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MEETING REPORT

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Proceedings of the ISEV symposium on "HIV, NeuroAIDS, drug abuse & EVs"

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ABSTRACT

Extracellular vesicles (EVs) are globular, membrane bound nanovesicles (30–100 nm range) that are shed both during normal cellular functioning and under pathological conditions by most cell types. In recent years, there has been significant interest in the study of these vesicles as conduits for the delivery of information between cells from both analogous and disparate tissues. Their ability to carry specialised cargo including signalling mediators, proteins, messenger RNA and miRNAs characterises these vesicles as primary facilitators of cell-to-cell communication and regulation. EVs have also been demonstrated to play important roles in the field of cancer biology and metastasis. However, significant knowledge gaps exist in the role these vesicles play in the context of HIV infection and drug abuse. To foster discussion in this area a satellite symposium on "HIV, NeuroAIDS, Drug Abuse & EVs", was held in conjunction with the annual meeting of the International Society for Extracellular Vesicles (ISEV) in Bethesda, in April 2015. Experts in HIV and drug abuse fields were invited to share their findings on the role of EVs in HIV-1 infection and drug addiction. Additional discussion included current areas of research in EV biology in HIV infection and drug abuse.

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Introduction

In conjunction with the annual meeting of the International Society for Extracellular Vesicles (ISEV), a satellite symposium on "HIV, NeuroAIDS, Drug Abuse & EVs" was held on 23 April 2015 in Bethesda, MD, USA. The workshop was organised by Dr Shilpa Buch from the University of Nebraska Medical Center in cooperation with Dr Kenneth Witwer from the Johns Hopkins University, Dr Jeymohan Joseph from the National Institute of Mental Health and Dr John Satterlee from the National Institute on Drug Abuse. Dr Witwer as the local Chair for ISEV2015 provided the introductory remarks and opened up the session. The satellite symposium was open to all the ISEV attendees.

Cellular crosstalk underlies almost all pathological conditions, especially within the CNS. While various agents have been identified as initiators of the disease process, it is now becoming clear that spread of inflammation and toxicity is a key hallmark feature of disease progression. In this light, spread of disease process by exosomes is gaining momentum. Exosomes are globular, membrane bound extracellular nanovesicles (30–-100 nm range) that are shed from almost all types of cells both during normal cellular functioning, and specifically in response to cellular stressors.[1–4] These small vesicles originally thought to be "junk" cellular debris, were first described by Trams et al. [5] when they observed smaller membrane bound vesicles within the larger endosomes (later termed multi-vesicular bodies; MVBs). An electron micrographic study related the now-termed "exosomes" to sheep reticulocytes when they were shown to be released into the extracellular environment following the fusion of the multi-

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vesicular bodies with the plasma membrane.[6] Release of these small vesicles into the extracellular environment was the proposed mechanism by which reticulocytes secreted the transferrin receptor. This proposed mechanism was further supported by *in vitro* analyses of sheep reticulocytes, which demonstrated selective loss of certain proteins from the maturing cells.[7] An understanding of their role in various cell types has evolved immensely since the turn of the century. They are no longer viewed as waste bags; instead, exosomes are thought to play an important role as cargo-carrying vesicles mediating communication among diverse cells and tissues, including the CNS.[8] Exosomes are known to carry nucleic acids (RNA, miRNA and DNA), functional proteins (including viral material) and other cellular products.[8–10]

The human immunodeficiency virus (HIV) is a lentivirus (a subgroup of retrovirus) that slowly works to destroy the host's immune system by invading T-cells, eventually hijacking them and using cellular machinery to perpetuate this process. This course of infection eventually leads to acquired immunodeficiency syndrome (AIDS), which subjects the host to increased risk of opportunistic infections. The development of combined antiretroviral therapy (cART) has been a major advancement in the control of AIDS.[11] The use of cART has significantly increased the life span and health of individuals living with HIV-1. However, despite the successful control of virus replication by antiretroviral therapies, there continues to be an increase in the number of individuals afflicted with HIV-associated neurological disorders (HAND), likely due to the increased longevity of individuals infected with HIV-1 as well as the existence of viral sanctuaries like the CNS, despite the presence of cART. These neurological disorders manifest as a spectrum from mild, in which the condition may go undetected and does not impair a person's daily functionality, to moderate, wherein the patient experiences progressive cognitive impairment affecting the ability to function normally.

