Characterization of Platelet–Monocyte Complexes in HIV-1–Infected Individuals: Possible Role in HIV-Associated Neuroinflammation

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Characterization of Platelet–Monocyte Complexes in HIV-1–Infected Individuals: Possible Role in HIV-Associated Neuroinflammation

Meera V. Singh,* Donna C. Davidson,* Joseph W. Jackson,* Vir B. Singh,* Jharon Silva,* Servio H. Ramirez, † and Sanjay B. Maggirwar*

HIV-1–associated neuroinflammation persists even with effective combined antiretroviral therapy, and it is associated with the presence of activated monocytes/macrophages within the CNS. To infiltrate the CNS, monocytes transmigrate across the selectively permeable blood–brain barrier, which is compromised during HIV-1 infection. Interestingly, platelet-derived excess soluble CD40 ligand found in the plasma and cerebrospinal fluid of HIV-1–infected individuals with cognitive impairment has previously been implicated in increased blood–brain barrier permeability. In this study we show that soluble CD40 ligand also promotes the formation of complexes between inflammatory monocytes and activated platelets (PMCs), which are detected by flow cytometry as monocytes that express excess of CD61, a platelet marker, and that these complexes are increased in individuals with HIV-1 infection. PMCs exhibit an enhanced ability to adhere to human brain microvascular endothelial cells as compared with monocytes alone, and they migrate across the transendothelial barrier. These complexes can be found marginalized in the lumens of postcapillary venules in postmortem brain tissue derived from cases of HIV-1–associated encephalitis. The extravasation of monocytes across the brain endothelium may exacerbate neuroinflammation, indicating that enhancing this event via platelet interaction may be a contributing factor in the development of cognitive impairment. Thus, dampening platelet activation, and in turn PMC formation, with antiplatelet agents may prove beneficial in developing adjunctive therapies for use in combination with combined antiretroviral therapy in an effort to reduce HIV-1–associated neurologic deficit. The Journal of Immunology, 2014, 192: 000–000.

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Abbreviations used in this article: ART, antiretroviral therapy; BBB, blood–brain barrier; BMVEC, brain microvascular endothelial cell; CART, combined antiretroviral therapy; HAND, HIV-1–associated neurocognitive disorder; HIV-1, HIV type 1; HIV-E, HIV-1–associated encephalitis; PMC, platelet–monocyte complex; PSGL-1, P-selectin glycoprotein ligand 1; sCD40L, soluble CD40L; VNR, vitronectin receptor.

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that mediate adherence to endothelial cells, and chemokine receptors such as CX3CR1, which increase responsiveness to fractalkine (16).

Previous reports have shown that the CD16^{+} phenotype is also induced by the interaction of monocytes with activated platelets. Activated platelets form transient complexes with monocytes in circulation, termed platelet–monocyte complexes (PMCs), and these complexes are elevated in diseases involving inflammation such as cardiovascular disease (17) and type 2 diabetes (reviewed in Ref. 18). Interestingly, PMCs were found to be a more sensitive marker of platelet activation and predictor of myocardial infarction as compared with levels of P-selectin, also known as CD62P, which is the traditional hallmark of platelet activation (19). Moreover, the extent to which PMCs develop is predominantly dependent on the platelet activation status (20, 21), and only to a very limited extent on monocyte activation (22).

Previous studies from our group (21) and others (23) have demonstrated that HIV-1 infection induces an increase in platelet activation, despite thrombocytopenia. Infection, as well as cognitive impairment during infection, is associated with an increase in plasma levels of soluble CD40 ligand (sCD40L) (24), for which platelets are the major source (25). Consistent with this notion, recent reports from our group indicate that excess sCD40L contributes to blood–brain barrier (BBB) permeability in the context of HIV-1 (26), and that PMCs are elevated in individuals with HIV-1 infection despite suppressive combined ART (cART) (21). Collectively, these findings underscore the importance of PMCs in the pathogenesis of HIV-1–associated illnesses, and they led us to the underlying hypothesis that during HIV-1 infection, the increase in platelet activation and subsequent release of sCD40L promote the formation of PMCs, which in turn have a higher propensity to cross the BBB, thereby exacerbating HIV-1–associated neuroinflammation.

The findings in the present study indicate that HIV-1–infected individuals exhibit increased levels of PMCs as compared with uninfected individuals, and that sCD40L activates platelets in a manner that promotes the formation of complexes with monocytes via engagement of P-selectin and P-selectin glycoprotein ligand 1 (PSGL-1) on platelets and monocytes, respectively. Consistently, mice treated with CD40L also exhibit a significant increase in Gr-1^{hi} (the functional equivalent of CD16^{+} monocytes in mice) (24), for which platelets are the major source (25). Consistent with this notion, recent reports from our group indicate that excess sCD40L contributes to blood–brain barrier (BBB) permeability in the context of HIV-1 (26), and that PMCs are elevated in individuals with HIV-1 infection despite suppressive combined ART (cART) (21). Collectively, these findings underscore the importance of PMCs in the pathogenesis of HIV-1–associated illnesses, and they led us to the underlying hypothesis that during HIV-1 infection, the increase in platelet activation and subsequent release of sCD40L promote the formation of PMCs, which in turn have a higher propensity to cross the BBB, thereby exacerbating HIV-1–associated neuroinflammation.

