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CLINICAL RESEARCH

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

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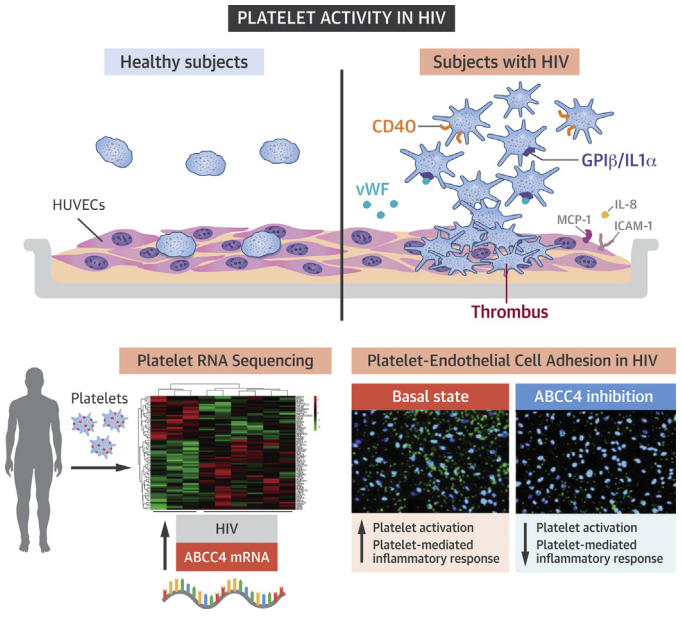
VISUAL ABSTRACT



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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.**

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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

S1P = 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)

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).

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Platelets play a major role in hemostasis, with increased platelet activity contributing to the pathogenesis of atherothrombosis (17). In addition to their well-known functions in hemostasis and thrombosis, platelets play an important role in inflammation and immune activation (18). Activated platelets synthesize and release a host of pleiotropic inflammatory mediators, including interleukin-1β, CD40L, microparticles, and tissue factor, which interact with leukocytes and endothelial cells (19–22). Platelets are discoid, anucleate cells generated from bone marrow megakaryocytes that retain megakaryocyte-derived mRNAs and translational machinery for protein biosynthesis (23–25). Platelet RNA expression profiling has been used in subjects with sickle cell disease, obesity, systemic lupus erythematosus, and CVD (26–30). Transcriptional profiling may yield novel mechanistic insights, unbiased by pre-existing disease hypotheses. We recently found the platelet transcriptome can: 1) distinguish healthy subjects with hyperreactive versus hyporeactive platelets; and 2) support a mechanistic link between platelet activity and CVD (31).

In the present study, we performed unbiased transcriptome profiling in platelets from subjects with HIV under ART and healthy controls. The platelet transcriptome was analyzed and single-transcript models constructed to identify candidate mRNAs with differential expression. We then sought to validate and study the mechanism of our top candidate transcript using ex vivo and in vitro cellular and molecular assays.

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

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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 A^C·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 QIAzol (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 QIAzol. 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 QIAzol 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),

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/m^2	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/mm^3)	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 (10^6/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 Biotechnology, 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, Axiocam 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 imaged randomly, and all cells in each field were manually counted for the number of green dots per cell.

MEPACRINE ASSAY. Washed platelets (10^8/ml) were diluted 1:40 in Hanks' balanced salt solution. Ceefourin 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.

SPHINGOSINE-1-PHOSPHATE MEASUREMENT. 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 diethylamide 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 # 51333009, 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).