A growing body of evidence suggests that exosomes play a vital role in neuroinflammatory diseases. These small vesicles are likely important in CNS communication as most CNS cells secrete them.[8] Cell–cell communication via exosomes could play an important role in pathogenesis due to its ability to quickly transmit disease-causing agents from one cell to another. Indeed, exosomes have been associated with numerous neuroinflammatory diseases including Parkinson's, Alzheimer's, and Creutzfield-Jacob diseases. Continued research into the role these vesicles play in disease progression is important for finding effective preventative and therapeutic options.

Summary of presentations

Dr Kenneth Witwer noted that retroviruses and extracellular vesicles were intimately related and that retroviruses can be seen as a hijacked extracellular vesicle.[12] From another perspective, the extracellular vesicle is a virus-like particle, transferring proteins, nucleic acids, and other cargoes to recipient cells.[2,13,14] With the advent of ultracentrifuge technology in the first part of the twentieth century,[15] methods were rapidly developed for virus and other subcellular particle purification.[16,17] Perhaps the earliest indications of EVs were particles obtained from uninfected, negative control preparations. Hints as to the roles of these particles were revealed in the 1940s, when Chargaff and West reported that ultracentrifuged fractions of blood from healthy individuals could confer clotting properties to the blood of haemophiliacs.[18] Two decades later, Peter Wolf described sudanophilic (lipid) particles derived from platelets, famously referring to them as "platelet dust".[19] He further described the necessity of diluting plasma or serum before centrifugation to allow optimal pelleting of particles, a principle now well known to the EV researcher.[20,21] Much more was learned about EVs – in bone,[22] in body fluids,[23] in cancers,[24] and in the immune system [25] – over the next several decades.

Around the turn of the century and in following years, viruses and EVs met again on three fronts. First, with the finding (a re-discovery of sorts) that viruses and EVs copurify by stepped ultracentrifugation,[26,27] leading to adaptation of new density gradient methods (and others) for virus/EV separation.[26,28,29] Second, with the idea that the HIV particle evades the immune system by mimicking a native exosome.[12] And third, with the rise of the exRNA hypothesis: the notion that EVs, like viruses, could shuttle RNA from a cell of origin to a recipient cell.[2,30,31] In this environment, the stage was set for new investigations into the relationships of EVs and retroviruses.

Dr Jeymohan Joseph gave a talk on "NIMH priorities in NeuroAIDS and exosome research". He provided a brief overview of the NeuroAIDS research programmes and priorities supported by the HIV Neuropathogenesis, Genetics and Therapeutics Branch, Division of AIDS Research. This Branch supports an integrated programme of studies to investigate the pathophysiology and genetic factors that contribute to HAND. Support is also provided for developing novel therapeutic strategies to mitigate the CNS

complications of HIV infection, using information gathered from research into HIV neuropathogenesis. He then outlined the following areas of focus relating to extracellular vesicles and HAND that have been identified by NIMH as priority areas:

(1) assessing changes in the physiology of exosome release and cargo content from CNS-derived cells in the setting of HAND;
(2) studying the impact of exosomes as novel conduits for cell–cell communication in the setting of HAND (for example, assessing the effect of exosomes derived from macrophages/astrocytes on neuronal apoptosis or neuroprotection);
(3) studying the impact of highly active antiretroviral therapy (HAART) on the content, release and effect of exosomes derived from HIV-1 infected cells of the CNS;
(4) studying the impact of exosome-mediated intercellular signalling on the development and maintenance of neural circuits in adult and paediatric populations in the setting of HIV-1 infection of the CNS;
(5) assessing the potential role of exosomal cargo as biomarkers to serve as clinical diagnostics for HAND;
(6) assessing the role of exosomes as delivery vehicles for CNS-targeted therapeutics; and
(7) studying the role of exosomes in cross talk between the periphery and CNS with particular focus on inflammatory mediators.

