucleotide homeostasis through a CAMP-protein kinase A (PKA)-mediated mechanism. Platelet ABCC4 inhibition attenuated platelet activation and effector cell function by reducing the release of inflammatory mediators, such as sphingosine-1-phosphate. ABCC4 inhibition may represent a novel antithrombotic strategy in HIV-infected subjects on antiretroviral therapy. (J Am Coll Cardiol Basic Trans Science 2018;3:9–22) © 2018 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Human immunodeficiency virus (HIV)-infected subjects are at significant risk for myocardial infarction and other forms of cardiovascular disease (CVD), even after controlling for traditional risk factors (1,2). Due to antiretroviral therapy (ART), HIV-infected individuals are living longer, but CVD has become a leading cause of death (3–5). The mechanism by which HIV infection increases the risk of CVD is not fully known. Possible mechanisms increasing the risk of CVD involve chronic inflammation (6,7), immune dysregulation (8), metabolic changes (9), increased coagulation (10), dyslipidemia (11), and endothelial dysfunction (12). Data from our group and others have demonstrated that platelets in persons with HIV reveal a basally activated state, which suggests that pathological platelet activation may contribute to HIV-mediated CVD (13–16).

Methods

**Study Persons.** The study was conducted in accordance with policies of the New York University Langone Medical Center Institutional Review Board, Bellevue Hospital Center, and the central office of the New York City Health and Hospital Corporation. Peripheral blood was drawn (3.8% sodium citrate tubes) with written consent, from healthy controls and HIV-infected subjects with HIV RNA viral load <200 copies/ml for ≥3 months on ART. Exclusion criteria included age <18 and >80 years, nonsteroidal anti-inflammatory drug (NSAID) use in the past week (including aspirin), antiplatelet or antithrombotic...
drug use, CVD, chronic kidney disease, steroids or immunosuppressive agents, active drug or alcohol use, known anemia (hemoglobin <8 mg/dl), or thrombocytopenia (<100 × 10^3/µl) or thrombocytosis (>500 × 10^3/µl).

**PLATELET PREPARATION, LYSATES AND SUPERNATANTS COLLECTION.** Platelet-rich plasma was added to 1:10 acid-citrate-dextrose solution, centrifuged (1,000 g, 10 min) and platelet pellet resuspended in Tyrode’s buffer and 1 µmol/l PGE_1 (Sigma-Aldrich, St. Louis, Missouri). Platelets were counted on a Coulter ACF-T diff2 Hematology Analyzer (Beckman Coulter, Brea, California) and adjusted to the desired concentration by addition of Tyrode’s buffer or endothelial or monocyte starvation medium. Cells were rested 30 min before thrombin activation. Resting or activated platelets were pelleted (14,000 g, 3 min) and lysed in 1% Triton X-100 (Thermo Fisher Scientific, Waltham, Massachusetts) in Tyrode’s buffer containing protease inhibitor cocktail.

**CELL CULTURE.** Human umbilical vein endothelial cells (HUVECs) (Lonza, Basel, Switzerland) were cultured in endothelial growth medium (Lonza) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, California). For all experiments, HUVECs were used between passages 3 to 5. THP-1 cells (ATCC, Manassas, Virginia) were grown in RPMI 1640 medium (Corning, Corning, New York) supplemented with 10% fetal bovine serum.

**PLATELET-HUVEC COINCUBATION.** HUVECs, serum-starved overnight (0.5% bovine serum albumin[BSA] in basal medium), were incubated with either untreated or stimulated (0.05 U/ml thrombin, 5 min) platelets (1:100 ratio, 2 h, 37°C). Unbound platelets were washed away and HUVECs lysed in TRIzol (Qiagen, Hilden, Germany). Where indicated, platelets were pre-treated (30 min, 37°C) with the highly selective ATP binding cassette subfamily C member 4 (ABCC4) inhibitor Ceefourin 2 (3-chloro-5-4-methylphenyl)-7-(trifluoromethyl)pyrazolo[1,5-a]pyrimidine-2-carboxylic acid; Abcam, Cambridge, United Kingdom) (32) before coincubation.

**PLATELET-MONOCYTE COINCUBATION.** Platelets, resuspended in serum-free RPMI 1640 containing 10 µg/ml polymyxin B sulfate (Sigma-Aldrich) were either left untreated or stimulated (0.25 U/ml thrombin, 5 min) before coincubation with THP-1 (1:100 ratio) in polystyrene round-bottom tubes (2 h, 37°C). THP-1 were pelleted (120 g, 5 min) and stored in TRIzol. Where indicated, platelets were pre-treated with Ceefourin (30 min, 37°C) before coculture.

**PLATELET PURIFICATION.** As previously described (33), platelets were subjected to negative selection based on magnetic cell sorting using human CD45^+ and GLY A^+ depletion kit (EasySep, STEMCELL Technologies, Vancouver, British Columbia, Canada). All purified platelets were lysed in TRIzol for RNA isolation. A relative purity (platelet/leukocyte ratio, 1 × 10^7) of platelet cell populations by flow cytometry and gene expression was obtained (31), consistent with other groups measuring platelet RNA expression (27).

**RNA SEQUENCING.** RNA sequencing (RNA-Seq) was performed in leukocyte-depleted platelet RNA from 6 subjects with HIV and 3 controls. Raw sequencing data were received in FASTQ format. Read mapping was performed using TopHat version 2.0.9 (Center for Computational Biology at Johns Hopkins University, Baltimore, Maryland) against the hg19 human reference genome. The resulting BAM alignment files were processed using the HTSeq version 0.6.1 Python framework (Python Software Foundation, Beaverton, Oregon) and respective hg19 GTF (gene transfer format) gene annotation, obtained from the UCSC Genome Browser database. The Bioconductor package DESeq2 (release version 3.2) was used to identify differentially expressed genes. This package provides statistics for determination of differentially expressed genes using a model based on the negative binomial distribution. The resulting values were then adjusted using the Benjamini and Hochberg’s method for controlling false discovery rate. Genes with a nominal p value ≤0.01 were determined differentially expressed.