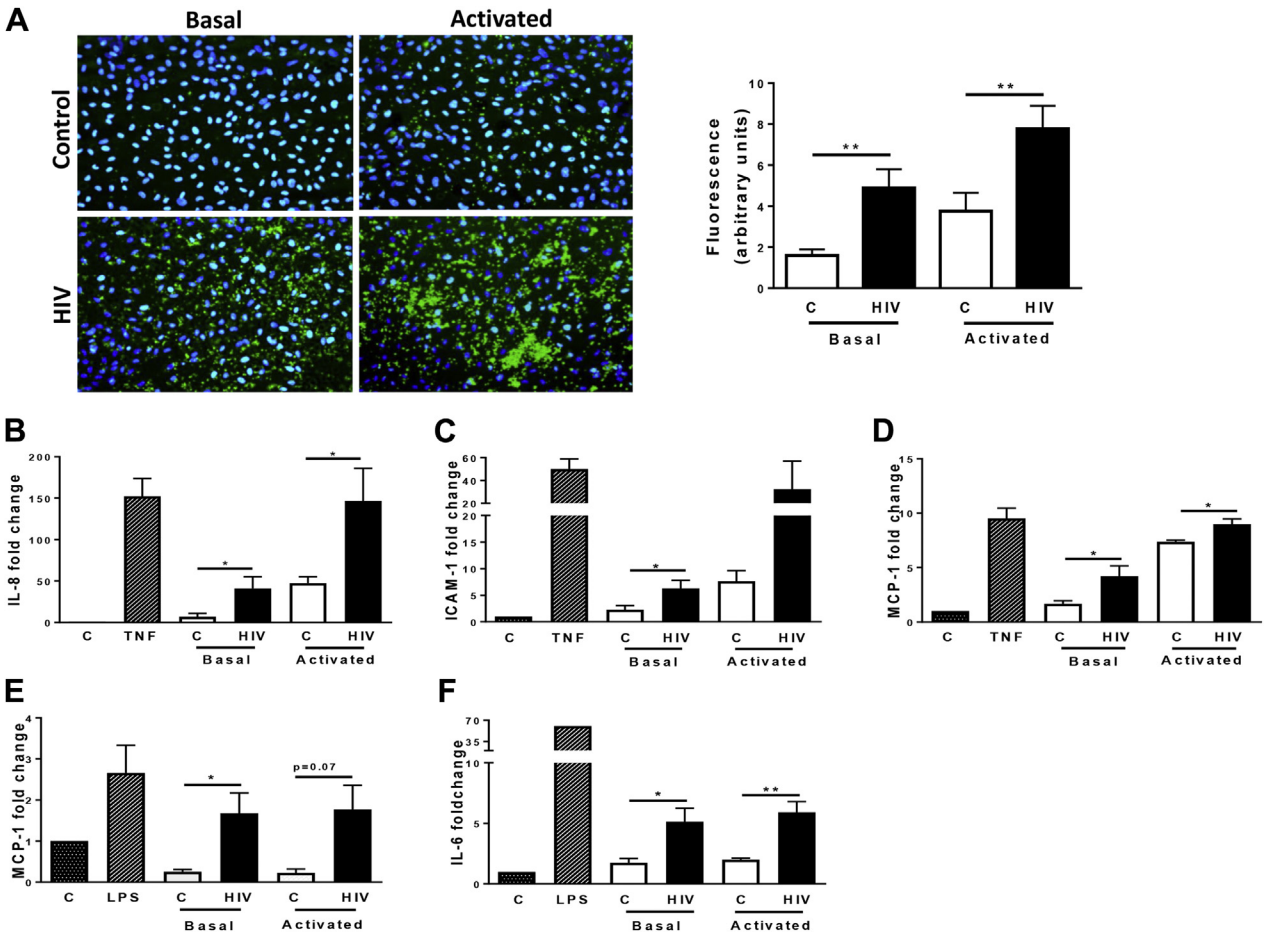
Methods for quantitative polymerase chain reaction (qPCR), Western blot, flow cytometry, and cyclic adenosine monophosphate (cAMP) measurement available in the Supplemental Methods.

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

FIGURE 1 Endothelial and Monocytic Activation by Platelets Is Enhanced in HIV Subjects

