Short Communication: Apoptotic Membrane Microparticles Quantified by Fluorescent Bead-Based Assay Are Elevated in HIV and SIV Infections

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Abstract

Apoptotic membrane microparticles (MMPs) derived from dying cells of multiple cell origins are highly immunostimulatory and are indicative of global immune activation and cell death in a variety of diseases. In this study, we developed a flow cytometric bead assay to quantify annexin-V⁺ apoptotic (MMPs) in plasma from humans and rhesus macaques. With a combination of flow cytometry and pan-fluorescent beads, MMPs were enumerated in plasma specimens by adding a constant ratio of beads to initial fluid volumes and then calculating MMP/mL based on MMP-to-bead ratios. Using this straightforward assay, we found that circulating MMP quantifications were highly reproducible and similar in number between normal rhesus macaques and humans subjects. However, MMPs increased two- to threefold during HIV and simian immunodeficiency virus (SIV) infections and were positively associated with T cell immune activation. Collectively, we present a rapid bead-based assay for both humans and macaque models to quantify MMPs that could be an instigator and predictor of immune activation, which is a primary source of HIV/SIV disease.

Keywords: fluorescent beads, membrane microparticles, exosomes, HIV, SIV

ORIGINALLY IDENTIFIED 50 YEARS AGO, membrane microparticles (MMPs) are derived from cellular membranes, generally range in size from 0.1 to 1 μm in diameter,¹ and are a major component of the growing family of extracellular vesicles (EVs).² Although normal release of MMPs is thought to be a tightly regulated process that can mediate intercellular communication, there is significant evidence that MMPs can be formed following cytoskeletal collapse.^{3,4} So-called apoptotic MMPs can be derived from any cell type, but differ from other EV in that MMPs reform the micelle from apoptotic cellular debris and thus have highly variable contents.⁵

Regardless of source, MMPs are generally indicative of cell death and tissue breakdown, and are highly immunostimulatory. Indeed, a wide range of chronic inflammatory and infectious pathologies as varied as atherosclerosis, diabetes, multiple cancers, malaria, and HIV-1 infection cause increased apoptotic and immune reactive MMPs.^{6–9} While pure isolation is critical for characterization, immunostimulatory,

and assessment assays, a method for rapid quantification is desirable for screening. Such analyses could provide rapid evaluation of global cellular death and help predict immune activation.

A total of 20 persons were analyzed in this study and the median ages and sex were as follows: 46 years, four males and six females among seronegative subjects; 39 years, four males and one female among viremic HIV⁺ subjects; and 42 years, four males and one female among virally suppressed HIV⁺ subjects on cART. Plasma HIV-1 RNA levels were determined using the Amplicor ultrasensitive HIV-1 monitor assay at UAB hospitals (version 1.5; Roche Diagnostics Systems) and absolute CD4⁺ T cell counts were determined in UAB diagnostic laboratories as described previously.¹⁰ All cART subjects had viral loads of <50 copies/mL, plasma, while viremic HIV⁺ subjects had a median viral load of 5.08×10^4 copies/mL, plasma (range 2.30×10^4 to 3.41×10^5 copies). Both the University of Alabama at Birmingham (UAB) and Harvard University IRB reviewed and gave

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approval for the studies herein and written informed consent was obtained from each of the study subjects.

A total of 25 juvenile male rhesus macaques (*Macaca mulatta*) of Indian genetic background were used for sampling in this study. All animals were housed at the New England Primate Research Center and maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and the Guide for the Care and Use of Laboratory Animals. Chronically SIVmac251-infected rhesus macaques were all infected greater than 200 days and had a median viral load of 1.5×10^4 copies/mL, plasma (range 1.90×10^4 to 1.20×10^6 copies).

Plasma was collected by venipuncture using standard clinical procedures and processed immediately over Ficoll to separate lymphocytes, erythrocytes, and plasma using standard

protocols. Following initial plasma separation, an additional centrifugation at 1500 g, 20°C for 15 min was performed to produce platelet poor-plasma. Plasma was then stored at -80°C until MMP isolation, whereupon plasma was first briefly centrifuged at 4000 g, 20°C for 5 min to remove any residual platelets or cellular debris. Next plasma was centrifuged at 20,000 g, 20°C for 20 min to pellet MMP. MMP pellets were washed in Ca^{++} -binding buffer (necessary for annexin-V binding and to mitigate any EDTA effects) and then centrifuged again at 20,000 g, 20°C for 20 min, before resuspending in Ca^{++} -binding buffer plus annexin-V FITC and incubated 30 min at room temperature. Aggregation of MMP was not routinely observed, but could be a minor confounding factor with larger samples. MMPs were then fixed with no wash in 1% PFA made in $1 \times \text{Ca}^{++}$ -binding