RNA sequencing data from platelet samples and subjects’ clinical characteristics have been submitted to GEO (accession number GSE99737). Gene Set Enrichment Analysis (GO) of transcripts differentially modulated between HIV and controls was performed.

**FLUORESCENCE MICROSCOPY.** Adhesion of platelets to HUVECs was performed as described earlier in the text, with an additional step. Briefly, freshly isolated platelets were stained with 3 µmol/l CellTracker Green CMFDA Dye (Life Technologies, Carlsbad, California) and left untreated or treated with 0.05 U/ml thrombin for 5 min. After incubation with HUVECs, cells were fixed with 3.7% paraformaldehyde (10 min, room temperature[RT]) and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline[PBS](10 min, RT). Coverslips were mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, California) and examined on an EVOS FL Imaging System microscope (Thermo Fisher Scientific). In some experiments, platelets were treated with Ceefourin after staining with CellTracker.

For ABCC4 and f-actin double staining, coverslips were coated with 40 µl of human collagen type I (1 mg/ml, Sigma-Aldrich), incubated (1.5 h, 37°C),
PHOSPHOLIPID EXCHANGE. Platelet-rich plasma (PRP) was obtained by centrifugation of whole blood (5 min, 300 × g) at 18°C. Washed platelets were collected off of forward and side scatter properties.

Sphingosine-1-phosphate (S1P) was analyzed, with minor modifications, as described (34). Twenty-five or 50 μl of sample (sera and supernatants, respectively) were extracted by vortexing in a 1:30 v/v solution of diethylamylamine 10%/dichloromethanol: methanol 1:1 containing sphingomyelin C12 (d18:1/12:0, 120 μmol/l) as internal standard. External standards were quantified and processed identical to samples. The analyses were carried out in organic acid-resistant deep 96-well plates (Agilent Square 96-well, 2 ml # 5133009, Agilent, Santa Clara, California).

STATISTICS. Data were analyzed using standard descriptive and multivariable methods. Data were expressed as mean ± SEM or median (25th, 75th percentile), as appropriate. The statistical significance between 2 groups was determined by parametric (Student t test) or nonparametric (Mann Whitney U test) testing, as appropriate. Unadjusted and multivariable linear regression analysis was used to determine the impact of HIV status on platelet mRNA ABCC4 expression, controlling for potentially influential demographic and biological covariables, including duration of HIV, CD4 count, and HIV therapy type. Probability values <0.05 were considered statistically significant. Analyses were performed using SAS (version 9.3, SAS Institute, Cary, North Carolina) and GraphPad Prism (version 7.00 for Windows, GraphPad Software, La Jolla, California).

Methods for quantitative polymerase chain reaction (qPCR), Western blot, flow cytometry, and cyclic adenosine monophosphate (cAMP) measurement available in the Supplemental Methods.

RESULTS

SUBJECTS DEMOGRAPHICS. Median age of HIV-infected subjects was 53.5 (range 29 to 68) years. Nearly 82% of the population was black, and 57% were men. Fifty percent were current smokers. Mean CD4+ T-cell count was 665.6 (range 214 to 1,727). Mean years of HIV-1 diagnosis was 19.7 (range 5 to 32) years, and mean years on effective ART therapy with a suppressed HIV-1 RNA viral load was 14.4 (range 1 to 26) years. An overview of clinical characteristics and treatment at the time of blood sampling is presented in Table 1 and Supplemental Table 1.

ENDOTHELIAL CELL AND MONOCYTE ACTIVATION BY PLATELETS FROM HIV-INFECTED SUBJECTS. We and others have previously shown that platelet activity is increased in the setting of HIV (13–16). We now sought to determine the effects of HIV-related

TABLE 1 Study Population Characteristics

|                          | Controls (n = 7) | HIV+ (n = 55) |
|--------------------------|-----------------|---------------|
| Age, yrs                 | 42.1 ± 8.5      | 53.5 ± 7.8    |
| Female                   | 57.1            | 42.6          |
| BMI, kg/m²               | 25.6 ± 1.8      | 27.0 ± 5.5    |
| Race                     |                 |               |
| White                    | 71.4            | 18.5          |
| Black                    | 28.6            | 81.5          |
| Asian                    | 0               | 0             |
| Other                    | 0               | 1.9           |
| Ethnicity                |                 |               |
| Hispanic                 | 28.6            | 11.1          |
| Smoking status           |                 |               |
| Current                  | 0               | 50.0          |
| Former                   | 14.3            | 40.0          |
| Never                    | 85.7            | 10.0          |
| CD4+ T-cell count (c/mm³) | 0              | 665.6 ± 353.2 |
| Years of HIV duration    | 0               | 19.7 ± 7.0    |
| Years on antiretroviral therapy | 0    | 14.4 ± 6.3    |

Values are mean ± SD or %. *All variables were significantly different between HIV and controls (p < 0.05) except for BMI (0.51).

BMI = body mass index; HIV = human immunodeficiency virus.

washed twice with PBS, and blocked by BSA (2 mg/ml, 1 h, 37°C). After another wash with PBS, coverslips were incubated with 20 μl of platelet suspension (10⁴/ml, 30 min, RT), washed with PBS 3 ×, and fixed by formaldehyde in PBS (1%, 30 min, RT). Platelets were then permeabilized with 0.5% Triton X-100 in PBS (15 min, RT) and blocked by 1% BSA in PBS (30 min, RT). ABCC4 staining was carried out using the ABCC4 antibody (1:500, overnight, 4°C; Abcam) and secondary fluorescein isothiocyanate antibody (1:200, 1 h, RT, Santa Cruz Biototechnology, Austin, Texas). Phalloidin staining, was added with ABCC4 secondary antibody (1:40, Thermo Fisher Scientific). Imaging was performed on a Zeiss AxioObserver with 63× N.A. 1.40 lens, Axioscam 503 mono, and narrow pass fluorescent filter blocks (Carl Zeiss, Oberkochen, Germany). For ABCC4 quantification, platelets from controls and HIV-infected subjects were compared. Fifty cells of each condition were imaged. Fields were manually counted for the number of green dots per cell.