The findings in the present study indicate that HIV-1–infected individuals exhibit increased levels of PMCs as compared with uninfected individuals, and that sCD40L activates platelets in a manner that promotes the formation of complexes with monocytes via engagement of P-selectin and P-selectin glycoprotein ligand 1 (PSGL-1) on platelets and monocytes, respectively. Consistently, mice treated with CD40L also exhibit a significant increase in Gr-1^{hi} (the functional equivalent of CD16^{+} monocytes in humans) inflammatory monocyte complexes as compared with saline-treated mice. Using an in vitro coculture system, as well as brain specimens derived from individuals with HIV-E, we now reveal that monocyte extravasation into the CNS is accentuated by the increased adherence of PMCs to brain microvascular endothelial cells. Importantly, we also show that these complexes can be detected as marginalized to the vessel lumen and in perivascular cuffs in brain tissue derived from individuals with HIV-E. Taken together, these results shed light on the underlying mechanisms that may contribute to the pathogenesis of neuroinflammation and HIV-E, thus revealing novel therapeutic targets. Currently, there remains a critical lack of adjunctive therapies for the management of HIV-1–associated neurocognitive disorders; however, without addressing the underlying neuroinflammation, no realistic control of these complications can be achieved.

Materials and Methods

Ethics statement

The Research Subjects Review Board at the University of Rochester Medical Center approved studies involving human samples. All study participants were adults and blood samples were obtained after written informed consent, in accordance with the Declaration of Helsinki. Mouse experiments were carried out in accordance with the Animal Welfare Act and the National Institutes of Health (NIH) guidelines, and the University Committee on Animal Resources of the University of Rochester Medical Center approved the animal protocol (protocol no. 2005-161). The facilities and programs of the Vivarium and Division of Laboratory Animal Medicine of the School of Medicine and Dentistry are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Patient samples

Persons with (n = 36) and without (n = 37) HIV-1 infection (without any occurrence of cardiovascular disease for at least one preceding year) were enrolled in the study, and blood samples were drawn into acid citrate dextrose–buffered vacutainers (BD Biosciences, San Jose, CA). All persons with HIV-1 infection were on ART at the time of the draw. Six of the HIV-1–infected individuals were coinfected with hepatitis C, one was infected with tuberculosis, and one was infected with human papillomavirus. Patient demographics are as outlined in Table I.

Flow cytometry and ImageStream analysis

PMCs were detected in whole blood, using a previously described method (21), within 1 h of the blood draw. In brief, 100 μl blood was fixed and RBCs were lysed and then stained with 10 μl anti-CD14-PE, 3 μl anti-CD16-PE-Cy7, 10 μl anti-CD62P FITC (all obtained from BD Biosciences, San Jose, CA), and 3 μl anti-CD61-Alexa Fluor 647 (from AbD Serotec, Oxford, U.K.). Following the staining, samples were acquired using a flow cytometer (Accuri C6; Accuri Cytometers, Ann Arbor, MI). Whole blood leukocytes and monocytes were gated based on forward and side scatter. Further monocytes (CD14^{+} cells) were divided into two subtypes based on CD16 expression. PMCs were defined as monocytes that were positive for CD61, a platelet-specific marker. The same tubes were also analyzed for platelet activation using CD62P (also known as P-selectin) expression. Fluorescence minus one controls were used to define various gates, and sizing beads (Mega Mix; BioCytex, Marseille, France) were used to delineate the platelet gate (0.9–3 μm).

Representative samples from HIV^{+} (n = 5) and HIV^{−} (n = 6) donors were also acquired and analyzed using ImageStream (Amnis, Seattle, WA). For these studies, 10 μl anti-CD16-Pac B (from BD Biosciences) was used in place of anti-CD16-PE-Cy7. Ideas software (Amnis) was used to analyze the ImageStream data. The gating strategy used was as described above. Initial analysis of the data indicated two types of PMCs: one in which platelets were attached to the surface of monocytes (henceforth called type 1 complexes), and another in which there was no visible platelet attached, but the monocyte itself expressed the platelet marker CD61 (henceforth called type 2 complexes). The internalization feature of the Ideas software, which measures and plots the distance between fluorescently labeled platelets and monocytes, was used to determine the level of PMCs. Table I. Demographic and clinical characteristics of study participants

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HIV^{+}</th>
<th>HIV^{−}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age ± SD, y</td>
<td>34 ± 13</td>
<td>48 ± 13</td>
</tr>
<tr>
<td>Race, N (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>3 (7)</td>
<td>24 (51)</td>
</tr>
<tr>
<td>White</td>
<td>38 (86)</td>
<td>15 (32)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>1 (2)</td>
<td>3 (6)</td>
</tr>
<tr>
<td>Unspecified</td>
<td>2 (5)</td>
<td>5 (11)</td>
</tr>
<tr>
<td>Gender, N (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>24 (55)</td>
<td>36 (78)</td>
</tr>
<tr>
<td>Female</td>
<td>20 (45)</td>
<td>11 (22)</td>
</tr>
<tr>
<td>Drug Use, N (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>43 (98)</td>
<td>21 (45)</td>
</tr>
<tr>
<td>Cocaine</td>
<td>1 (2)</td>
<td>11 (24)</td>
</tr>
<tr>
<td>Marijuana</td>
<td>0 (0)</td>
<td>12 (26)</td>
</tr>
<tr>
<td>Heroin</td>
<td>0 (0)</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Nicotine</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Alcohol</td>
<td>1 (2)</td>
<td>8 (17)</td>
</tr>
<tr>
<td>Mean CD4 Count ± SD, Cells/mm^3</td>
<td>NA</td>
<td>585 ± 240</td>
</tr>
<tr>
<td>Viral Load</td>
<td></td>
<td></td>
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<tr>
<td>Undetectable, N (%)</td>
<td>NA</td>
<td>26 (57)</td>
</tr>
<tr>
<td>Detectable, Mean ± SD, RNA Copies/ml</td>
<td>NA</td>
<td>128 ± 132</td>
</tr>
</tbody>
</table>