FIG. 1. Bead-based quantification of MMPs in plasma in SIV-infected rhesus macaques and HIV-infected human subjects. **(A)** Representative gating strategies showing fluorescence profiles of beads and Annexin-V⁺ MMPs. **(B)** MMP quantification comparison using variable starting volumes of plasma. **(C)** MMP quantification in naive and untreated or cART HIV-1-infected subjects. Horizontal lines indicate medians. **(D)** MMP quantification in naive and SIV-infected rhesus macaques. **(E, F)** Correlation of HLA-DR expression on CD4⁺ **(E)** and CD8⁺ T cells with circulating MMP in plasma. Human samples are shown in black, rhesus macaque in blue. * $p < .05$; ** $p < .01$; Mann-Whitney U test for cross-sectional comparisons. Spearman correlation test was used for **(E, F)**. MMP, membrane microparticle; SIV, simian immunodeficiency virus.

buffer and transferred to tubes containing 50,050 lyophilized polyfluorescent beads and run on an LSRII. Unstained controls and irrelevant FITC-conjugated antibodies were used to determine background fluorescence and gating. Triton-X-induced MMPs were used as positive controls. Analyses were performed using FlowJo 9.1, and calculations were made using the formula: absolute count of MMP/ μL = (events in MMP region/events in region containing absolute count beads) $\times$ (beads per tube/test plasma volume in μL).

Initially, flow cytometry panels were optimized to identify apoptotic MMPs in both rhesus macaques and humans and used in combination with fluorescent beads. As shown in Figure 1A, MMPs were identified as plasma events $<1.0\ \mu\text{m}$ in diameter that also costained for annexin-V. Beads were then readily identified by their high SSC properties and fluorescence. For quality control, we quantified MMPs, using varying initial volumes of plasma, and found that while some variation occurred at lower volumes ($250\ \mu\text{L}$), in general, quantification was quite reproducible regardless of initial volume (Fig. 1B). Although MMPs were readily identifiable by this method, it is important to note that quantification of individual MMP size below $1.0\ \mu\text{m}$ is not possible due to discrimination limitations of cytometers. Next, we evaluated differences in humans and rhesus, both normal and those infected with HIV-1 and simian immunodeficiency virus (SIV), respectively. In seronegative humans a median of 4.31×10^7 MMP/mL of plasma (range 2.08×10^7 to 7.44×10^7) were quantified, and similarly in rhesus macaques a median of 4.93×10^7 MMP/mL of plasma (range, 6.23×10^6 to 1.15×10^8) (Fig. 1C, D) were found. The human sexes were nearly identical with seronegative males and females exhibiting median 4.30×10^7 and 4.32×10^7 MMP/mL of plasma, respectively. These strikingly similar values suggest a conservation of circulating levels of MMP in primates and that rhesus macaques closely model human phenomena. However, in HIV-1 infected persons, median MMP numbers increased to 1.31×10^8 and 1.03×10^8 , in untreated and treated patients, respectively (Fig. 1C). Again similarly in chronically SIV-infected rhesus macaques, median MMP numbers increased to 1.52×10^8 MMP/mL of plasma (Fig. 1D). Collectively, these data indicated that apoptotic MMP increase in frequency in HIV infection and may remain elevated even with virus suppression by cART, and is recapitulated in SIV-infected macaque models. Interestingly, virus loads did not significantly correlate with MMP or circulating CD4^+ T cell counts in either species (data not shown), suggesting that although infection is the inducing factor for increased MMP, it is not necessarily directly contributing to MMP formation. Interestingly, and importantly, MMP numbers did strongly correlate with HLA-DR expression on both CD4^+ and CD8^+ T cells in both species (Fig. 1E, F), suggesting MMP may either be a source or a predictor of immune activation.

Herein, we present a rapid and reproducible assay that could be useful for large numbers of specimens and could be used to monitor changes in MMP during HIV and SIV infections. Although this assay provides that it is difficult to determine exact origins of MMP, higher frequencies overall are known to be associated with increases in overall apoptosis and thus could provide substrate for formation of these particles in lentivirus infections. Thus, since MMP are associated with activated T cells, but not virus loads, this assay may

also be a useful predictor of an alternative mechanism of overall immune activation.

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Author Disclosure Statement

No competing financial interests exist.

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