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Research Paper

HIV-Nef and ADAM17-Containing Plasma Extracellular Vesicles Induce and Correlate with Immune Pathogenesis in Chronic HIV Infection

Jung-Hyun Lee [a], Stephan Schierer [a], Katja Blume [a], Jochen Dindorf [a], Sebastian Wittki [a], Wei Xiang [b], Christian Ostalecki [a], Nina Koliha [c], Stefan Wild [c], Gerold Schuler [a], Oliver T. Fackler [d], Kalle Saksela [e], Thomas Harrer [f], Andreas S. Baur [a,*]

[a] Department of Dermatology, University Hospital Erlangen, Friedrich-Alexander-University of Erlangen-Nürnberg, Translational Research Center, Ulmenweg 12, 91054 Erlangen, Germany
[b] Department for Biochemistry and Molecular Medicine, Friedrich-Alexander University of Erlangen-Nürnberg, Fahrstraße 17, 91054 Erlangen, Germany
[c] Miltenyi Biotec GmbH, Friedrich-Ebert-Straße 68, 51429 Bergisch Gladbach, Germany
[d] Department of Infectious Diseases, Integrative Virology, University Hospital Heidelberg, Im Neuenheimer Feld 324, 69120 Heidelberg, Germany
[e] Department of Virology, 00014 University of Helsinki, PO Box 21, Haartmaninkatu 3, Finland
[f] Department for Internal Medicine 3, University Hospital Erlangen, Friedrich-Alexander-University of Erlangen-Nürnberg, Ulmenweg 18, 91054 Erlangen, Germany

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ABSTRACT

Antiretroviral therapy (ART) efficiently suppresses HIV replication but immune activation and low CD4 T cell counts often persist. The underlying mechanism of this ART-resistant pathogenesis is not clear. We observed that levels of plasma extracellular vesicles (pEV) are strongly elevated in HIV infection and do not decline during ART. Surprisingly, these vesicles contained the viral accessory proteins Nef and Vpu, which are assumed to be not expressed under efficient ART, as well as pro-inflammatory effectors, including activated ADAM17. HIV pEV were characterized by the presence of activated αvβ3 and absence of CD81 and Tsg101. Correlating with immune activation, peripheral monocytes ingested large amounts of pEV, giving rise to an increased population of $CD1c^+$ $CD14^+$ cells that secreted inflammatory cytokines. Importantly, the pro-inflammatory content, particularly ADAM17 activity, correlated with low T cell counts. Preliminary evidence suggested that HIV pEV derived from peripheral mononuclear cells and from an unknown myeloid cell population. In summary we propose an important role of pro-inflammatory pEV in chronic HIV infection due to ongoing viral Nef activity.

1. Introduction

HIV is targeting predominantly T cells, most of which are killed rapidly after infection (Perelson et al., 1996; Ho et al., 1995; Doitsh et al., 2014), but leave behind a small reservoir of largely inactive proviruses (Eriksson et al., 2013; Eisele and Siliciano, 2012). Despite this seemingly negligible amount of latent HIV present under ART, signs of the infection persist, including immune activation, low CD4 counts and increased risk for a number of co-morbidities (Deeks et al., 2013). Moreover, HIV replication resumes quickly when ART is discontinued, although a cellular and humoral immune response is present (Davey et al., 1999).

Evidence is accumulating that extracellular vesicles not only play a role in cancer (Greening et al., 2015) but also in viral infections (Meckes, 2015). The membrane-enclosed viral-like structures are found at high levels in plasma, contain numerous effectors including enzymes, proteins, and RNAs (Raposo and Stoorvogel, 2013; Konadu et al., 2015) and are secreted continuously by cells with activated endo- and exocytosis (Baur, 2011). The latter may in part explain why tumor- as well as HIV-infected cells shed high numbers of EV (Skog et al., 2008; Muratori et al., 2009). In viral infections they seem to facilitate the spread of the virus, a finding that has also been suggested for HIV (Feng et al., 2013; Arenaccio et al., 2014; Meckes, 2015).