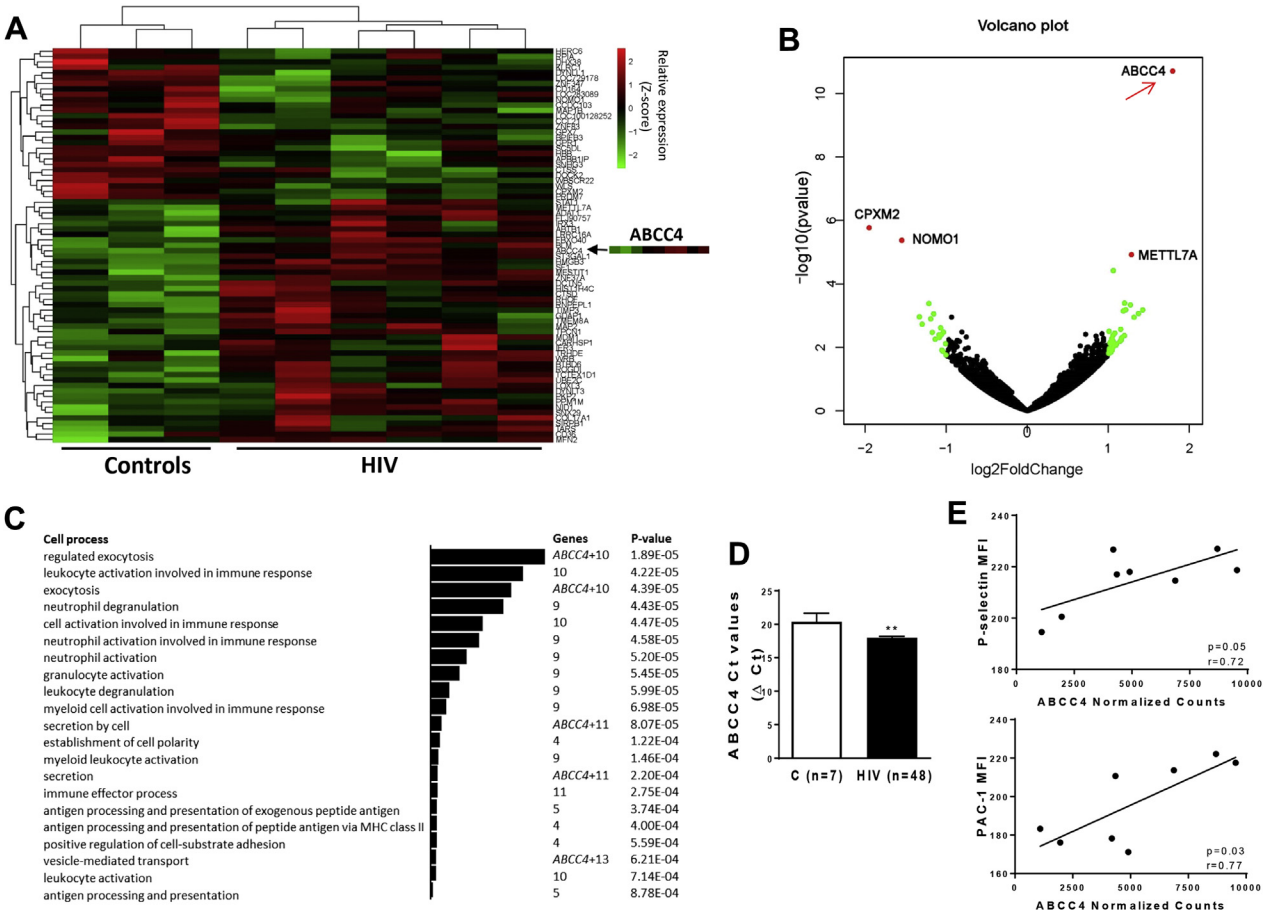


(A) Immunofluorescence microscopy of platelet adhesion to HUVECs in healthy and HIV subjects. Freshly isolated platelets **(green)** from controls (C) and HIV subjects (HIV) were stained with CellTracker dye and left untreated (Basal, **left panels**) or treated with thrombin (0.05 U/ml for 5 min; Activated, **right panels**) before incubating with HUVECs. HUVEC nuclei are stained with DAPI **(blue)**. The images are representative of 3 subjects for each group. Magnification, 20×. **p < 0.01. **(B to D)** HUVECs were coincubated with resting (Basal) or activated platelets from HIV (n = 6 to 7) and healthy controls (n = 6 to 7) for 2 h. Interleukin (IL)-8 **(B)**, intercellular adhesion molecule (ICAM)-1 **(C)**, and monocyte chemotactic protein (MCP)-1 **(D)** expression was assessed by quantitative polymerase chain reaction (qPCR). Values, normalized on 18S5 RNA represent fold change versus respective control. TNF (10 ng/ml) represents the positive control. *p < 0.05. **(E and F)** THP-1 were cultured with HIV platelets (HIV, n = 7) or control platelets (C, n = 4) at basal (Basal) or after stimulation (thrombin 0.25 U/ml, 5 min) for 2 h. MCP-1 **(E)** and IL-6 **(F)** expression was assessed by qPCR and normalized on 18S5 RNA. Lipopolysaccharide (100 ng/ml) was used as positive control. Values represent fold increase versus respective control. *p < 0.01, **p < 0.05, and p = 0.07. HIV = human immunodeficiency virus; HUVEC = human umbilical vein endothelial cell.

