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Investigating the role of ankyrin-rich membrane spanning protein in Human Immunodeficiency Virus Type-1 Tat-induced microglia activation

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Abstract

Long-term persistence of Human Immunodeficiency Virus Type-1 (HIV) in the central nervous system (CNS) results in mild to severe neurocognitive impairment in a significant proportion of the HIV infected population. These neurological deficits are known as HIV-Associated Neurocognitive Disorders (HAND). Microglia are CNS resident immune cells that are directly infected by HIV, and consequently secrete proinflammatory molecules that contribute to HIV-induced neuroinflammation. Indeed, the number of activated macrophage and microglia in the brain is more highly correlated with cognitive impairment than the amount of neuronal apoptosis. Ankyrin-rich membrane spanning protein (ARMS/Kidins220) is a multidomain transmembrane protein that is involved with neurotrophin signaling in the CNS. We have previously established the role of ARMS in mediating neuronal survival via a neurotrophin-dependent mechanism. Recent reports also have suggested that ARMS is involved with cell signaling in multiple immune cell types. In this study we aim to investigate the role of ARMS in HIV Tat-mediated microglial cell activation by employing *in vitro* methods. Following ARMS depletion by a lentivirus encoding ARMS-specific shRNA, we observed a marked reduction in the HIV Tat-induced proinflammatory response, associated with loss of tumor necrosis factor alpha production and nuclear factor-kappa B (NF- κ B) activation. Furthermore, co-immunoprecipitation studies suggested that ARMS physically interacts with inhibitory kappa B kinase subunits in order to facilitate NF- κ B activation. Our results establish the role of ARMS in microglial activation by HIV Tat and warrant additional studies to better understand these molecular mechanisms, which may uncover novel therapeutic targets for the treatment of HAND.

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Conflict of Interest

Vir Singh, Alicia Wooten, Joseph Jackson, Sanjay Maggirwar, and Michelle Kieba declare that they have no conflict of interest.

Authors' Contributions

VBS, AKW, JWJ, MK performed the experiments. VBS, SBM, MK participated in the design of the study. VBS, SBM, MK conceived of the study, and participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript. AKW, JWJ, MK, SBM presented preliminary results in the form of an abstract for this project at the 19th Annual Society for NeuroImmune Pharmacology Conference which was published in the Journal of Neuroimmune Pharmacology (Wooten, 2013).

Keywords

ARMS; Microglia; HIV Tat; HAND

Introduction

In the post-highly active antiretroviral therapy (HAART) era, Human Immunodeficiency Virus Type-1 (HIV) patients are living much longer, but despite effective control of the virus, many patients suffer from systemic, chronic inflammation (Deeks *et al*, 2013a; Deeks *et al*, 2013b; Neuhaus *et al*, 2010). This chronic inflammation is responsible for the development of various secondary disorders, including cardiovascular disease (Hsue *et al*, 2012) as well as HIV-associated neurocognitive disorders (HAND) (Gill and Kolson, 2014). Importantly, a recent study showed that HAND persists in more than 50 % of HIV infected subjects (Heaton *et al*, 2010). HAND is further categorized into asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), and HIV associated dementia (HAD) with respective increases in levels of impairment. Fortunately, in the post-HAART era, the mild forms of HAND, ANI and MND, are much more common than HAD, however, it was recently reported that ANI increases the risk for symptomatic decline (Grant *et al*, 2014). HAND is characterized by loss of blood brain barrier (BBB) integrity, synaptodendritic injury, astrogliosis, and microglial activation (Clifford and Ances, 2013), all of which contribute to neuronal damage followed by cognitive deficits and impaired daily functioning. Microglia are the central nervous system (CNS) resident immune cell type (Streit and Kincaid-Colton, 1995), and together with monocyte-derived macrophages, are the major source of productive HIV infection in the brain (Gorry *et al*, 2003; Kaul *et al*, 2005). Indeed, the extent of HIV infection in microglia determines the severity of HAND (Kaul *et al*, 2005; Minghetti *et al*, 2004).