Individuals with (n = 36) and without (n = 37) HIV-1 infection were enrolled in the study. All HIV-1–infected individuals were on ART. Six HIV^{+} individuals were coinfected with hepatitis C, one with tuberculosis, and one with human papillomavirus. Unless otherwise stated, the values indicate numbers with the percentage of total study population in parentheses.
rochromes of interest (i.e., CD14 for monocytes and CD61 for platelets) was used to differentiate between these two types of complexes and to measure the relative percentage of these two types among the total PMC population. Additionally, the spot count feature was used to measure the number of platelets per monocyte in a complex. This feature measures pixel intensity of a particular fluorochrome after subtracting the background (in this case CD61 expression on monocytes) and examines whether this connects to a particular spot on the image (i.e., a platelet).

Cell culture

Monocytes were isolated using a MACS Pan Monocyte Isolation Kit (Miltenyi Biotec, San Diego, CA) as per the manufacturer’s instructions with minor modifications. In all experiments except for electron microscopy, biotin-labeled Ab against CD41 (250 ng/25 million PBMCs; Abcam, Cambridge, MA) was added to the Ab mixture provided with the kit to remove contaminating platelets and existing PMCs. The resulting monocytes contained <10% residual PMCs (data not shown). Monocytes used for electron microscopy contained 35–40% PMCs (data not shown). Monocytes were cultured in RPMI 1640 supplemented with 10% FBS and 2% penicillin/streptomycin/glutamine.

Whole blood from HIV⁻⁻ donors was centrifuged at 250 × g for 15 min, and platelet-rich plasma was collected. Following addition of PGI₂, the platelet-rich plasma was centrifuged at 1000 × g for 10 min to pellet the platelets. The platelet pellet was then washed and resuspended using Tyrode’s salt solution (Sigma-Aldrich, St. Louis, MO) supplemented with acid citrate dextrose anticoagulant and PGI₂. Subsequently, washed platelets were centrifuged once again at 1000 × g for 10 min, and the remaining purified platelet pellet was resuspended in Tyrode’s salt solution without supplements. The purity of isolated platelets was determined using a Sysmex KX 21N hematology analyzer and was found to be 99% pure.

Primary human brain microvascular endothelial cells (BMVECs; Applied Cell Biology Research Institute, Kirkland, WA) were cultured in DMEM/F12 supplemented with 10% FBS, 2% penicillin/streptomycin/glutamine, and 100 ng/ml endothelial cell growth supplement (BD Biosciences). Cells up to passage 10 were used for performing experiments.

Scanning electron microscopy

Monocytes isolated from the whole blood of HIV⁻⁻ donors were allowed to adhere onto poly-L-lysine–coated coverslips for 2 h and were subsequently placed into 0.1 M sodium cacodylate-buffered 2.5% glutaraldehyde at 4°C for overnight fixation. The cells on the cover glasses were postfixed using the same buffer in 1.0% osmium tetroxide and then transitioned through a graded series of ethanol to 100% (×3). The last change was allowed to evaporate off of the cover glasses overnight in a fume hood. The cover glasses were then mounted onto aluminum stubs and sputter coated with gold. Imaging was performed using a Zeiss Auriga field emission scanning electron microscopy with an attached Gatan digital camera system.

Transmission electron microscopy

Monocytes were isolated, as described above, from whole blood obtained from HIV⁻⁻ donors and were allowed to adhere to a two-chamber slide for 2 h, following which media was removed and immediately replaced with room temperature fixative composed of 0.1 M sodium cacodylate–buffered 2.5% glutaraldehyde. The slides were fixed for 1 h at room temperature and then at 4°C overnight. The plastic chambers were removed, and slides were rinsed in the same buffer, postfixed using Tyrode’s salt solution (Sigma-Aldrich, St. Louis, MO) supplemented with acid citrate dextrose anticoagulant and PGI₂. Subsequently, washed platelets were centrifuged once again at 1000 × g for 10 min, and the remaining purified platelet pellet was resuspended in Tyrode’s salt solution without supplements. The purity of isolated platelets was determined using a Sysmex KX 21N hematology analyzer and was found to be 99% pure.

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In vitro whole blood treatment with recombinant human sCD40L

Blood from HIV⁻⁻ donors (n = 4) was treated with either ADP at 10 μM for 30 min or with recombinant human (rh)sCD40L (R&D Systems, Minneapolis, MN) at 1 μg/ml for 2 h at 37°C. When indicated, blood was pretreated with either 8 μg/ml MK13 (neutralizing Ab against CD40L) (24) or 5 μg/ml 9E1 (neutralizing Ab against P-selectin; R&D Systems) for 15 min, followed by the addition of rhCD40L. After the treatment, blood was processed and analyzed as described above for the detection of CD14⁻⁺ PMCs.