Dr Vincent C. Bond talked on "Cytokines associated with exosomes in HIV-infected individuals". He presented data showing that THP-1 cells when transfected with HIV-1 negative regulatory factor (Nef) or exposed to exosomal Nef protein demonstrated increased surface expression of IL-15/IL-15Rα in a dose-dependent manner. Peripheral blood mononuclear cells (PBMCs) exposed to exosomal Nef showed increased cell surface expression of interleukin 15 receptor alpha subunit (IL-15Rα) on CD14+ monocytes, and CD38 on central and effector memory CD4 and CD8 T-lymphocytes. Exposure of PBMCs to exosomes purified from plasma of viremic HIV-1 infected individuals significantly increased cell surface expression of IL-15Rα on CD14+ monocytes compared to untreated cells, suggesting exNef contributes to immune activation via induction of IL-15/IL-15Rα in monocytes and macrophages.

His work examined purified exosomes for levels of 21 cytokines and chemokines. These were compared to similar cytokine/chemokines in the exosome depleted plasma. Most cytokines were markedly enriched in exosomes from HIV+ individuals relative to exosomes from negative controls and to exosome depleted plasma. The authors demonstrated the biological relevance of these plasma exosomes, exposing naive PBMCs to exosomes purified from HIV+ patients. Their group observed induction of CD38 expression on naive and central memory CD4 and CD8 + T cells relative to plasma exosomes from negative controls.

In their study, they observed that most cytokines were not in free form, but rather were associated with exosomes. Additionally, cytokines were actively and selectively enriched in plasma exosomes (distinct from virions) isolated from HIV-1 infected individuals. Interestingly, this mechanism has remarkable similarity to a host mechanism leading to immune privilege during pregnancy. This process has been found to be hijacked during carcinogenesis leading to immune modulation/inflammation and ultimately allowing tumour growth. It was thus suggested that this mechanism likely contributed to inflammation and viral propagation via bystander cell activation.

Dr Shilpa Buch gave a talk on "Trans-activator of transcription (Tat) and opiates modulate HAND: blaming the messengers". Impairment of microglial functions, such as activation and migration, comprise the underlying features of HAND. HIV Tat has been well documented to activate glial cells. The author's previous studies demonstrated that HIV Tat via induction of miR-9, a highly conserved and brain enriched miRNA (miR), plays a pivotal role in regulating the microglial inflammatory response in the context of HAND. The goal of the current study was to examine whether HIV Tat mediated release of miR-9 released from astrocytes, which could also lead to impairment of other microglial functions such as cell migration. The authors demonstrated upregulation of miR-9 in the brains of SIV/HIV-infected subjects. These findings were further validated by *in situ* hybridisation, demonstrating increased expression of miR-9 in the astrocytes in the brains of SIV-infected macaques compared with uninfected controls. The authors further demonstrated that EVs released from Tat treated astrocytes could shuttle miR-9, which in turn, could be taken up by neighbouring microglia leading to microglial migration, as evidenced by increased migration in Boyden chambers. The authors identified miR-9 as a positive regulator of microglial migration via downregulation of the key target protein, monocyte chemotactic protein-induced protein 1 and its downstream signalling, the β-catenin pathway. In agreement with the in vitro results, the in vivo data demonstrated increased microglial migration towards the lipopolysaccharides (LPS) injection site in the brains of mice administered exogenous miR-9 compared with the

control group. These data not only shed light on the mechanism(s) underlying the roles of EV miRs on microglial function(s) but also serve as a foundation for future development of nanovesicle and miR-based therapeutics for the treatment of cognitive decline observed in HAND.