Once infected, microglia release various factors including cytokines, chemokines, prostaglandins, and excitatory amino acids (EAA). Non-infected microglia can be activated by exposure to viral proteins including Tat, or by exposure to soluble factors released by infected cells (Minghetti *et al*, 2004; Yadav and Collman, 2009). A number of studies have suggested that HIV Tat can activate microglia via the nuclear factor-kappa B (NF- κ B) pathway, leading to viral transcription (Jeang *et al*, 1999) as well as the release of various inflammatory cytokines and chemokines (Minghetti *et al*, 2004; Nicolini *et al*, 2001). Notably, many, if not all, viral and cellular factors released from both infected and activated microglia contribute to a neurotoxic environment in the CNS. Altogether, this strongly suggests a central role for microglia in the pathogenesis of HAND.

Ankyrin repeat rich membrane spanning protein (ARMS), also known as Kidins220, hereafter referred to as ARMS, is a conserved integral membrane protein that was initially identified as a substrate for protein kinase D (PKD) in neurons (Iglesias *et al*, 2000). ARMS was then further characterized as the downstream target of neurotrophin- and ephrin-mediated signaling (Kong *et al*, 2001). ARMS is a multi-domain protein; it contains four transmembrane domains and cytoplasmic N- and C-terminals. ARMS contains an N-terminal ankyrin rich domain, a C-terminal PDZ binding motif, a kinesin-1 interacting motif

(KIM), a sterile alpha motif (SAM) domain, and a proline rich domain (Neubrand *et al*, 2012), all of which can facilitate protein-protein interactions. Thus, ARMS has potential for diverse interacting partners and as such may mediate the activation of a variety of signaling cascades. In support of this, ARMS has been shown to interact with all three Trk receptors (Trk A, Trk B, and Trk C), the p75 receptor, and the vascular endothelial growth factor (VEGF) receptor, and plays an important role in neurotrophin and VEGF signaling (Cesca *et al*, 2012; Kong *et al*, 2001). Further, a recent study demonstrated that ARMS can also interact with the T-cell receptor (TCR) to mediate the T-cell response via sustained activation of extracellular signal-regulated kinase (ERK) signaling (Deswal *et al*, 2013). In addition, a report using an ARMS knockout mouse model demonstrated that embryos lacking ARMS expression die at birth due to severe defects in neuronal and vascular development, including cardiac malformation (Cesca *et al*, 2011). Clearly ARMS is essential for life as it functions to integrate multiple signaling cascades.

Our lab was the first to show that ARMS plays an important role in NF- κ B signaling elicited by brain-derived neurotrophic factor (BDNF) in neurons (Sniderhan *et al*, 2008). NF- κ B is a family of dimeric transcription factors that regulate a wide array of cellular functions including cell survival, proliferation, and inflammation (Oeckinghaus and Ghosh, 2009). Depletion of ARMS by ARMS-specific short hairpin RNA (shRNA) or a dominant negative ARMS mutant abolished NF- κ B activation triggered by BDNF. This study suggested ARMS to be a key regulator of neuronal survival and function mediated by neurotrophin receptors. Although ARMS is most strongly expressed in various neuronal cell types (Chen *et al*, 2012; Kong *et al*, 2001), its expression and function have recently been demonstrated in multiple peripheral immune cell types in mice (Li *et al*, 2013; Ni *et al*, 2011) as well as in human T-cells (Deswal *et al*, 2013; Jean-Mairet *et al*, 2011), and dendritic cells (Riol-Blanco *et al*, 2004).

On the basis of these findings we anticipate a broader role for ARMS in HIV-induced neurocognitive impairment, importantly, as a mediator of NF- κ B signaling in microglia. The above-mentioned study performed in our lab (Sniderhan *et al*, 2008) showed that ARMS is important for NF- κ B signaling, thus we aim to investigate the role of ARMS in Tat-induced microglial activation. In the present study we demonstrate that ARMS is expressed in microglia and its depletion via ARMS-specific shRNA inhibits HIV Tat-induced production of tumor necrosis factor alpha (TNF α). We further show that ARMS can physically interact with the inhibitory kappa B kinase (IKK) complex in order to facilitate Tat-induced microglial activation via NF- κ B signaling. Finally, we show that microglial function is impaired in the absence of ARMS as determined by reduced cell viability as well as suppressed CD40 expression. Considering the central role of microglia in the pathogenesis of HAND, a better understanding of the molecular mechanisms underlying HIV-induced microglia activation may lead to novel therapeutic targets for the treatment of HAND.