MEPACRINE ASSAY. Washed platelets (10⁸/ml) were diluted 1:40 in Hanks’ balanced salt solution. Cee-fourin or vehicle was added to platelets to a final concentration of 10 μmol/l (30 min, 37°C). Platelets were then stimulated with thrombin (0, 0.05 or 0.25 U/ml, 5 min, 37°C). Mepacrine (10 μmol/l, Sigma-Aldrich) was added (30 min, 37°C) to stain dense granules. Samples, diluted 1:2 in Hanks’ balanced salt solution were run on the flow cytometer. In these experiments, 10,000 platelets were collected off of forward and side scatter properties.
platelet activation on endothelial cells and monocytic cell line because these cell types play important roles in CVD pathogenesis (35,36). Labeled platelets isolated from HIV and healthy individuals were cocultured with HUVECs for 2 h in unstimulated (basal) conditions and following incubation with thrombin (0.05 U/ml). In healthy subjects, few unstimulated platelets adhered to HUVECs (Figure 1A). By contrast, platelets in their basal state isolated from subjects with HIV had increased adhesion to endothelial cells (Figure 1A). Thrombin stimulation further increased platelet adhesion in both groups, with a more pronounced effect in the HIV group (Figure 1A). Moreover, we observed that thrombin-activated platelets from subjects with HIV surrounded HUVECs forming rosette-like aggregates (Figure 1A).

We next evaluated whether adherent platelets induced mRNA expression of endothelial cells and monocytic cells activation markers. Consistent with prior reports (37–41), we found that incubating endothelial cells or monocytic cells with healthy donor platelets that were activated by thrombin resulted in up-regulation of inflammatory gene expression (Figures 1B to 1F). Platelets isolated from
subjects with HIV (vs. controls) induced the expression of interleukin (IL)-8, intercellular adhesion molecule (ICAM)-1, and monocyte chemotactic protein (MCP)-1 in HUVECs. These differences were observed from platelets in their basal state and after treatment with thrombin (Figures 1B to 1D). Platelets isolated from subjects with HIV were also able to induce expression of IL-6 and MCP-1 in THP-1 cells (Figures 1E and 1F). Altogether, these data demonstrate that platelets from HIV-infected subjects cause endothelial cell and monocyte activation.

DIFFERENTIALLY EXPRESSED PLATELET mRNA IN HIV. To characterize transcriptional changes associated with the hyperreactive platelet phenotype observed in persons with HIV, we conducted an unbiased RNA-Seq analysis of platelet mRNA from 6 HIV and 3 control subjects. Across the 9 platelet samples, there was an average of 23 million mapped reads per sample with an average unique mapping rate of 78.6%. Using a cutoff of normalized counts $>1$ averaged across the 9 samples, we found 11,988 expressed transcripts (Supplemental Figure 1). We identified candidate transcripts differentially expressed in platelets between HIV and controls (Figure 2A, Supplemental Table 2). The volcano plot of log2 (fold change) versus log10 (adjusted p values) from differentially expressed
genes between the 2 cohorts is reported in Figure 2B. Using filtering criteria of a p value <0.01, GO analysis was performed on 73 differentially expressed transcripts (Supplemental Table 3).

Platelets from persons with HIV had increased expression of genes involved in secretion and exocytosis and leukocyte activation in immune responses (Figure 2C, Supplemental Table 3). The gene encoding ABCC4 (ATP binding cassette subfamily C member 4, also called ABCC4) was the most up-regulated gene in the platelet transcriptome of HIV-infected subjects versus controls (3.5-fold change, p < 0.0001). Consistent with pathways analysis, ABCC4 is a protein-coding gene known to play an important role in platelet degranulation and activation (Figure 2C). Therefore, we selected ABCC4 as our candidate transcript for further analyses.

We validated ABCC4 up-regulation in HIV platelets by qPCR; ABCC4 expression was increased 2.3-fold in HIV versus controls (Supplemental Figure 2). To determine the robustness of our findings, ABCC4 platelet mRNA expression was assessed in a larger cohort of HIV-infected persons (n = 48) and healthy controls. Consistently, ABCC4 was significantly up-regulated in the HIV population compared with the control group, supporting our platelet RNA-Seq data (Figure 2D). In unadjusted analyses, HIV status was significantly associated with higher platelet mRNA ABCC4 expression. Additional variables associated with higher ABCC4 platelet expression were male sex and Hispanic ethnicity (Supplemental Table 4). No significant difference in ABCC4 expression was noted for hepatitis B, hepatitis C, CD4 count, smoking status, HIV duration, or type of ART (Supplemental Table 3). After adjustment for age, sex, race/ethnicity, and body mass index, persons with HIV had significantly higher ABCC4 than controls (β = −2.09; p = 0.001) (Supplemental Table 5).

Because platelet activity is increased in persons with HIV, we examined whether expression of platelet ABCC4 mRNA was associated with platelet activity. Platelet surface expression of P-selectin (r = 0.72; p = 0.046) and activated Integrin αIIβ3 (r = 0.77; p = 0.025) significantly correlated with ABCC4 platelet mRNA expression (Figure 2E).