Dr Norman Haughey gave a talk on "Brain-derived exosomes regulate the peripheral immune response to brain injury". He presented his findings that adoptive transfer of EVs into the striatum induced a liver cytokine response, and activated leukocytes, which, in turn, transmigrated to the site of brain injection in a manner that was indistinguishable from the response following a simple injection of IL-1β into the striatum. The protein and/or miR cargo of EVs was thus responsible for the observed effects on leukocyte migration. The next step involved stimulation of isolated neurons, astrocytes, microglia and oligodendrocytes with IL-1β (and a plethora of other stimuli), to monitor the release of EVs. The goal of the study was to investigate whether there was a soluble mediator released from the brain that could enter into the periphery. Herein efforts were focused on astrocytes since these cells have an intimate association with endothelial cells of the blood–brain barrier (BBB). The premise of the study was that if EVs released from astrocytes regulated the peripheral immune response to an inflammatory brain lesion, then these nm-sized particles would have to efficiently cross the BBB. It was found that astrocytes isolated from mice expressing GFP under the control of a GFAP promoter released EVs that were GFP positive. This cellular labelling of EVs provided a means to easily track the fate of astrocyte shed EVs. Using the transwell BBB system to track the transit of GFP+ EVs shed from astrocytes, it was found that the astrocyte EVs shed in response to IL-1β rapidly crossed to the luminal side of the barrier without disrupting the trans-barrier resistance. These results thus suggested that EVs shed from astrocytes could rapidly enter into the circulation without a gross disruption of the integrity of the BBB. Ultrastructural examination of these transwell systems by electron microscopy showed that IL-1β stimulation induced the formation of multivesicular bodies in astrocytes that was followed by the release of EVs that entered into endothelial cells. The cellular entrance of EVs appeared to occur largely by a macropinocytosis mechanism with a lesser contribution by endocytosis. Most of the EVs simply appeared at either the extracellular or intracellular surface of endothelial cells as single particles with a few encapsulated in what appeared to be endocytic bodies. The authors confirmed these results *in vivo* showing that striatal injection of IL-1β into GFAP-GFP mice resulted in the release of GFP labelled EVs that entered into circulation. Ultrastructural examination of these brain tissues by electron microscopy and immunogold labelling of GFP showed gold-labelled particles the size of EVs located in the cytosol of endothelial cells adjacent to the site of IL-1β injection. These gold-labelled particles were observed in small numbers in saline injected mice, with a low-basal level of GFP labelled nm-sized particles in liver, lung and spleen, suggesting thereby a possible constitutive release of EVs from astrocytes into the peripheral circulation. This basal release could simply be the removal of cellular debris for transportation to peripheral macrophages for degradation, or it could represent a continuous communication of brain with the peripheral systems. In either case, the numbers of labelled EVs in these tissues was dramatically increased following IL-1β injection into the striatum. It was thus concluded that a constitutive low-level release of EVs from astrocytes is increased in response to IL-1β, and that these EVs rapidly cross the BBB, and are localised to multiple tissues including the liver, lung and spleen.

To determine the mechanism(s) by which EVs regulate the peripheral immune response to inflammatory brain lesions a proteomic, lipidomic, and nanostring (miR) analysis of EVs released from astrocytes in response to IL-1β was performed. It was determined that the cargo of EVs was complex and contained hundreds of distinct proteins, lipids and miRs. It was reasoned that a focus on any one of these components would be unlikely to provide adequate information on how these complex particles regulated immune function. Bioinformatics approaches interrogating the entire molecular composition of EVs was thus used to assess the cargo. This approach identified multiple putative pathways including PPARs. As PPAR is highly expressed in liver and tissues that oxidise fatty acids,[32,33] and regulates cytokine expression through NF-κB,[34] efforts were focused on assessing this pathway in keeping with previous reports by Daniel Anthony et al.,[35–40] demonstrating an acute phase response in liver and that NF-κB was required to prime neutrophils for transmigration into the brain parenchyma following IL-1β induced brain lesion.[35–40] Immunoprecipitation of chromatin (ChIP) isolated from liver samples of mice following striatal injection of IL-1β demonstrated increased binding of the NF-κB subunit c-Rel to the promoter regions of *CCL2, IL-1β*, and *TNFα*, but not to *IL-17*, consistent with the increased expression of inflammatory cytokines. Administration of the PPARα antagonist fenofibrate just prior to the induction of an inflammatory brain lesion with IL-1β prevented not only the binding of NF-kB binding to *CCL2, IL-1β* and *TNFα* promoters but also the induction of inflammatory cytokines transmigration of leukocytes into the brain parenchyma. Involvement of PPAR was thus confirmed in mice administered IL-1β in conjunction with the nSMase2 inhibitor altenusin. In this study leukocyte influx is normally blocked, but peripheral

administration of the PPAR-α antagonist GW6471 re-established leukocyte recruitment to the site of IL-1β induced lesion. These findings thus suggested that peripheral activation of leukocytes in response to an inflammatory brain lesion can be regulated by astrocyte shed EVs that enter into peripheral circulation to regulate the liver acute cytokine response through suppression of PPARα.