Our previous work suggested that large amounts of extracellular vesicles (EV) are secreted by HIV-infected cells (Lee et al., 2013; Muratori et al., 2009), which was confirmed by additional work (Lenassi et al., 2010; Narayanan et al., 2013; Shelton et al., 2012). Vesicle secretion was induced by the viral pathogenesis factor Nef and linked to Nef-mediated activation of ADAM17 at the plasma membrane. From there, both factors were shuttled into EV in a Paxillin-dependent mechanism. These in vitro vesicles were able to induce the release of TNF when ingested by resting PBMC. So far it was not clear whether these mechanisms occurred in vivo, what effect(s) they would induce and whether they were linked to HIV-associated immune pathogenesis. Here we demonstrate that HIV pEV contain a number of pro-inflammatory factors as well as the viral accessory proteins Nef and Vpu, are persistently upregulated despite ART and correlate significantly with pathogenesis in chronic infection. Importantly our results point to a so far not recognized cell compartment with ongoing viral activity under ART.

* Corresponding author.
E-mail address: andreas.baur@uk-erlangen.de (A.S. Baur).

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2. Materials and Methods

2.1. Nef Antibodies and Detection Reagents

Different anti-Nef antibodies and reagents were used: (1) anti-Nef JR6, a mouse monoclonal antibody (Abcam ab42358); (2) anti-Nef 2A3, a mouse monoclonal antibody (Abcam ab77172); (3 and 4) anti-Nef sheep serum, either as a purified biotinylated polyclonal antibody or non-labeled (both from NEXT Biomed, Helsinki); (5) anti-Nef polyclonal serum (provided by Mark Harris, Leed University). All Nef-antibodies were used to demonstrate the presence of Nef in pEV. For immunoblotting JR6 turned out to have the highest sensitivity and specificity as judged by the ratio of Nef vs. background staining. For immunohistochemistry we used the biotinylated Nef sheep serum and the JR6 antibody. To confirm Nef in pEV, we also employed the recombinant Neffin construct, comprised of a 118 aa llama Ig heavy chain variable domain fragment (VHH) fused to a ligand-tailored 57 aa SH3 domain (Jarviluoma et al., 2012) and immunoprecipitated Nef from plasma (data not shown).

2.2. Antibodies

The following antibodies were used for immunostaining, flow cytometry or immunoblotting: anti-ADAM10 (mouse monoclonal, Abcam ab73402), anti-ADAM10-PE (mouse monoclonal anti-ADAM17 (rabbit polyclonal, Cell Signaling 3976), anti-alpha-smooth muscle actin-FITC (Sigma-Aldrich, F3777), anti-TNF (rabbit monoclonal, Cell Signaling 6945), anti-Gag p24 (mouse monoclonal, Abcam ab9071), anti-CD81 (mouse monoclonal, BD Biosciences 555675), anti-Paxillin (mouse monoclonal, Millipore 05-417), anti-Tsg101 (mouse monoclonal, Santa Cruz Biotecnology sc-7964), anti-HLAI (mouse monoclonal, BD Biosciences 555551), anti-HLAII (mouse monoclonal, Abcam ab20181), anti-Vpu (rabbit polyclonal, Biozol FBX-VPU-101AP-100), anti-CD1c-PE (mouse monoclonal, Miltenyi Biotec, 130-90-007), anti-CD14-FITC (mouse monoclonal, Miltenyi Biotec, 130-080-701), anti-CD19-APC (mouse monoclonal, Miltenyi Biotec, 130-091-247), anti-Integrin αvβ3 (mouse monoclonal, Abcam ab78289), Propidium iodide (Genaxxon bioscience, M3181.0010), DAPI (4′,6-diamidino-2-phenylindole, Biomol ABD-17510). Primary antibodies were used at 1–2 μg ml^{-1} for immunoblotting, 2 μg ml^{-1} for immunofluorescence and 5–10 μg ml^{-1} for FACS analysis. The following secondary antibodies were used: Alexa Fluor 488 goat anti-mouse and Alexa Fluor 555 goat anti-rabbit IgG (both from Life Technologies) and anti-mouse IgG-HRP conjugate and anti-rabbit IgG-HRP conjugate (both from Cell Signaling).

2.3. DNA Constructs and Transfection

Expression plasmids for Nef and Nef-cofactors (hnRNPK, PKCδ, Lck) were described previously (Lee et al., 2013). The HIV-Δenv (pNL-4.3Δenv (Clavel et al., 1989)) expression plasmid was kindly provided by U. Schubert (Department of Virology, University of Erlangen). For immunoblotting experiments, plasmids were transfected with Lipofectamine® LTX with Plus™ Reagent (Invitrogen) according to the manufacturer's instructions, or using the classical calcium phosphate procedure. Cells were used for experiments 24–72 h after transfection.