**ABCC4 PROTEIN LEVELS IN PLATELETS OF HIV SUBJECTS.** ABCC4 transcriptional up-regulation in subjects with HIV was assessed and confirmed at the protein level. Western blot analysis of platelet lysates showed a significant increase of ABCC4 protein expression in HIV-infected subjects versus controls (p < 0.05) (Figure 3A). Immunofluorescence microscopy of collagen-spread platelets further confirmed increased ABCC4 expression in platelets isolated from subjects with HIV (Figure 3B).

**ABCC4 INHIBITION REDUCES PLATELET GRANULE RELEASE AND IMPAIRS CYCLIC NUCLEOTIDE HOMEOSTASIS IN HIV.** Because ABCC4 is involved in the transport of diverse endogenous compounds, including dense granule content, we sought to investigate dense granule release in controls and HIV-infected subjects. Accumulation of the fluorescent mepacrine is used as a dense granule marker (42,43).

Consistent with the role of thrombin in inducing dense granule release (44), thrombin stimulation decreased mepacrine staining (e.g., increase in dense granule release) in both groups in a concentration-dependent manner (Figure 4A). Notably, in the basal state, subjects with HIV showed decreased mepacrine staining compared with controls (Figure 4B), supporting our previous data of enhanced basal platelet activation in HIV (16).

Delta granule release was further characterized by the highly selective ABCC4 inhibitor, Ceefourin 2. Delta granule release was measured after incubation with Ceefourin in both HIV and control subjects. Pretreatment of HIV platelets with Ceefourin prevented granule release after thrombin stimulation. By contrast, Ceefourin had no effect on granule release in platelets from controls (Figure 4C). Our data suggest that ABCC4 inhibition would impair delta granule release in HIV-infected persons, for example, those with increased expression and function of ABCC4.

Upon platelet activation and certain pathophysiological conditions, ABCC4 translocates to the plasma membrane and alters platelet function by increasing transport of several substrates (45,46). In platelets, a rise in cyclic nucleotides prevents activation of signaling pathways. Thus, any change in the distribution or availability of cyclic nucleotides may interfere with platelet reactivity. We therefore analyzed whether ABCC4 overexpression in HIV was associated with platelet levels of intracellular cAMP and downstream signaling. A significant increase in the amount of secreted cAMP was observed in persons with HIV versus controls (p < 0.01) (Figure 4D). To determine whether these differences in cAMP secretion affected downstream signaling, we analyzed vasodilator-stimulated phosphoprotein (VASP) phosphorylation on Ser157, a preferential cAMP-dependent protein kinase phosphorylation site. Western blot analysis of platelet lysates in HIV-infected individuals and healthy subjects revealed that VASP phosphorylation was significantly reduced in HIV (Figure 4E), suggesting a decrease in cytosolic cAMP levels in HIV platelets. Altogether, these data...
suggest that ABCC4 overexpression observed in HIV contributes to decreased cytosolic cAMP levels in platelets, resulting in a decreased VASP phosphorylation, thus leading to enhanced platelet activation. **ABCC4 INHIBITION DECREASES PLATELET EFFECTOR CELL FUNCTION AND PLATELET S1P RELEASE IN HIV.** To investigate the potential role of ABCC4 in platelets as effector cells, we tested platelet adhesion to endothelial cells in the presence of Ceefourin. Pre-treatment with Ceefourin of platelets isolated from HIV-infected persons significantly reduced platelet adhesion to HUVECs in the basal state or following thrombin stimulation (47% and 39% reduction compared with controls, respectively [Figure 5A]), whereas no differences were observed in healthy subjects (Supplemental Figure 3). Consistent with reduced platelet adhesion to endothelial cells, ABCC4 inhibition in platelets resulted in the reduction of HUVEC activation markers IL-8, ICAM-1, and MCP-1 and THP-1 markers IL-6 and MCP-1 only in the HIV cohort (Figures 5B and 5C, Supplemental Figure 4). Altogether, these data suggest that ABCC4 inhibition in HIV may represent a useful mechanism to reduce platelet-induced endothelial cell and monocyte activation, by blocking the release of granule contents from platelets.

S1P, an immune modulating lipid mediator, has been reported to induce proinflammatory signaling pathways in the immune and vascular system (47–49). Platelets release S1P upon activation, and ABCC4 has been reported to mediate its release from platelets (50). We therefore sought to investigate whether platelets from HIV-infected subjects with ABCC4 overexpression released greater amount of S1P compared with controls. In the basal state, S1P levels were markedly increased in HIV platelet supernatants (Figure 5D). After platelet activation, S1P was measured in plasma of HIV and controls. S1P levels were significantly increased in plasma of subjects with HIV (p < 0.0001) (Figure 5G).
DISCUSSION

Prior studies have demonstrated increased cardiovascular risk in persons with virologically controlled HIV on ART (12,51). Nonetheless, the reason(s) for this heightened risk have yet to be fully clarified. Platelet activation and immune activation leading to a prothrombotic state have been proposed as significant contributors to CVD (6,16). Platelets isolated from persons with HIV have increased surface expression of P-selectin, activated glycoprotein IIb/IIIa, and increased aggregation in response to submaximal agonist simulation (16,52). In the current study, we expand on these findings and demonstrate an enhanced platelet effector role in subjects with HIV. Both in the basal state and following agonist stimulation, platelets from subjects with HIV were more adherent to endothelial cells and monocytic cells and were able to induce a proinflammatory effect on these cell types. These findings support the
role of platelets as inflammatory mediators in persons with HIV infection.

In pathological conditions in response to extracellular signaling, a transcriptional modulation during megakaryopoiesis may occur (53). Although anucleate, platelets retain megakaryocyte-derived cytoplasmic RNA and may translate small amounts of mRNAs as well as process miRNAs (54–56). Therefore, platelet transcript content may play a biological role beyond being remnant RNA derived from the megakaryocyte. Herein, we present an unbiased characterization of the transcriptome of platelets isolated from HIV subjects under ART using RNA-Seq, to identify mRNAs associated with increased platelet activity in HIV. Our platelet transcriptome analysis identified pathways differentially expressed between HIV and healthy individuals, including exocytosis and secretion, inflammatory response, and immune cell trafficking.