Methods

Reagents

HIV Tat 1–72 was obtained from Philip Ray (University of Kentucky, Lexington, KY, USA), and was used as previously described (Kiebalá and Maggirwar, 2011; Kiebalá *et al*,

2010). Briefly, this recombinant Tat protein was used at 100 nM (~800 ng/mL) concentration, unless otherwise noted. Soluble Tat levels in HIV patient sera have been previously measured up to 40 ng/mL (Westendorp *et al*, 1995; Xiao *et al*, 2000). It has also been shown that Tat can interact with endogenous glycosaminoglycans and heparan sulfates, thereby potentially lowering its measurable concentration *in vivo* (Chang *et al*, 1997). Therefore, it is likely that Tat concentrations surrounding HIV-infected cells are much higher (Hayashi *et al*, 2006). In addition, Tat's affinity for other proteins and glass/plastic, and its temperature susceptibility, make it impossible to accurately determine what fraction of the initial Tat dose actually reaches the experimental specimen, thus likely leading to an underestimation of Tat functions *in vitro* (Nath *et al*, 2000). Finally, Tat's effects *in vivo* are likely to occur over long-term chronic exposures. Chronic, low dose *in vivo* effects of any reagent are often appropriately modeled *in vitro*, by proportionately higher doses of that same reagent, over more acute time frames. For these reasons, and in order to be consistent with previous experiments from our laboratory, and others, we use 100 nM Tat for these experiments, which is at the lower end of the Tat dose range seen with many comparable studies, and which many studies have found to be an appropriate Tat dose by which to model Tat's *in vivo* effects *in vitro*.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polybrene and protein A/G+ agarose beads were purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA, USA). Hoechst 33342 was purchased from Life Technologies (Grand Island, NY, USA).

Cell culture

The murine microglial cell-line (BV-2) was obtained from Dr. R. Donato (University of Perugia, Perugia, Italy) (Blasi *et al*, 1990). The BV-2 cultures used in our experiments were 100 % positive for the microglia marker, mouse CD68 (macrosialin), as determined by flow cytometric analyses using Alexa Fluor® 647-conjugated anti-CD68 antibody (0.025 µg per 2.5×10⁵ cells in 100 µL volume; BioLegend Inc., San Diego, CA, USA; #137003). Human embryonic kidney 293 T (HEK 293 T) cells were obtained from Invitrogen/Life Technologies (Grand Island, NY, USA). BV-2 cells and HEK 293 T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % fetal bovine serum (FBS), 2 mM glutamine, and antibiotics, by standard procedures. Human microglial cells that were isolated from fetal human brain were obtained from Clonexpress (Gaithersburg, MD, USA) and were maintained in 50:50 DMEM:F-12 supplemented with 5 % FBS and 10 ng/mL of M-CSF.

Lentivirus production and BV-2 cell transduction

HIV-based pseudotyped lentivirus vectors encoding shRNA targeted to rat ARMS transcripts (nucleotides 1027–1045) were generated by placing an oligonucleotide sequence 5'-GCCACCAAGATGAGAAATA-3' under the control of the human U6 RNA polymerase III promoter (Figure 1B), using FG12 lentiviral vector (gift of Dr. David Baltimore), as described (Qin *et al*, 2003; Sniderhan *et al*, 2008). Briefly, HEK 293 T cells were transiently transfected using polyethylenimine (PEI) and a mixture of FG12 transfer plasmid containing

ARMS shRNA (or FG12 transfer plasmid without ARMS shRNA as a control), packaging plasmid pCMV4- 89.2, and VSV-G envelope plasmid in a 2.5:2.5:1 ratio. Lentiviral particles produced in the transfected cultures were collected at 48 h and 72 h post-transfection. Cell debris was removed via centrifugation at 1200 rpm for 10 minutes. To obtain a concentrated virus stock the supernatant was subjected to ultracentrifugation at 30,000 rpm for 2 h at 4 °C using a Beckman Coulter SW32 rotor (Indianapolis, IN, USA). Lentiviral particles were resuspended in a small volume (< 1 % of original volume) of serum-free DMEM. Titration of the virus was performed in BV-2 cells transduced with 10-fold serial dilutions, and virus titer was determined at 48 h post-transduction by counting the number of GFP-positive cells using flow cytometry. Transduction of BV-2 cells was initiated by adding virus at multiplicity of infection (MOI) 10, 50, 100 to the cells in the presence of 5 µg/mL polybrene (Santa Cruz Biotechnologies Inc., Santa Cruz, CA, USA).