Platelet–monocyte co-culture

Monocytes were isolated from HIV⁻⁻ donors as described above and incubated overnight at 37°C. The next day, platelets were isolated from the same donor and treated with 1 μg/ml rhCD40L, or were left untreated, for 20 min at 37°C. The platelets were washed with Tyrode’s buffer and mixed with monocytes at a ratio of 10:1. Monocytes with or without rhCD40L treatment were used as controls. Mixed cultures were incubated for 1 h at 37°C, following which half of the cells were fixed with 4% paraformaldehyde and stained with Abs against CD14, CD16, CD61, and CD62P expression was used as a marker of platelet activation. Fluorescence minus one controls were used for analysis, as described above.

Monocyte adhesion assay

This experiment was performed as described previously (27). Briefly, 2 × 10⁶ BMVECs per well were plated onto black-walled, transparent-bottom 96-well plates (Corning, Corning, NY) that had been coated with rat-tail collagen type I (BD Biosciences). After the formation of confluent monolayers, the BMVECs were cultured without growth factors for 4–6 h and were then either treated with rTNF-α (10 ng/ml; R&D Systems) to activate the endothelial cells or left untreated for 4 h. Monocytes and platelets were isolated separately from whole blood obtained from the same HIV⁻⁻ donor (n = 3). Monocytes were labeled with calcein AM green (5 μM/1 × 10⁶ cells for 45 min; Invitrogen Life Technologies, Carlsbad, CA). A coculture of monocytes and platelets that had been activated by rhCD40L (1 μg/ml) was established, as described above. Monocytes treated with or without rhCD40L treatment were used as controls. Mixed cultures treated with LPS (10 ng/ml), were used as controls. Twenty-four hours after treatment, 2.5 × 10⁵ monocytes per well were added onto the BMVECs and were allowed to adhere for 30 min at 37°C. The plates were subsequently washed three times with PBS to rinse away any unattached cells, and the relative fluorescence was measured on a Spectramax M3 fluorescence plate reader (Molecular Devices, Sunnyvale, CA). Relative fluorescence unit values obtained using only the monolayer of BMVECs were subtracted from all readings as background and results are represented as the fold difference between the number of monocytes that attached to BMVECs under the different experimental conditions and the number of nontreated monocytes that attached to the untreated endothelial cells.

Transendothelial migration assay

This assay was performed as described previously (27) with some modifications. BMVECs were plated on rat-tail collagen coated FluoroBlok-tinted tissue culture inserts (with 3-μm pores; BD Biosciences) at the density of 2.5 × 10⁴ cells/well. Platelet and monocyte cocultures were established as described before (n = 3). Platelets were activated using 1 μg/ml...
rhesCD40L in the presence or absence of 5 μg/ml 9E1 (neutralizing Ab against P-selectin; R&D Systems) for 20 min. The monocytes were either left untreated or were treated with 1 μg/ml rhesCD40L or with 5 μg/ml 9E1. The monocytes were alternatively mixed with activated platelets with or without 9E1 at the ratio of 1:10. One hour after treatment, 5 × 10^5 monocytes/treatment were stained with Abs against CD14-PE, CD16-PE-Cy7, and CD61-Alexa Fluor 647 and were acquired on an Accuri C6 flow cytometer to enumerate the percentage of CD14^+ PMCs. Two hours after treatment as well as 24 h after treatment, 5 × 10^5 monocytes/treatment were added to the upper chamber of FluoroBlok inserts in the presence or absence of MCP-1 (30 ng/ml; R&D Systems) in the lower chamber; that is, the monocytes were allowed to migrate with or without a chemokinetic gradient. The migration was allowed to continue up to 7 h at 37°C. After 7 h, the cells in the lower chamber were collected and stained with Abs against CD14-PE and CD16-PE-Cy7, as described previously. After staining, the cells were resuspended in 250 μl PBS. Twenty microliters per sample was acquired on an Accuri C6 flow cytometer at slow speed and at the same instrument settings for each experiment, and the number of CD14^+ monocytes/20 μl/sample was obtained. Final number of migrated cells was estimated by subtracting the number of CD14^+ monocytes that had migrated in absence of MCP-1 (passive migration) from the number of CD14^+ monocytes that had migrated in presence of MCP-1 (active migration). A fold change in monocyte migration was then calculated by dividing the final number of migrated monocytes per treatment by the final number of migrated cells in untreated monocytes.

**PMC detection by immunohistochemical staining of human brain sections**

Immunohistochemistry was performed on serial sections from postmortem paraffin-embedded brain tissue of cases obtain from the National Neuro-AIDS Tissue Consortium (Washington, DC). The area under observation included the basal ganglia and cortical regions from three HIV-E cases and from two seronegative age-matched controls (cases 1–3 and cases 8 and 10) (28). The immunofluorescence was performed using standard methodology. Briefly, serial sections (5 μm in thickness) were baked at 65°C for 20 min followed by deparaffinization and rehydration. The tissue was then subjected to Ag retrieval by incubating the sections at 100°C in 10 mM sodium citrate buffer (pH 6.0) for 20 min. To minimize autofluorescence, the sections were placed for ~8 hrs 38 cm apart from a fluorescent lamp using a 13-W compact fluorescent bulb. To block nonspecific Ab binding, the slides were incubated with 1% goat serum in 1× PBS and 0.1% Triton X-100 (Sigma-Aldrich). For immunolabeling, the following primary Abs were used: mAbs to human CD60 (diluted 1:300; Abcam) and polyclonal Abs to human CD61 (1:200; Cell Signaling Technology). Tissue sections were then rinsed, and secondary Abs to the m-mouse conjugated to Alexa Fluor 488 (diluted 1:500; Invitrogen) or donkey anti-rabbit conjugated to Alexa Fluor 594 (diluted 1:500; Invitrogen), were added for 1 h. The slides were mounted with ProLong antifade reagent containing DAPI (for detection of nucleus; Invitrogen). Images (at ×40 or ×100 objective magnification) were acquired using a CoolSNAP EZ CCD camera (Photometrics) coupled to a Nikon i80 Eclipse. Both the acquisition and pseudocolor conversion were performed using the NIS-Elements software from Nikon.