Notably, ABCC4 mRNA emerged as the most upregulated transcript in HIV compared with controls. ABCC4 has been shown to play an important role in conveying several molecules that control multiple

![Image](50x332 to 522x651)

**FIGURE 5** ABCC4 Contributes to Endothelial and Monocyte Activation and Increased S1P Release From Activated Platelets in HIV

(A) Immunofluorescence microscopy of platelet adhesion to HUVECs in subjects with HIV. Stained platelets (green) were left untreated (Untreated) or treated with Ceefourin2 (Ceefourin, 10 μmol/l) for 30 min. Thrombin (0.05 U/ml, 5 min) was then added (Activated). HUVEC nuclei are stained with DAPI (blue). The images are representative of 3 subjects for each group. Values are represented as percentage of respective controls. **p < 0.01 and *p < 0.05. Magnification ×20.**

(B and C) Gene expression analysis of HUVEC inflammatory genes IL-8, ICAM-1, and MCP1, and THP-1 markers IL-6 and MCP-1. HUVECs and THP-1 were cocultured with untreated or pre-treated (Ceefourin, 10 μmol/l) platelets from HIV-infected subjects at basal (upper graphs) and after stimulation with thrombin (0.05 U/ml, bottom graphs). Results were normalized on 18S5 RNA. Values represent fold change after normalization to basal or activated condition in 3 different subjects. **p < 0.01 versus Basal and *p < 0.05 versus Activated.

(D) Sphingosine-1-phosphate (S1P) in platelet supernatants of HIV (n = 16) and controls (n = 8) at the basal state was analyzed by high-performance liquid chromatography-mass spectrometry (HPLCMS/MS) and normalized for protein content. *p < 0.05.

(E) S1P quantification in platelet supernatants of subjects with HIV (n = 17) and healthy controls (n = 7) before and after stimulation with thrombin (0.05 U/ml, 5 min). Levels are reported after normalization to respective basal levels. *p < 0.05.

(F) Platelets from healthy subjects (n = 5) were left untreated or stimulated with thrombin in presence or absence of Ceefourin (10 μmol/l). S1P levels were quantified in platelet supernatants, and values expressed as percentage of resting platelets. *p < 0.05.

(G) S1P quantified in plasma of subjects with HIV (n = 40) and controls (n = 16) by HPLCMS/MS. Abbreviations as in Figure 1.
cardiovascular processes, including smooth muscle cell proliferation, cardiomyocyte contractility, and platelet activation (57–59). Recent findings indicate that he-mostasis and thrombosis are affected in ABCC4-deficient mice, with ABCC4 promoting platelet aggregation by modulating the cAMP-protein kinase A (PKA) signaling (44). These results are in line with studies showing that the absence or inhibition of ABCC4 affects platelet activation and aggregation (60,61). Therefore, ABCC4 was chosen as a candidate for further analyses and was validated in a larger cohort of persons with HIV and controls. In this second larger cohort, increased ABCC4 gene expression was also demonstrated, and remained significant after multivariable adjustment. Although HIV status was associated with platelet expression of ABCC4 mRNA, no association was noted for different HIV ART regimens, length of HIV diagnosis, smoking status, and CD4 count.

We and others have previously noted that platelet activity is in a heightened state in persons with HIV on ART (16,52,62,63). It was therefore not surprising that ABCC4 overexpression in HIV significantly correlated with platelet activation markers P-selectin and PAC-1, supporting a link between ABCC4 and platelet activity in these persons.

Localization of ABCC4 in platelets has been debated and remains uncertain. ABCC4 was demonstrated to be highly expressed on the membrane of dense granules, which facilitates ADP accumulation and export (42,46). It was also proposed that ABCC4 localization in platelets can be shifted from granules to the plasma membrane in certain high-risk conditions, including platelet activation (45,64). Other groups found that ABCC4 is localized primarily to the plasma membrane in platelets (65,66). We investigated dense granule ADP accumulation and release in HIV-infected
subjects and healthy controls. We confirmed increased platelet activity in HIV-infected individuals, compared with controls in the basal state. The export of ADP and granule mediators was evaluated in both groups using the highly selective ABCC4 inhibitor, Ceefourin 2 (32). In previous reports, ABCC4 platelet inhibition used the MK571 antagonist (65,67,68). However, MK571 has been noted to be a non-specific ABCC4 inhibitor as well as a potent leukotriene D4 receptor and MRP1 antagonist (69). In persons with HIV infection, Ceefourin inhibited dense granule release after thrombin activation, suggesting that ABCC4 impairs dense-granule release in HIV.

ABCC4 has been reported to be an endogenous regulator of intracellular cAMP and cAMP-mediated signaling pathway in platelets (44,65,68). ABCC4 inhibition was found to protect from hypoxemia-induced pulmonary hypertension in a murine model by increasing intracellular cAMP levels and preventing activation of cAMP-mediated pathways (68). In murine platelets, absence of ABCC4 attenuated collagen-mediated aggregation and impaired thrombus formation by producing an elevation in cAMP level (65). Therefore, we sought to investigate whether ABCC4 modulated platelet activation in HIV through a cAMP-mediated mechanism. We measured platelet cAMP secretion and cAMP cytosolic levels through VASP phosphorylation on Ser157. Our data demonstrated that platelet ABCC4 is an important contributor to platelet activity in HIV, by impairing cAMP homeostasis.