Dr Fatah Kashanchi gave a talk on "Exosomes from latent HIV infections: spreading the message beyond infection". He stated that nearly one third of HIV-infected individuals develop neurocognitive deficits despite adequate cART and excellent virological control in the blood. This range of neurocognitive deficits is collectively referred to as HAND, and in general, the number of productively infected cells in the brain is small compared to the amount of neuronal damage, and the neurons are not infected with the virus. He stated that HIV may traffic into the brain via blood monocytes (Trojan horse phenomenon) early in the course of infection long before symptoms of AIDS appear, and in fact the virus can be detected in the cerebrospinal fluid (CSF) soon after a primary infection. This is consistent with data from a humanised mouse model. Microglia, perivascular and meningeal macrophages, astrocytes, and neural stem cells are sites of viral infection in the human brain and they potentially secrete exosomes. Exosomes play a key role in intercellular communication, as well as in the pathogenesis of neurodegenerative diseases, including neuronal degeneration. However, the role of exosomes released from HIV-1 infected cells in the neurological impairments associated with HIV infection is still unclear. HIV-1 and other viruses encode multiple small, regulatory non-coding RNAs (ncRNAs) and miRs that regulate host gene expression via exosomes. They have previously shown that exosomes containing non-coding RNA, called trans-activating response (TAR) element RNA, enhance susceptibility of undifferentiated naive cells to HIV-1 infection. The current studies indicated that exosomes from HIV-1 infected primary cells are highly abundant with TAR RNA as detected by RT-real-time PCR. Interestingly, up to 0.1–1.0 million copies of TAR RNA per microlitre were also detected in human infected serum and also from HIV-1 infected humanised mice, suggesting that TAR RNA may be very stable *in vivo*. TAR RNA was also present in four out of 10 CSF samples (generous gift of Dr A. Nath; NIH) tested from HIV-1 infected patients under cART. Incubation of exosomes from HIV-1 infected cells with primary macrophages resulted in a dramatic increase of proinflammatory cytokines, IL-6 and TNF-β, indicating that exosomes containing TAR RNA could play a direct role in control of cytokine gene expression and HAND.[9,41] The intact TAR molecule was able to bind to PKR, TLR3 effectively, whereas 5′ and 3′ stems bound best to TLR3, 7 and 8, and none to PKR. Binding of TAR to PKR did not result in its phosphorylation and, therefore, TAR may be a dominant negative decoy molecule. The TAR RNA molecule has a 23 base pair stem, which is not sufficient to activate PKR. Interestingly, the entire 9.5 kb RNA genome does not have a straight 30 base pair stem and loop structure, potentially acting as dominant negative for PKR (or related innate immune complexes). Furthermore, the single-stranded 5′ or 3′ processed stem RNA binding to TLRs activates the NF-κB pathway and regulates cytokine expression. Finally, the binding of RNA to the TLRs can activate a new form of IKK-β, which may contribute to the release of free p50 and p65 NF-κB and ultimately contribute to cytokine activation.[42] Collectively, these results imply that exosomes containing TAR RNA could directly affect the proinflammatory cytokine gene expression (potentially in naive uninfected cells) and may explain a possible mechanism of inflammation observed in HIV-1 infected patients. The effects may be further enhanced in tissues vs. blood stream, where the uninfected cells are constantly exposed to neighbouring infected exosomes. This may ultimately result in an initial phase of activation, but over time with continued exposure may cause exhaustion and cell death. This bell-shape curve effect of the infected exosomes was discussed.