**Statistical Analysis**

GraphPad Prism (version 4, GraphPad Software, La Jolla, CA) was used to perform all statistical analyses. Unpaired t tests were used to compare data obtained from HIV-1–infected and uninfected samples. For multiple groups statistical significance was determined using one-way ANOVA followed by Bonferroni’s test. Pearson correlation test was used to correlate PMCs and platelet activation. A p value <0.05 was considered significant.

**Results**

**HIV-1 infection leads to elevated levels of CD16^+ PMCs, despite cART**

Previous studies of cardiovascular diseases have shown that complexes between activated platelets and monocytes are inflammatory in nature and induce monocyte maturation (reviewed in Ref. 17). Because HIV-1 infection is a chronic inflammatory disease and is known to cause platelet activation, we conducted a pilot study to assess the levels of PMCs in HIV-1–infected individuals (21). Because we found that there was a significant increase in PMCs in HIV-1–infected individuals, we recruited more participants. Whole blood samples obtained from HIV^+ (n = 36) and HIV^- (n = 37) individuals were stained and subsequently analyzed using flow cytometry. HIV-1–infected individuals showed significantly increased levels of CD16^+ PMCs as compared with HIV-1–uninfected individuals (p = 0.013, R^2 = 0.08369; Fig. 1A). In contrast, CD16^+ PMCs and platelet–granulocyte complexes did not differ between the two groups (Fig. 1B, 1C). Platelets obtained from HIV-1–infected donors appeared activated as compared with uninfected samples, and these platelets exhibited increased expression of CD62P (P-selectin) on their surface (p = 0.036; Fig. 1D). Consistently, the percentage of CD62P^+ platelets in both HIV^+ and HIV^- population demonstrated a positive correlation with the percentage of CD16^+ PMCs (p = 0.008, R^2 = 0.0888; Fig. 1E). We also observed a significant increase in the percentage of CD16^+ monocytes in HIV-1–infected individuals, which correlated positively with the CD16^+ PMCs, whereas there was no difference in CD16^- monocytes (data not shown). Additional representative samples from HIV^+ (n = 5) and HIV^- (n = 6) individuals were also stained and analyzed using ImageStream (Amnis) and demonstrated a similar increase in CD16^+ PMCs (data not shown).

**Morphological characteristics of PMCs**

Corresponding images generated from the ImageStream analysis of the PMCs indicated the presence of two types of complexes: those with one or more platelets attached to the surface of the monocyte (type 1; Fig. 2A), and another in which the monocytes express CD61 internally or on its surface (type 2; Fig. 2B). In all samples (HIV^+ and HIV^-) the percentage of type 1 complexes was significantly higher than type 2 complexes (p = 0.0008; Fig. 2C), and the percentage of CD16^+ type 1 complexes was increased in HIV-1–infected individuals (p < 0.05; Fig. 2D) and that of type 2 complexes showed a similarly elevated trend toward an increase (p = 0.07, Fig. 2E). The type 1 complexes were primarily observed with one or two platelets per monocyte; however, several cells demonstrated more than two platelets that had attached (range of one to four). The average number of platelets attached did not differ significantly between HIV-1–infected and uninfected samples (Fig. 2F). Consistently, images obtained using scanning electron microscopy and transmission electron microscopy also indicated that there were one to three platelets per monocyte (Fig. 2G, 2H).

The presence of type 2 complexes is very intriguing, as there were no platelets physically attached to these monocytes. Monocyte may express some level of CD61 on their surface, as a subunit of the vitronectin receptor (VNR). The exact level of expression of this receptor on primary monocytes is not well understood. A study by Weerasinghe et al. (29) detected low levels of VNR on monocytes. Another report by Lefrenie et al. (30) demonstrated total absence of VNR in primary monocytes unless these cells were further cultured in the presence of M-CSF for 3–5 d. It is noteworthy that the monocytes in our experiments are unexposed to such external stimuli. Furthermore, our transmission electron microscopy images of the type 1 complexes illustrate that the platelet and monocyte membranes remain in close proximity when complexed (Fig. 2H), which may facilitate the transfer of platelet microparticles (which often expresses CD61) (31–33) to the monocyte. This seems to be a more plausible explanation for the presence of type 2 complexes, as we did not observe fusion between the membranes of the two cell types.

**sCD40L induces PMC formation via the interaction of platelet P-selectin with monocyte PSGL-1**

Previously we reported that HIV-1–infected individuals with cognitive impairment have increased levels of sCD40L in their plasma and cerebrospinal fluid as compared with infected individuals...
without cognitive impairment (24). To investigate the role of sCD40L in the platelet–monocyte interaction, wild-type C57BL/6J mice were injected with either saline or rmsCD40L (0.2 µg/g body weight), and PMC formation was subsequently analyzed in whole blood. CD40L-treated animals demonstrated a significant increase in total PMCs (*p = 0.023, Fig. 3A), which was more pronounced in Gr-1\textsuperscript{hi} inflammatory complexes (**p = 0.0001, Fig. 3B). As expected, sCD40L treatment induced a significant increase in platelet activation (*p = 0.008, Fig. 3C) as compared with saline-treated animals.