We then demonstrated that ABCC4 inhibition in platelets attenuated endothelial cell and monocyte activation, both in the basal state and after activation. These data support a new role for platelet ABCC4 in mediating, not only platelet function, but also the platelet-mediated effector cell function in HIV. The mechanism linking platelet ABCC4 expression and monocyte/endothelial cell activation is unknown, but signaling lipid S1P is a likely intermediary. Platelets are known to release high levels of a multifunctional S1P upon direct activation of protein kinase C signaling (e.g., thrombin) or during blood clotting (70,71). Cellular S1P secretion requires active transport across the membrane by ATP-dependent carriers, such as ABCC4 (50). A link between platelet S1P in inflammatory processes and immune response has already been suggested (48,72,73). In our study, we demonstrated an enhanced basal secretion of S1P from platelets in HIV, consistent with a hyperreactive platelet phenotype in the basal state. Moreover, ABCC4 inhibition was able to reduce S1P levels following thrombin-induced platelet activation, suggesting a role of ABCC4 as a mediator of S1P release in subjects with HIV who showed a basally active platelet phenotype. A model depicting the role of ABCC4 on platelet function and regulation of inflammatory response of endothelial cells and monocytic cell line in HIV disease is reported in Figure 6.

As previously mentioned, ABCC4 is involved in the development and progression of pulmonary artery hypertension (PAH) (68). Many clinical studies have demonstrated an association between PAH and HIV infection (74), both before and after ART (75,76). The impact of ABCC4 on PAH incidence and severity in persons with HIV is unknown and deserves further investigation.

Finally, NSAIDs, including aspirin, increase ABCC4 expression (45,77). ABCC4-mediated aspirin extrusion from the platelet cytosol causes an incomplete COX-1 inhibition in persons after coronary artery bypass graft surgery (45). In the current study, NSAID use was an exclusion criterion for participation. A recent randomized trial from our group demonstrated no significant benefit on immune activity or vascular health from low-dose aspirin in persons with HIV (78). Whether this neutral effect was mediated, in part, by ABCC4 overexpression in HIV-infected persons is unknown. A pilot trial of a different antiplatelet therapy, clopidogrel, in persons with HIV is ongoing.

CONCLUSIONS

Our study is the first to identify increased levels of ABCC4 mRNA as a novel mediator regulating platelet function in persons with HIV. Moreover, we provide insights into the molecular mechanisms by which ABCC4 mediates a hyperreactive platelet phenotype and platelet effector cell function in the setting of HIV. These findings may have important clinical implications in HIV-infected persons. In fact, by acting on the extrusion of cyclic nucleotides and inflammatory mediators, ABCC4 inhibition might represent a novel antithrombotic strategy for virologically suppressed HIV-infected subjects on ART.

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**COMPETENCY IN MEDICAL KNOWLEDGE:** HIV infection significantly increases the risk of myocardial infarction and other forms of CVD. Multiple factors including pathological platelet activation contribute to this enhanced risk. However, the mechanism by which HIV infection increases platelet activity is unknown.

**TRANSLATIONAL OUTLOOK:** The characterization of the platelet transcriptome profile in persons with virologically controlled HIV on antiretroviral therapy revealed ABC4 as a central mediator of platelet activity and platelet-mediated proinflammatory response in endothelial cells and monocytic cell line. Targeting ABC4 in HIV-infected subjects may represent a novel antithrombotic strategy in persons with HIV.