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

FIGURE 2 Platelet Transcriptome Profiling in HIV



(A) Heat map from hierarchical clustering of differentially expressed genes between controls and HIV platelets. Genes with $p < 0.01$ were considered for the analysis. The *ABCC4* heat map is highlighted. **(B)** The data for all genes are plotted as log2 fold change versus the log10 of the p value. The top genes that are significantly different (sorted by p value) are indicated. **(C)** GO enrichment analysis (2 unranked list) of differentially expressed genes ($p < 0.01$). **Bars** indicate significantly enriched GO terms associated with cellular process and cell function. **(D)** ABCC4 mRNA expression in platelets from healthy volunteers ($n = 7$) and HIV ($n = 48$) subjects. qPCR analysis was reported expressing the ABCC4 Ct values after normalization with 18S5 RNA. **$p < 0.01$. **(E)** ABCC4 expression (RNA-Seq normalized counts) correlates with surface P-selectin and PAC-1 (activated Integrin $\alpha_{IIb}\beta_3$) staining in persons with HIV and controls, measured by flow cytometry ($r = 0.72$; $p = 0.05$ and $r = 0.77$; $p = 0.03$, respectively). Ct = concentration-time product; MFI = median fluorescence intensity; MHC = major histocompatibility complex; PAC-1 = activated glycoprotein IIb/IIIa receptor; other abbreviations as in Figure 1.

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 ($\beta = -2.9$; $p = 0.001$) (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 $\alpha_{IIb}\beta_3$($r = 0.77$; $p = 0.025$) 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**).

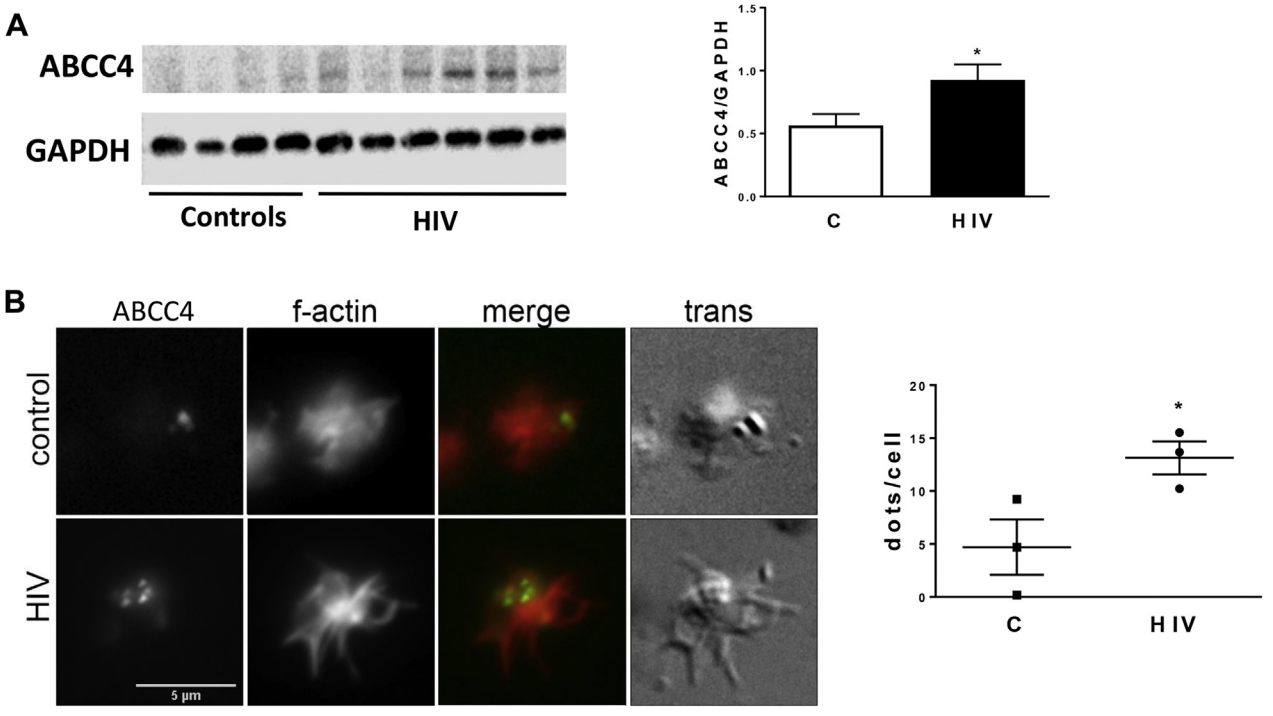
ABCC4 INHIBITION REDUCES PLATELET GRANULE RELEASE AND IMPAIRS CYCLIC NUCLEOTIDE HOMEOSTASIS IN HIV. Because ABCC4 is involved in the transport of diverse endogenous compounds, including dense granule content, we sought to investigate dense granule release in controls and HIV-infected subjects. Accumulation 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