In line with this, whole blood treatments using samples from HIV\textsuperscript{−} donors also showed that treatment with rhsCD40L increases the degree of CD14\textsuperscript{+} PMC formation (p = 0.006, Fig. 3D) and platelet activation (p < 0.0001, Fig. 3E) to levels comparable to ADP treatment (positive control, p = 0.006 for platelet activation and p = 0.0009 for PMCs). This effect was reduced by the addition of MK13, a neutralizing Ab raised against CD40L, indicating that the increase in PMCs was specifically due, in part, to CD40L. Similarly, 9E1, a blocking Ab raised against P-selectin, also ameliorated PMC formation, implying the involvement of the P-selectin–PSGL-1 interaction in PMC formation. Additionally, whereas MK13 treatment abrogated P-selectin (CD62P) expression by platelets, 9E1 had no effect on this marker (**p = 0.001, Fig. 3D, 3E).

**FIGURE 1.** Detection of PMCs in HIV-1–infected and uninfected individuals. One hundred microliters whole blood obtained from HIV\textsuperscript{+} (n = 36) and HIV\textsuperscript{−} (n = 37) was fixed, stained with Abs against CD14, CD16, CD61, and CD62P (P-selectin), and subsequently acquired on an Accuri C6 flow cytometer. (A) CD16\textsuperscript{+} PMCs were significantly increased in HIV-1–infected individuals. (B) There was no difference in CD16\textsuperscript{+} PMCs between the two groups. (C) Platelet–granulocyte complexes did not differ between the groups. (D) Platelets from HIV-1–infected individuals expressed more CD62P (P-selectin) than platelets obtained from HIV\textsuperscript{−} donors. (E) Platelet CD62P expression correlated positively with CD16\textsuperscript{+} PMC percentages. In (A)–(D), samples were compared using an unpaired t test. *p < 0.05.

**FIGURE 2.** Morphological characterization of PMCs. (A–F) One hundred microliters whole blood obtained from HIV\textsuperscript{+} (n = 5) and HIV\textsuperscript{−} (n = 6) donors was fixed, stained with Abs against CD14, CD16, and CD61, and acquired on an Amnis ImageStream flow cytometer. (A and B) Representative images of type 1 and type 2 CD16\textsuperscript{+} PMCs, respectively. Original magnification ×40. (C) Type 1 complexes were more prevalent in HIV-1–infected and uninfected samples. (D and E) HIV-1–infected individuals contain significantly higher percentages of type 1 and type 2 PMCs. (F) The average number of platelets per monocyte in a PMC did not differ between the two study groups. (G and H) Monocytes were isolated from PBMCs derived from HIV-1–seronegative subjects (n = 3) and were allowed to adhere to the culture dishes for 2 h. Cells were further processed for scanning electron microscopy (G) and transmission electron microscopy (H). (G and H) Original magnification ×800 [insert in (H) ×6000]. The red circles in (H) indicate platelets interacting with the monocyte. In (A)–(F), samples were compared using an unpaired t test. *p < 0.05, ***p < 0.001.
Monocytes in complex with activated platelets exhibit an enhanced proinflammatory and promigratory phenotype

To further investigate the effect of the interaction of monocytes with platelets on monocyte activation, we developed a coculture system using platelets and monocytes isolated from HIV-1–infected donors. Platelets were either treated using rhsCD40L, or left untreated, and analyzed for P-selectin (CD62P) expression to verify activation status (Fig. 4A). The platelets were subsequently mixed with monocytes, and the cocultures were then used for the detection of CD14+ PMCs, as well as analysis of cell surface expression of CCR2, PSGL-1, and CD40 on the complexed monocytes. Single-cell type cultures were also treated in parallel and used as controls. Platelets that were pretreated with rhsCD40L induced significantly higher levels of PMC formation upon mixture with monocytes, as compared with platelets that had been left untreated (p = 0.029, Fig. 4B). This effect of platelets on PMC formation was not due to direct action of rhsCD40L on monocytes because such treatment failed to increase CD16 expression on monocytes (data not shown). Monocytes in complex with activated platelets showed elevated expression of CCR2 (p = 0.007, Fig. 4C), PSGL-1 (p = 0.048, Fig. 4D), and CD40 (p = 0.017, Fig. 4E). Additionally, in some experiments, the platelet–monocyte cocultures were allowed to migrate through a monolayer of BMVECs that were either nonactivated or were activated using TNF-α for 30 min. Monocytes that had been cocultured with activated platelets demonstrated enhanced adhesion to endothelial cells, regardless of endothelial cell activation status (p < 0.01, Fig. 5A).

Interaction of monocytes with activated platelets promotes transendothelial migration of monocytes

Because monocytes in complex with activated platelets showed increased adherence to BMVECs, we sought to investigate whether these cells also migrate more efficiently through the transendothelial barrier. We established platelet–monocyte cocultures as described previously. Additionally, this time we used 9E1, which is a P-selectin blocking Ab, to prevent P-selectin–dependent formation of PMCs. One hour after treatment, cells were stained and acquired to enumerate the percentages of CD14+ PMCs. Consistent with the data shown in Fig. 3D, coculturing of monocytes treated with activated platelets, Fig. 5B). Twenty-four hours later the cells were allowed to migrate through a monolayer of BMVECs. It was our speculation that the incubation of PMCs for 24 h will lead to enhanced expression of CCR2, PSGL-1, and CD40, followed by increased adhesion to BMVECs (as shown in Figs. 4C–E, 5A). As expected, higher numbers of CD14+ monocytes that were treated with activated platelets migrated through the BMVEC monolayer (p < 0.01 as compared with untreated monocytes). Interestingly, this population of monocytes appeared to be predominantly CD16+ (>65%; data not shown). Alternatively, the migration of monocytes was significantly reduced when PMC formation was blocked with the use of 9E1 Ab (p < 0.05 as compared with monocytes treated with activated platelets, Fig. 5B).