2.4. ADAM17/α-Secretase Activity Assay

The assay was performed essentially as described previously (Lee et al., 2013) using a commercial, SensoLyte®520 α-Secretase Activity Assay Kit (AnaSpec 72085), according to the manufacturer's instructions. Briefly, we placed sucrose gradient purified pEV (the equivalent of 1 ml plasma) on a 96-well, black, flat bottom plate (Greiner 655900) and added a 5-FAM (fluorophore) and QXL™ 520 (quencher) labeled FRET peptide substrate for continuous measurement of enzyme activity. Upon cleavage of the FRET peptide by the active enzyme, the fluorescence of 5-FAM is recovered and continuously monitored at excitation/emission = 490 nm / 520 nm by a preheated (37 °C) TECAN infinite M200 Pro plate reader.

2.5. Patient Material

Patient material was obtained from patients of the HIV-clinic (headed by T. Harrer) at the Department of Medicine 3, University Hospital Erlangen. Plasma was drawn from patients after informed consent and approval of the local ethics committee. All procedures were pursued in accordance with the Declaration of Helsinki, with the patient's Guardian Informed Consent and the approval from the Institutional Review Board. At the time of sampling, non-viremic HIV patients were under ART for prolonged periods without detectable viral load (<20 viral copies/ml), while viremic patients (for viral copy number see Supplement Table S1) were untreated or had just started treatment. CD4 and CD8 counts (cells/μl blood) were determined by the Department of Medicine 3 and viral copies number (copies/ml blood) by the Department of Virology in Erlangen. In general, 6–7 ml of plasma was obtained from each individual per visit.

2.6. Immunostaining, Confocal Microscopy and FACS Analysis

For detection of Nef by immunofluorescence, monocytes were separated from the non-adherent fraction (NAF) by plastic adherence on cell culture flasks and cultured in RPMI (Sigma-Aldrich) including supplements. In HIV pEV incubation experiments, 3.0×10^5 monocytes were seeded in a 12 well plate and 10 μg of sucrose gradient purified HIV pEV were added and incubated overnight at 37 °C under 5% CO_2. Then monocytes were harvested and $1.0–2.5 \times 10^5$ cells were seeded on a Poly-(L)-Lysine (Sigma Aldrich) coated cover slips. The cells adhered for 2 h at 37 °C under 5% CO_2 and were fixed thereafter (3% PFA for 30 min at room temperature followed with three washes with PBS/1% BSA). Then cells were permeabilized (0.1% Triton X-100/1% BSA) and immunostained by standard procedures (primary and secondary antibodies). Finally, the cells were washed 30 min with PBS/1% BSA and mounted with Entellan (Merck 1079610100). Fixed samples were imaged with a laser scanning confocal microscope (LSM-780; Zeiss) equipped with a 63× objective. For Alexa488 the illumination was set at 488 nm and emissions were collected between 506 and 583 nm. For Alexa555 the illumination was at 561 nm and emission collected between 574 and 667 nm. Detecting DAPI, illumination was set to 405 nm and emission collected between 410 and 495 nm.

For FACS analysis, $CD1c^+$ cells from at least 1.0×10^7 PBMC were isolated following the protocol of the $CD1c^+$ ($BDCA\text{-}1^+$) Dendritic Cell Isolation Kit (Miltenyi Biotec 130-090-506), but without depletion of CD19 cells. Cells were stained with fluorochrome-conjugated antibodies and flow cytometric analysis was done using a FACS Canto II flow cytometer (BD Bioscience). Data were analyzed with the FCS Express 4 (De Novo Software) software. $CD1c^+$ $CD14^+$ cells were analyzed in the $CD19^-$ gated fraction of cells, as CD1c is also expressed on a subset of B cells. CD19 MACS beads (Miltenyi Biotec 130-050-301) and CD14 Magnetic particles (BD Bioscience 557769) were used for positive selection following the magnetic separation protocol of the manufacturer.

2.7. Micro-RNA Microarray

The pEV were purified from equal volumes of pooled or non-pooled platelet poor plasma supplemented with BHRF1-2* miRNA as spike-in control (see below) and pelleted. The pEV pellets were then dissolved in 700 μl of Qiazol and total RNA was isolated using Qiagen miRNeasy Mini Kits (Qiagen 217004). The extracted RNA was sent on dry ice to Miltenyi Biotec. 100 ng total RNA was concentrated to 50 ng/μl and Cy3-labeled using Agilent's miRNA Complete Labeling and Hyb Kit (Agilent Technologies, 5190-0456). After purification through Micro Bio Spin Columns (Bio Rad, 732-6221) the total RNA samples were