ELISA

TNFα levels were measured in culture supernatants (pre-cleared by brief centrifugation) using a mouse TNFα ELISA kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions, as previously described (Kiebalá and Maggirwar, 2011; Kiebalá et al, 2010; Sui et al, 2007). This kit has a minimum sensitivity threshold of 8 pg/mL. Briefly, 100 µL of cell culture supernatant was incubated in a 96-well plate pre-coated with a TNFα-specific monoclonal antibody for 1.5 h. After extensive washing, binding of TNFα was detected by incubation with biotinylated antibodies, followed by streptavidin-peroxidase; colorimetric enzyme assays were performed to detect bound TNFα.

Viability assay

BV-2 cells (2.5×10^4) were plated in a 48-well plate and were transduced with increasing amounts (MOI 10, 50, 100) of either ARMS shRNA expressing lentivirus, or FG12 control lentivirus for 72 h. The MTT assay was performed as previously described (Kiebalá and Maggirwar, 2011). Briefly, 10X MTT was added to the wells and the cells were incubated for an additional 1 h at 37 °C. After removal of the media, MTT solvent (4 mM HCl, 0.1 % NP40, in isopropanol) was added to dissolve the formazan dye. The optical density was measured at 570 nm and the percent cell survival was determined compared to the non-transduced (NT) sample.

Immunoblot analysis

Immunoblot analysis was performed as previously described (Kiebalá and Maggirwar, 2011; Kiebalá et al, 2010; Sui et al, 2007). Following the indicated treatments, whole cell lysates were prepared in ELB buffer (50 mM HEPES (pH 7), 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 10 mM NaF, 0.1 mM Na₃VO₄, 50 µM ZnCl₂, supplemented with 0.1 mM PMSF, 1 mM DTT, and a mixture of protease and phosphatase inhibitors). Cellular debris was removed by high-speed centrifugation. Lysates were fractionated on 7.5 % SDS-PAGE gels and protein was electrophoretically transferred to Hybond ECL nitrocellulose membrane (GE Healthcare Bio-Sciences Corporation, Piscataway, NJ, USA). The membranes were analyzed for immunoreactivity with primary antibodies raised against IκBα (1:1000; # sc-371), RelA (1:1000; # sc-8008), RelB (1:1000; # sc-226), CD40 (1:1000; # sc-9096), or α-Tubulin (1:1000; # sc-8035; all from Santa Cruz Biotechnologies

Inc., Santa Cruz, CA, USA), or RelA P-S536 (1:1000; # 3033; from Cell Signaling Technology, Danvers, MA, USA). Polyclonal antibodies to ARMS were raised against the C-terminus region (amino acids 1535–1715) (1:1000; from Cocalico Biologicals, Inc., Reamstown, PA, USA) (Sniderhan *et al.*, 2008). Bound antibodies were detected by species-specific, IRDye-conjugated secondary antibodies (1:20,000, LI-COR BioSciences, Lincoln, NE, USA). Membranes were then subjected to Odyssey infrared imaging (LI-COR BioSciences, Lincoln, NE, USA).

Indirect immunofluorescence analysis

BV-2 (1.2×10^5) cells were grown on chamber slides for 18 h. Cells were transduced with ARMS shRNA expressing or FG12 control lentiviral particles at MOI 100 for 48 h followed by 1 h 100 nM Tat treatment. Subsequently, cells were fixed and stained as described (Sui et al., 2007). Briefly, cells were fixed with 4% formaldehyde for 10 min., followed by washing in phosphate buffered saline (PBS). For intracellular staining, cell membranes were permeabilized with 0.1 % Triton X-100 in PBS. Cells were treated with 5 % FBS for 60 min. (to eliminate nonspecific binding) followed by overnight incubation with anti-RelA antibody (1:100; # sc-372; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were washed with PBS and incubated with the appropriate Alexa Fluor® secondary antibody (Life Technologies, Grand Island, NY, USA), for 60 min. After washing in PBS, Hoechst 33342 (Life Technologies, Grand Island, NY, USA) was added at 0.2 µg/mL to visualize cell nuclei and immunofluorescence was observed with fluorescent microscopy.