FIGURE 3 Enhanced ABCC4 Protein Expression in HIV



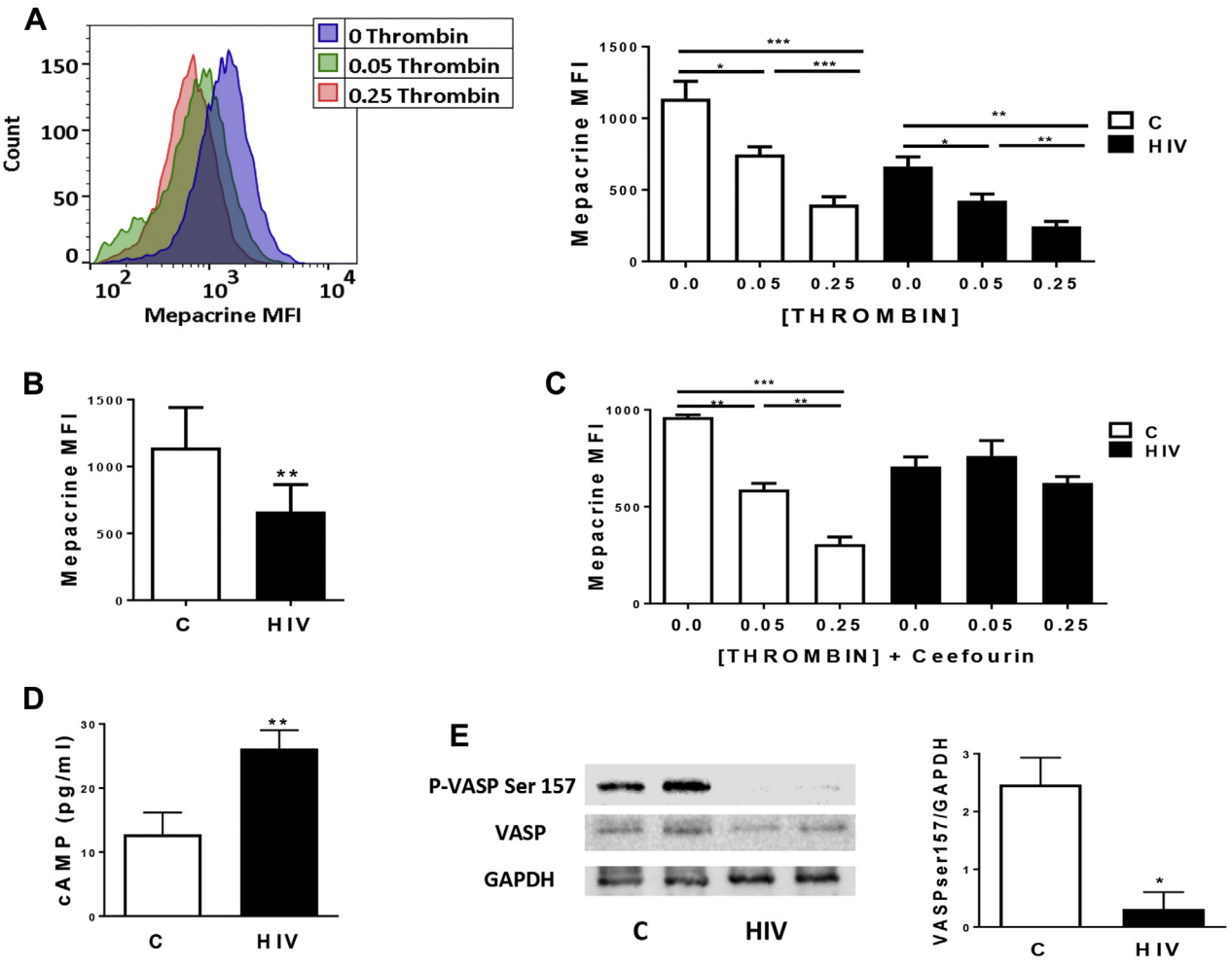
(A) Immunoblots of ABCC4 in platelet lysates from controls (C) (n = 8) and HIV (n = 14) subjects. Thirty micrograms of protein were loaded, and GAPDH was used as a loading control. Quantification after normalization is reported. *p < 0.05. **(B)** Fluorescence microscopy showing ABCC4 expression **(green)** and f-actin **(red)** in HIV (n = 3) and controls (n = 3). Image is representative of a single platelet for each group. The quantification reports ABCC4 green dots/cell counted in 3 subjects per group (>50 cells for each sample). *p<0.05. Scale bar: 5 μm. GAPDH = glyceraldehyde 3-phoshate dehydrogenase; other abbreviations as in Figure 1.