Immunohistochemical evidence of PMCs in brain tissue from cases of HIV-E

We next sought to evaluate whether PMCs could be detected in the CNS of individuals that had prominent neuropathology due to HIV-1 infection. Postmortem tissues derived from basal ganglia and the cortical region of three patients with HIV-E and two...
HIV−seronegative patients were double immunolabeled for CD68 (monocyte/macrophage marker) and CD61. Clinical, pathologic, and demographic details of these cases have been published before (28, 34). As shown in Fig. 6, the brain specimens obtained from patients with HIV−E contained evidence of PMCs, both in the lumen (Fig. 6C, 6D, 6F; only basal ganglia regions are shown) and marginalized (attached to the endothelium) to the brain microvasculature (Fig. 6E, 6F). Of note, there was an increased presence of type I PMCs featuring multiple platelets on a single monocyte/macrophage (Fig. 6C–G). In contrast, no PMCs were observed in control brain samples derived from HIV−seronegative subjects (Fig. 6A, 6B). Although CD68+ cells in the perivascular space were seen in perivascular cuffs and disseminated throughout the brain parenchyma, no platelets were detected alone or in complex with monocytes within the perivascular space or in the parenchyma. As previously noted, these tissues have extensive neuroinflammation, showing microgliosis (as revealed by Iba-1 Abs) and infiltrating CD68+ cells that are also immunoreactive for the HIV-1 p24 core Ag (34). These results suggest that the increased presence of PMCs is associated with HIV−induced neuroinflammation, thus offering the possibility that PMCs contribute to immune cell migration and breach of the BBB during infection.

**Discussion**

HIV−associated neuroinflammation and HIV−E are associated with excess immune activation, which outweighs the amount of virus present within the CNS (7). The inflammatory response from resident, as well as infiltrating, monocytes and macrophages is known to fuel the neuropathogenesis of these disorders (12). To transmigrate into the CNS, monocytes must traverse the BBB, formed by endothelial cells that line the cerebral vasculature. Immune transendothelial migration can alter the normal function of the neurovascular unit, which consists of endothelial cells, astrocytes, pericytes, and neurons. We have previously indicated that sCD40L secreted by aberrantly activated platelets is implicated in the impairment of the BBB during HIV−infected infection (26), thereby providing monocytes with an increased chance of thwarting this barrier. Furthermore, almost all cells of the monocytic lineage found within the CNS of HIV−infected individuals are CD16+ proinflammatory monocytes (12). Importantly, CD16+ monocytes are well known for their invasiveness across endothelial barriers. Interestingly, the findings in the present study now serve to bridge these reports, wherein we demonstrate that during HIV−infected infection, activated platelets interact more frequently with monocytes that consequently promote a proinflammatory and promigratory phenotype and cause an increase in the migration of these monocytes across the transendothelial barrier. Thus, these monocytes, with an enhanced capacity to transmigrate, are better equipped to extravasate through a compromised BBB and, consequently, contribute to neuroinflammation.

Consistent with this notion, we now demonstrate that HIV−infected individuals enrolled in our study showed the occurrence of increased circulating PMCs, especially in the CD16+ inflammatory monocyte subset, as well as increased platelet activation, despite successful cART. Studies have shown an increased incidence of milder forms of HAND in patients treated with cART (35). Thus, these studies highlight the need for the development of adjunctive therapies to supplement cART that could limit the neurologic complications associated with infection. The data reported in the present study, as well as previous studies from our group, indicate that modulation of platelet activation, and more specifically the release of sCD40L, may be a novel therapeutic target for these disorders. We previously demonstrated that the formation of PMCs was an event subsequent to platelet activation, and not monocyte activation (21), whereas we now report that PMCs have an enhanced migratory and invasive phenotype.
sCD40L is almost entirely platelet derived (25, 26), and in the present study, the role of CD40L signaling in the generation of PMCs was corroborated via in vitro coculture of monocytes with CD40L-activated or nonactivated platelets. These experiments demonstrated that monocytes in complex with CD40L-activated platelets express higher levels of CCR2, a chemokine receptor important for MCP-1–driven chemotaxis of monocytes into the CNS. PSGL-1, which is necessary for the interaction of monocytes with not only platelets, but also with endothelial cells, and CD40, which is a costimulatory molecule and the receptor for CD40L, were also elevated in monocytes incubated with CD40L-activated platelets. Monocytes that were exposed to CD40L-activated platelets also demonstrated an increased propensity to adhere to endothelial cells and migrate across the BMVEC monolayer as compared with nonactivated monocytes. Corroborating the in vitro results, immunohistochemical analysis of brain tissue samples from patients with HIV-E showed an increased infiltration of PMCs lining the brain endothelium (lumen) within the basal ganglia and cortical regions, as compared with tissue from uninfected controls.