Dr Lynn Pulliam gave a talk on "Monocyte immune activation in HIV can alter end organ function via exosomes". She commented that the monocyte is a critical first line response to the peripheral immune reaction to HIV infection. The previous findings from Dr Pulliam's group showed that circulating monocytes from HIV-infected individuals undergoing antiretroviral therapy had a type 1 interferon profile with increased levels of LPS in the plasma. While the interferon profile in some individuals correlates with cognitive impairment, the levels of LPS do not, suggesting that while LPS is present, it is not entirely responsible for neural cell dysfunction. The authors were interested in exploring how the activated monocytes might influence neural cell function, in particular how exosomes from activated monocytes might cause subtle changes in neural cells. Exosomes normally interact with recipient cells and maintain homeostasis; however, activation not only alters the parent cell but also the exosomes they secrete. Preliminary characterisation of monocyte-derived exosomes showed that when monocytes were stimulated with IFNα, LPS or a combination of the two, there was no difference in exosome size or amount secreted. Because exosomes contain abundant miR, we were interested in comparing the monocyte profile with macrophages. Using miR arrays, we determined that the most abundant miR in monocytes is miR-223, with this miR being decreased when monocytes differentiate into macrophages.[43,44]

We next looked at miRs in activated monocytes and their exosomes using qPCR. Their profiles were similar with the most significant change being an increased abundance of miR146a/b and miR155 in monocytes and exosomes from LPS and I/L treatments.[45] Finally, the impact of activated monocyte-derived exosomes on primary human astrocytes was examined by staining for p65 as an indicator of NF-kB activation. Exosomes from monocytes with no activation showed minimal p65 staining and looked the same as astrocytes not treated with exosomes. Exosomes from IFN-treated monocytes had a strong cytoplasmic p65 response with little to no p65 nuclear translocation. Exosomes from LPS-treated monocytes showed increased cytoplasmic and nuclear translocation of p65; the I/L-activated monocyte exosomes had almost exclusively nuclear p65 staining with little to no cytoplasmic staining. The preliminary interpretation is that IFN-activated exosomes may elicit a protective response whereas IFN in the presence of LPS promote nuclear translocation. The results showed that activated monocytes can secrete exosomes with altered miR profiles and that these exosomes can activate NF-kB. The downstream consequences of this activation are being investigated. These preliminary findings highlight the impact that activated peripheral monocyte exosomes can have on distant neural cells without the parent cell being present.

Conclusions

Experts in the field focused on different aspects of exosome biology in keeping with the NIH priorities outlined by **Drs Joseph** and **Satterlee**. **Dr Bond**'s work revealed that plasma cytokines from infected individuals did not exist in the free form, rather were associated with exosomes. Additionally, cytokines are actively and selectively enriched in plasma exosomes (distinct from virions) from infected individuals. **Dr Buch**'s group found that exosomes released from astrocytes contained abundant levels of various miRs, and that exosomal miRs could be taken up by microglia, resulting in their activation and thereby contributing to neuroinflammation. **Dr Haughey**'s work implicated that EVs regulate leukocyte activation in the peripheral immune system, thereby underscoring the intricacies of bidirectional communication system. **Dr Pulliam**'s findings showed that activated monocytes could secrete exosomes with altered miR profiles and that these exosomes could activate the transcription factor NF-kB in astrocytes. Interestingly, **Dr Kashanchi** presented that exosomes containing TAR RNA could directly affect the proinflammatory cytokine gene expression profile, and that this could explain a possible mechanism of inflammation observed in HIV-1 infected patients. Overall, there were interesting presentations followed by stimulating discussion. All experts agreed that the expanding knowledge concerning exosome biogenesis, composition, function, and use continues to provide new insights into the normal physiology as well as disease pathogenesis. These small vesicles are secreted by many cell types, including nearly all the CNS cells, and are key in regulating cell–cell communication. Such a mechanism can have important implications in the context of disease progression and pathogenesis. This workshop focused mainly on the possible link between exosomes, HIV-1 pathogenesis, HIV-associated disease and drug abuse. The ability to target miRs involved in HIV-1 pathogenesis that are released by the exosomes could provide a novel means of controlling the infection/inflammation, which in turn could mitigate many of the complications associated with neurocognitive disorders. Further study is needed concerning the application of exosome therapeutics, both through these vesicles as a drug delivery mechanism and, as therapeutic targets themselves, in the context of battling HIV-1 infection and its associated CNS complications.

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