**REFERENCES**

1. Freiberg MS, Chang CC, Kuller LH, et al. HIV infection and the risk of acute myocardial infarction. JAMA Intern Med 2013;173:614–22.
2. Vos AG, Hulzebosch A, Grobbée DE, Barth RE, Klijpstein-Grobusch K. Association between immune markers and surrogate markers of cardiovascular disease in HIV positive patients: a systematic review. PLoS One 2012;7:e0169986.
3. Friis-Moller N, Thiebaut R, Reiss P, et al. Predicting the risk of cardiovascular disease in HIV-infected patients: the data collection on adverse events of anti-HIV drugs study. Eur J Cardiovasc Prev Rehabil 2010;17:491–501.
4. Triant VA. HIV infection and coronary heart disease: an intersection of epidemics. J Infect Dis 2012;205 Suppl 3:535–61.
5. Nou E, Lo J, Grinspoon SK. Inflammation and cardiovascular disease in HIV. Lancet Diabetes Endocrinol 2016;4:598–610.
6. Nou E, Lo J, Grinspoon SK. Inflammation, immune activation, and cardiovascular disease in HIV. AIDS 2016;30:1495–509.
7. Triant VA, Meigs JB, Grinspoon SK. Association of C-reactive protein and HIV infection with acute myocardial infarction. J Acquir Immune Defic Syndr 2009;51:268–73.
8. Lichtenstein KA, Armon C, Buchacz K, et al. Low CD4+ T cell count is a risk factor for cardiovascular disease events in the HIV outpatient study. Clin Infect Dis 2010;51:435–47.
9. Hadigan C, Meigs JB, Corcoran C, et al. Metabolic abnormalities and cardiovascular disease risk factors in adults with human immunodeficiency virus infection and lipodystrophy. Clin Infect Dis 2001;32:130–9.
10. Kuller LH, Tracy R, Beloso W, et al. Inflammatory and coagulation biomarkers and mortality in patients with HIV infection. PLoS Med 2008;5:e203.
11. Riddler SA, Smit E, Cole SR, et al. Impact of HIV infection and HAART on serum lipids in men. JAMA 2003;289:2979–82.
12. Torriani FJ, Komarow L, Parker RA, et al. Endothelial function in human immunodeficiency virus-infected antiretroviral-naive subjects before and after starting potent antiretroviral therapy: the ACTG (AIDS Clinical Trials Group) study S152s. J Am Coll Cardiol 2008;52:569–76.
13. Allass O, Samaran R, Jenabian MA, et al. Differential synthesis and release of IL-18 and IL-18 binding protein from human platelets and their implications for HIV infection. Cytokine 2017;90:144–54.
14. DAD Study Group, Friis-Moller N, Reiss P, et al. Class of antiretroviral drugs and the risk of myocardial infarction. N Engl J Med 2007;356: 1723–35.
15. Hauguel-Moreau M, Bocca F, Boyd A, et al. Platelet reactivity in human immunodeficiency virus infected patients on dual antplatelet therapy for an acute coronary syndrome: the EVEREST-HIV study. Eur Heart J 2017;38: 1676–86.
16. O’Brien M, Montenont E, Hu L, et al. Aspirin attenuates platelet activation and immune activation in HIV-1-infected subjects on antiretroviral therapy: a pilot study. J Acquir Immune Defic Syndr 2013;63:280–8.
17. Davi G, Patrono C. Platelet activation and atherothrombosis. N Engl J Med 2003;357:2482–94.
18. Rondina MT, Weyrich AS, Zimmerman GA. Platelets as cellular effectors of inflammation in vascular diseases. Circ Res 2013;112:1506–19.
19. Gawaz M, Langer H, May AE. Platelets in inflammation and atherogenesis. J Clin Invest 2005;115:3378–84.
20. Klinger MH, Jelkmann W. Role of blood platelets in the regulation of the type I interferon system is strongly associated with vascular disease. Blood 2010;116:1951–7.
21. Raghavachari N, Xu X, Harris A, et al. Amplified expression profiling of platelet transcriptome reveals changes in arginine metabolic pathways in patients with systemic lupus erythematosus: up-regulation of the type I interferon system is strongly associated with vascular disease. Blood 2010;116:1951–7.
22. Freedman JE, Larson MG, Tanrioverdi K, et al. Platelet transcriptional and protein expression in patients with systemic lupus erythematosus: up-regulation of the type I interferon system is strongly associated with vascular disease. Blood 2010;116:1951–7.
23. Montenont E, Echagarruga C, Allen N, Araldi E, Suarez Y, Berger JS. Platelet WDR1 suppresses platelet activity and is associated with cardiovascular disease. Blood 2016;128:2033–42.
24. Cheung L, Fleming CL, Watt F, et al. High-throughput screening identifies Ceefoorin 1 and Ceefoorin 2 as highly selective inhibitors of multidrug resistance protein 4 (MRP4). Biochem Pharmacol 2014;91:97–108.
25. Plé H, Landry P, Benham A, Coarfa C, Gunaratne PH, Provost P. The repertoire and features of human platelet microRNAs. PLoS One 2012;7:e50746.
34. Bu H, Leohr JK, Kuo MS. Analysis of sphingolipids in extracted human plasma using liquid chromatography electrospray ionization tandem mass spectrometry. Anal Biochem 2012;423:187-94.
35. Jackson SP. Arterial thrombosis—insidious, unpredictable and deadly. Nat Med 2011;17:1423-36.
36. von Hundelshausen P, Weber C. Platelets as immune cells: bridging inflammation and cardiovascular disease. Circ Res 2007;100:27-40.
37. Weyrich AS, Elstad MR, McEver RP, et al. Activated platelets signal chemokine synthesis by human monocytes. J Clin Invest 1996;97:1525-34.
38. Passacquale G, Vamadevan P, Pereira I, Hamid C, Corrillig V, Ferro A. Monocyte-platelet interaction induces a pro-inflammatory phenotype in circulating monocytes. PLoS One 2011;6:e25595.
39. Nhek S, Clancy R, Lee KA, et al. Activated platelets induce endothelial cell activation via an interleukin-1beta pathway in systemic lupus erythematosus. Arterioscler Thromb Vasc Biol 2017;37:707-16.
40. Kojima H, Kanada H, Shimizu S, et al. CD226 mediated platelet and megakaryocyte cell adhesion to vascular endothelial cells. J Biol Chem 2003;278:36748-53.
41. Bombeli T, Schwartz BR, Harlan JM. Activation of adherent platelets to endothelial cells: evidence for a GPIblllalpha-dependent bridging mechanism and novel roles for endothelial intercellular adhesive molecule 1 (ICAM-1), alphavbeta3 integrin, and GPIbalpha. J Exp Med 1998;187:329-39.
42. Jedlitschky G, Tirschmann K, Lubenow LE, et al. The nucleotide transporter MRP4 (ABCC4) is highly expressed in human platelets and present in vascular endothelial cells. J Biol Chem 2003;278:36751-64.
43. Gordon N, Thom J, Cole C, Baker R. Rapid detection of hereditary and acquired platelet storage pool deficiency by flow cytometry. Br J Haematol 1995;89:117-23.