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. Pretreatment 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 increased in both groups (Figure 5E). To confirm whether ABCC4 inhibition affects S1P levels, we measured S1P release in supernatants of Ceefourin-pre-treated platelets. ABCC4 inhibition significantly reduced S1P levels following platelet activation (Figure 5F). Finally, 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).

FIGURE 4 **ABCC4 Affects Dense Granule Release and Platelet cAMP Homeostasis in HIV**

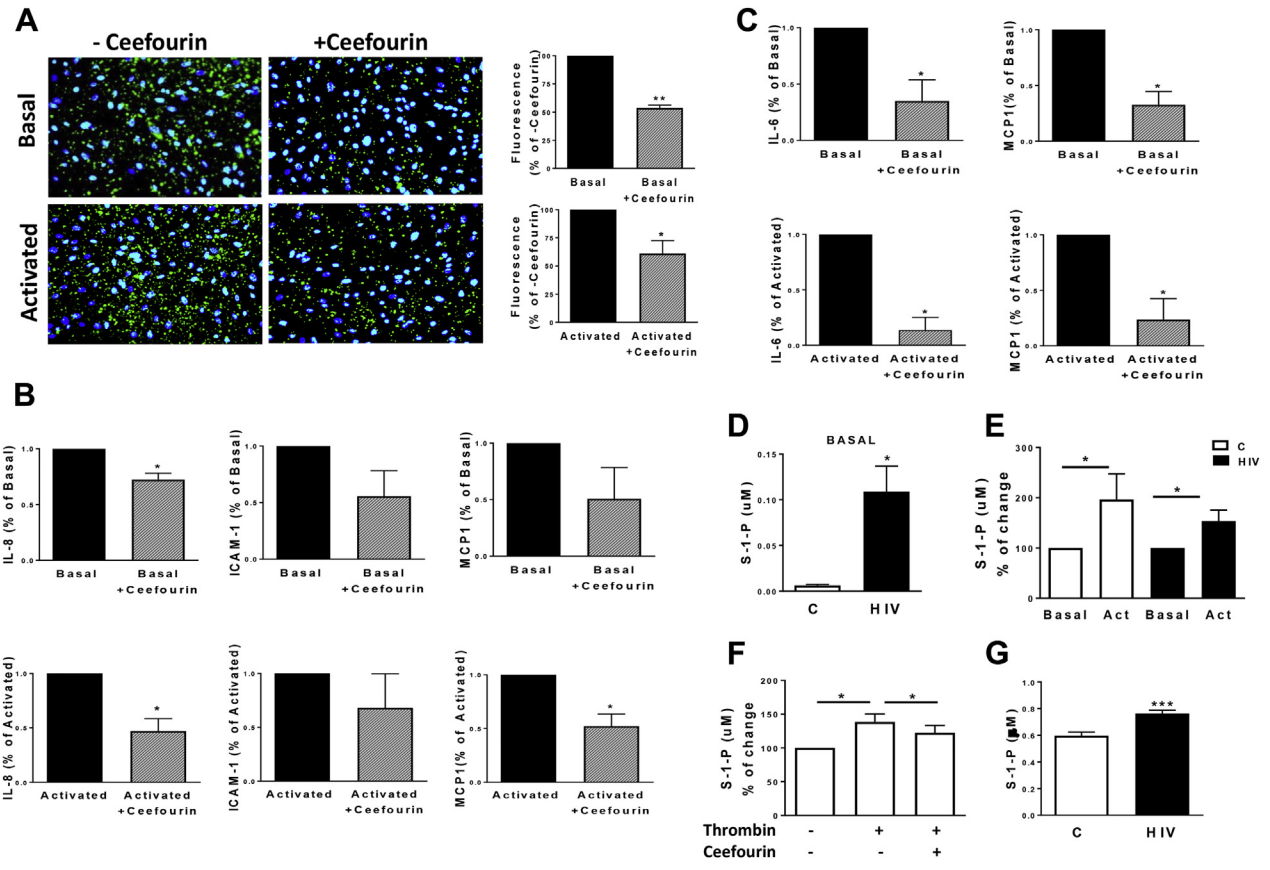


(A) Mepacrine staining was assessed via flow cytometry in platelets from controls (C) and HIV before and after activation with 0.05 or 0.25 U/ml thrombin. A representative histogram of 1 healthy control is shown. Quantitative analysis of 8 controls and 9 HIV is shown. *$p < 0.05$ and ***$p < 0.0001$. **(B)** Mepacrine staining via flow cytometry of platelets from controls ($n = 9$) and HIV ($n = 8$) at basal state. **$p< 0.01$. **(C)** Platelets from controls ($n = 5$) and HIV subjects ($n = 4$) were pre-treated with Ceefourin 2 (Ceefourin, 10 μmol/l), activated with 0, 0.05, or 0.25 U/ml thrombin and assessed for mepacrine staining. **$p < 0.01$ and ***$p < 0.0001$. **(D)** cAMP was quantified in the supernatants of activated (0.05 U/ml thrombin, 5 min) platelets from controls ($n = 9$) and subjects with HIV ($n = 13$). Levels are reported as pg/ml. **$p < 0.01$. **(E)** Platelets from HIV ($n = 3$) and controls ($n = 3$) were harvested for Western blot analysis. Blots were probed with anti P-VASP Ser 157 and anti-VASP, and loading was controlled with anti-GAPDH. Quantitative analysis was obtained by densitometry using ImageJ 1.51n software (NIH, Bethesda, Maryland). *$p < 0.05$. cAMP = cyclic adenosine monophosphate; GAPDH = glyceraldehyde 3-phoshate dehydrogenase; MFI = mean fluorescence intensity; VASP = vasodilator-stimulated phosphoprotein; other abbreviations as in Figure 1.