These data are consistent with the notion that excess levels of sCD40L, and thus platelet activation, found in the plasma of HIV-1 patients (24, 26, 36, 37) contributes to the pathogenesis of neurologic disorders presumably via formation of PMCs, leading to invasion of the CNS by activated monocytes/macrophages. Indeed, our recent report also highlighted the ability of BMVECs to respond to CD40L in a manner that promotes monocyte attachment and migration, as exposure of primary human BMVECs to sCD40L in vitro leads to increased expression of adhesion molecules, ICAM-1 and VCAM-1. The same study also showed that the receptor for CD40L, CD40, was found to be highly expressed on endothelial cells in brain tissue collected from HIV-E patients (28). Consistently, other studies speculated that the interaction between platelets and monocytes increases the expression and activity of αβ1 and αβ2 integrins on monocytes (15, 38), thereby exhibiting increased ability of primary and secondary tethering to endothelial cells and other inflammatory cells (39, 40).

Interestingly, short-term treatment with low-dose aspirin, a known antiplatelet agent, was able to attenuate platelet and immune activation in virologically suppressed HIV-1–infected individuals (41). Furthermore, Gremmel et al. (42) have shown that prasugrel, an ADP receptor antagonist, reduces agonist-induced platelet activation and platelet–leukocyte interactions. Similarly, the clinically used mood stabilizer valproic acid exerts antiplatelet activity in a manner that attenuates sCD40L levels in HIV-1–infected individuals (36). It is noteworthy that the valproic acid treatment improves cognitive performance, as well as indices of brain metabolism, when tested in a controlled pilot study of HIV-1–infected individuals (43). Thus, dampening platelet activation, and in turn PMC formation, with antiplatelet agents such as aspirin, prasugrel, or valproic acid may prove a worthy avenue of pursuit in developing adjunctive therapies for use in combination with cART in an effort to reduce HIV-1–associated inflammatory illnesses such as HAND and cardiovascular disease. Indeed, PMCs are a known risk factor for cardiovascular disease, and HIV-1–infected individuals are at an increased risk for developing these secondary disorders (44, 45). Therefore, the use of antiplatelet agents as an adjunctive therapeutic strategy for HIV-1 infection may have benefits that are multifactorial.

Formation of PMCs appears to be a very rapid phenomenon, as injection of rnsCD40L caused an increase in total (CD115+) as well as Gr-1hi (inflammatory murine monocyte subset) PMCs in mice as early as 2 h posttreatment. Gr-1hi monocytes are the functional counterpart in mice for CD16+ monocytes in human, as these cells initiate inflammatory activity and accumulate at the sites of injury more abundantly than do Gr-1hi monocytes (46). Consistently, ex vivo treatments using rhsCD40L in whole blood samples obtained from HIV-1–seronegative individuals also induced the formation of PMCs within 1 h, an effect that was nullified by the use of a neutralizing Ab raised against CD40L (MK13). Blockade of P-selectin on platelets using the blocking Ab 9E1 did not affect platelet activation in response to rhsCD40L, but it significantly inhibited PMC formation, indicating the involvement of P-selectin–PSGL-1 interaction during PMC formation. Additional molecules known to be involved in platelet–monocyte interactions include EMPRIN (47), CX3CR1 (48), and monocytic CD115 with platelet P-selectin. However, the association of PSGL-1 with platelet P-selectin is considered to be the most critical interface, whereas other ligands play an additive role (17). Collectively, these results indicate that inhibition of sCD40L signaling with antiplatelet agents would be capable of attenuating the formation of PMCs, further highlighting the potential benefits of
antiplatelet therapies for the management of neuroinflammatory consequences of HIV-1 infection in patients. Presence of CD68+ monocytes/macrophages in the perivascular space and adjoining parenchymal regions within HIV-E brain is clearly evident; however, we were unable to detect PMCs and/or platelets that had extravasated into the brain of these patients. Platelets are occasionally shown to possess the ability to extravasate into tissues. For example, a study by Laidlaw et al. (49) reported the presence of platelet–leukocyte complexes in inflamed respiratory tissue of subjects with aspirin-exacerbated respiratory disease, whereas another report by Pitchford et al. (50) indicated that platelets migrate extravascularly in response to a sensitizing allergen and can participate directly in allergic tissue inflammation in a mouse model. In contrast to these observations, a recent report by van Gils et al. (51) suggested that platelets relocate with monocytic PSGL-1 to the rear of the monocyte, following adherence to the endothelium, subsequently detach from monocytes, and remain at the endothelial cell surface. Our data are consistent with those of van Gils et al. and suggest that the platelets may not leave the circulation; however, association with activated platelets in the periphery could prime monocytes to migrate into the CNS, with the release of the platelet behind in the vessel lumen.

Taken together, our findings substantiate the role of PMCs in HIV-1–associated neuroinflammation. In the post-cART era, the severity of neurologic diseases has been reduced; however, with the increased lifespan of HIV-1–infected individuals, mild and asymptomatic forms of neurocognitive impairment are increasing in prevalence (reviewed in Ref. 35). Although this means that the morbidity associated with neurologic complications has been lessened, milder forms of the disease can still interfere with the day-to-day functions of an individual, and can often be predictive of more severe forms of these disorders. Given the current absence of effective therapeutic options to address this aspect of the disease, the results presented in this study shed light on the underlying mechanisms that may drive the pathogenesis of HIV-1–associated neuroinflammation, thereby identifying novel targets for the development of adjunctive therapies and highlighting the potential utility of antiplatelet agents as such.

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Disclosures

The authors have no financial conflicts of interest.

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