44. Decouture B, Dreano E, Belleverie-Rolland T, et al. Impaired platelet activation and cAMP homeostasis in MRP4-deficient mice. Blood 2015;126:1823-30.
45. Mattiello T, Guerriero R, Lotti LV, et al. Aspirin extrusion from human platelets through multidrug resistance protein-4-mediated transport: evidence of a reduced drug action in patients after coronary artery bypass grafting. J Am Coll Cardiol 2011;58:56-61.
46. Jedlitschky G, Cattaneo M, Lubenow LE, et al. Role of MRP4 (ABCC4) in platelet adenine nucleotide-storage: evidence from patients with delta-storage pool deficiencies. Am J Pathol 2010;176:1097-103.
47. Zeng F, Xia Y, Yan W, et al. Sphingosine 1-phosphate signaling contributes to cardiac inflammation, dysfunction, and remodeling following myocardial infarction. Am J Physiol Heart Circ Physiol 2016;310:H250-61.
48. Rauch BH. Sphingosine 1-phosphate as a link between blood coagulation and inflammation. Cell Physiol Biochem 2014;34:185-96.
49. Aoki M, Aoki H, Ramanathan R, Hilt NC, Takabe K. Sphingosine-1-phosphate signaling in immune cells and inflammation: roles and therapeutic potential. Mediators Inflamm 2016;2016:860878.
50. Ulytch T, Bohm A, Polzin A, et al. Release of sphingosine-1-phosphate from human platelets is dependent on thromboxane formation. J Thromb Haemost 2011;9:790-8.
51. Liao JK. Linking endothelial dysfunction with endothelial cell activation. J Clin Invest 2013;123:540-1.
52. Holme PA, Muller F, Solum ND, Brostad F, Froland SS, Aukrust P. Enhanced activation of platelets with abnormal release of RANTES in human immunodeficiency virus type 1 infection. FASEB J 1998;12:79-89.
53. Tijssen MR, Ghevaert C. Transcription factors in late megakaryopoiesis and related platelet disorders. J Thromb Haemost 2013;11:593-604.
54. Rowley JW, Oler AJ, Tolley ND, et al. Genome-wide RNA-seq analysis of human and mouse platelet transcriptomes. Blood 2011;118:e101-11.
55. Nagalla S, Shaw C, Kong X, et al. Platelet microRNA-mRNA coexpression profiles correlate with platelet reactivity. Blood 2011;117:5189-97.
56. Edelstein LC, Bray PF. MicroRNAs in platelet production and activation. Blood 2011;117:5289-96.
57. Sassi Y, Lypiskaia L, Vandecasteele G, et al. Multidrug resistance-associated protein 4 regulates cAMP-dependent signaling pathways and controls human and rat SCM proliferation. J Clin Invest 2008;118:2747-57.
58. Sassi Y, Abi-Gerges A, Fauconnier J, et al. Regulation of CAMP homeostasis by the efflux protein MRP4 in cardiac myocytes. FASEB J 2012;26:1009-17.
59. Belleverie-Rolland T, Sassi Y, Decouture B, et al. MRP4 (ABCC4) as a potential pharmacological target for cardiovascular disease. Pharmacol Res 2016;107:381-9.
60. Jedlitschky G, Vogelgesang S, Kroezer HK. HDL1-P-glycoprotein (ABCC1)-mediated disposition of amyloid-beta peptides: implications for the pathogenesis and therapy of Alzheimer’s disease. Clin Pharmacol Ther 2010;88:441-3.
61. Borgognone A, Pulcinelli FM. Reduction of cAMP and cGMP inhibitory effects in human platelets by MRP4-mediated transport. Thromb Haemost 2012;108:955-62.
62. Satchell CS, Cotter AG, O’Connor EF, et al. Platelet function and HIV: a case-control study. AIDS 2010;24:649-57.
63. Corrales-Medina VF, Simkins J, Chirinos JA, et al. Increased levels of platelet microparticles in HIV-infected patients with good response to antiretroviral therapy. J Acquir Immune Defic Syndr 2010;54:217-8.
64. Jedlitschky G, Greinacher A, Kroeker HM. Transports in human platelets: physiologic function and impact for pharmacotherapy. Blood 2012;119:3394-402.
65. Cheepala SB, Pitre A, Fukuda Y, et al. The ABC4 membrane transporter modulates platelet aggregation. Blood 2015;126:2307-19.
66. Bai J, Lai L, Yeo HC, Goh BC, Tan TM. Multidrug resistance protein 4 (MRP4/ABCC4) regulates thrombus formation in vitro and in vivo. Eur J Pharmacol 2014;737:159-67.
67. Hara Y, Sasaki Y, Guibert C, et al. Inhibition of MRP4 prevents and reverses pulmonary hypertension in mice. J Clin Invest 2011;121:2888-97.
68. Reid G, Wielinga P, Zelcer N, et al. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. Proc Natl Acad Sci U S A 2003;100:9244-9.
69. Tantry US, Bonello L, Aradi D, et al. Consensus and update on the definition of on-treatment platelet reactivity to adenosine diphosphate associated with ischemia and bleeding. J Am Coll Cardiol 2013;62:2261-73.
70. Yatomi Y, Ohmori T, Rile G, et al. Sphingosine 1-phosphate as a major bioactive lysophospholipid that is released from platelets and interacts with endothelial cells. Blood 2000;96:3431-8.
71. English D, Welch Z, Kovala AT, et al. Sphingosine 1-phosphate released from platelets during clotting accounts for the potent endothelial cell chemotactic activity of blood serum and provides a novel link between hemostasis and angiogenesis. FASEB J 2000;14:2255-65.
72. Mahajan-Thakur S, Bohm A, Jedlitschky G, Schror K, Rauch BH. Sphingosine-1-phosphate and its receptors: a mutual link between blood coagulation and inflammation. Mediators Inflamm 2015;2015:83059.
73. Morris A, Gingo MR, George MP, et al. Cardiopulmonary function in individuals with HIV infection in the antiretroviral therapy era. AIDS 2012;26:731-40.
74. Speich R, Jenni R, Opravil M, Pab M, Russi EW. Primary pulmonary hypertension in HIV infection. Chest 1991;100:1268-71.
75. Sittbon O, Lascoux-Combe D, Delfraissy JF, et al. Prevalence of HIV-related pulmonary arterial hypertension in the current antiretroviral therapy era. Am J Respir Crit Care Med 2008;177:108-13.
76. Temperilli F, Di Franco M, Massimi I, et al. Nonsteroidal anti-inflammatory drugs in-vivo and in-vivo treatment and Multidrug Resistance Protein 4 expression in human platelets. Vascul Pharmacol 2016;76:11-7.
77. O’Brien MP, Hunt PW, Kitch DW, et al. A randomized placebo controlled trial of aspirin effects on immune activation in chronically human immunodeficiency virus-infected adults on virologically suppressive antiretroviral therapy. Open Forum Infect Dis 2017;4:ofw278.

**KEY WORDS** ABCC4, cardiovascular disease, HIV, platelet activity

**APPENDIX** For an expanded Methods section and supplemental tables and figures, please see the online version of this paper.