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

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



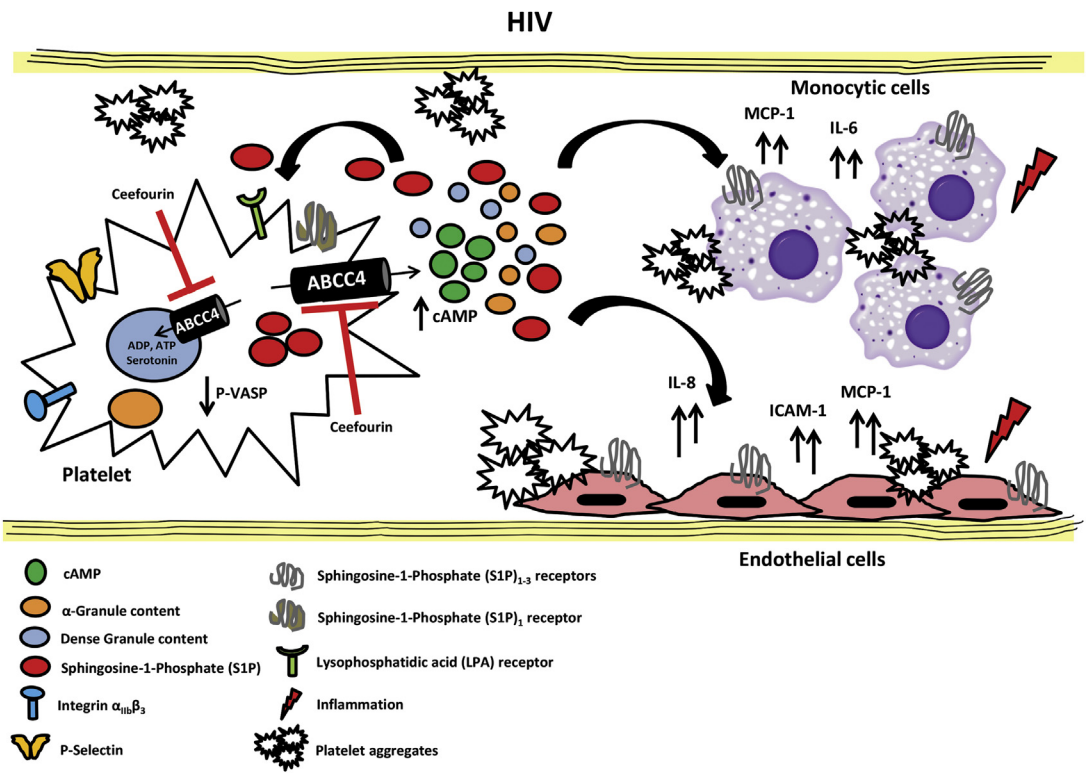
(A) Immunofluorescence microscopy of platelet adhesion to HUVECs in subjects with HIV. Stained platelets **(green)** were left untreated (Untreated) or treated with Ceefourin2 (Ceefourin, 10 μmol/l) for 30 min. Thrombin (0.05 U/ml, 5 min) was then added (Activated). HUVEC nuclei are stained with DAPI **(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 (C) (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.

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

FIGURE 6 Schematic Model of Platelet ABCC4 Role in HIV



In HIV, circulating platelets have increased expression of ABCC4 as consequence of enhanced platelet activation. ABCC4 overexpression on platelet plasma membrane contributes to increased excretion of platelet mediators in the extracellular space, including cAMP, which plays a crucial role in maintaining platelets in the inactive state. In turn, platelet inflammatory mediators activate endothelial cells and monocytic cells, and also platelets, promoting the formation of circulating platelet aggregates. The large amount of S1P released by activated platelets likely contributes to amplify platelet response and the inflammatory process in HIV. Platelet ABCC4 inhibition by Ceefourin in HIV prevents platelet activation by maintaining cAMP homeostasis. Moreover, the inhibition of ABCC4 has an important role in mediating platelet-cell interaction and the inflammatory response in HIV, by decreasing platelet-mediator release and activation of target cells in circulation. Abbreviations as in **Figure 1**.

cardiovascular processes, including smooth muscle cell proliferation, cardiomyocyte contractility, and platelet activation (57-59). Recent findings indicate that hemostasis 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 enhanced 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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PERSPECTIVES

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 ABCC4 as a central mediator of platelet activity and platelet-mediated proinflammatory response in endothelial cells and monocytic cell line. Targeting ABCC4 in HIV-infected subjects may represent a novel antithrombotic strategy in persons with HIV.

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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.