Transient transfection

HEK 293 T cells were transfected with plasmid DNA expressing full-length ARMS alone or in combination with plasmid DNA expressing IKK α , β , or γ using Lipofectamine LTX (Life Technologies, Grand Island, NY, USA). Cells were seeded at 1.8×10^5 cells/well in 24-well plates 18 h prior to transfection. Each transfection reaction contained a total of 1 µg plasmid DNA. After addition of the transfection mixture to the cells, they were incubated at 37 °C for 48 h.

BV-2 cells were transfected with a plasmid expressing the HIV long terminal repeat (LTR) region upstream of a luciferase reporter alone or in combination with FG12 vector expressing ARMS-specific shRNA or the empty FG12 vector as a control using Nucleofector (Amaxa/Lonza, Basel, Switzerland; Walkersville, MD, USA), as described (Kiebal and Maggior, 2011). For nucleofection, 10×10^6 cells were transfected with 10 µg total plasmid DNA. Transfected cells were plated at 1.0×10^5 cells/well in a 48-well plate and incubated at 37 °C for 24 h prior to treatment. Media was changed 4 h after transfection and again prior to treatment to reduce cell toxicity.

Luciferase assay

A luciferase reporter plasmid containing the HIV LTR region upstream of a firefly luciferase gene alone or in combination with FG12 vector expressing ARMS-specific shRNA or the empty FG12 vector as a control were transfected into BV-2 cells using Nucleofector (Amaxa/Lonza, Basel, Switzerland; Walkersville, MD, USA). Twenty-four hours post-transfection, cells were either left untreated or were incubated for 8 h with 100 nM Tat.

Luciferase assays were performed as described (Kiebala and Maggirwar, 2011; Kiebala et al, 2010). In brief, cell lysates were prepared using reporter lysis buffer (Promega Life Sciences, Madison, WI, USA), and luciferase activity was measured with a SpectraMax M3 Plate Reader (Molecular Devices, Sunnyvale, CA). In these assays, total protein amount as determined by Bradford assay (Bio-Rad, Hercules, CA, USA), was used to normalize the samples.

Immunoprecipitation

Immunoprecipitation was performed as previously described (Kiebala et al, 2010). Samples containing equal amounts of protein were pre-cleared by incubation with 2 µg of normal rabbit serum and 20 µL of a 50/50 protein A/G+ agarose beads solution (both from Santa Cruz Biotechnologies Inc., Santa Cruz, CA, USA) at 4 °C on a rotator for 1 h. After this pre-clear, the beads were removed by centrifugation and samples were transferred to tubes with IKK α/β specific antibody (2 µg per sample; # sc-7607; Santa Cruz Biotechnologies Inc., Santa Cruz, CA, USA). Samples were incubated with the antibodies at 4 °C for 18 h on a rotator. Thirty µL of 50/50 protein A/G+ agarose beads were added to the samples, after which they were incubated with rotation for an additional 2 h at 4 °C. The beads were then washed extensively with ELB lysis buffer supplemented with 0.1 mM PMSF, 1 mM DTT, and a mixture of protease and phosphatase inhibitors, and then boiled for 5 minutes in 20 µL of Laemmli buffer. Immunocomplexes were separated using SDS-PAGE (10 %), transferred to a nitrocellulose membrane, and probed with the indicated antibodies as described above.

Statistical analysis

Mean data values and standard error of the mean (SEM) were calculated for each variable. One-way ANOVA followed by Bonferroni's test for multiple comparisons was used to analyze data involving the analysis of multiple sample groups. A value of $p < 0.05$ was designated as statistically significant. These analyses were performed using Prism 4.0 software (GraphPad Software, La Jolla, CA, USA).

Results

ARMS expression and subsequent depletion in microglial cells

The expression and function of ARMS has been well characterized in neurons as well as during development, both of the CNS and of the cardiovascular system (Cesca *et al*, 2011; Kong *et al*, 2001). However, although ARMS is a conserved protein that is expressed in many different tissue types (Kong *et al*, 2001), little is known about the role of ARMS in non-neuronal cells. Very recently ARMS has been described to be important in immune cell types including dendritic cells and T-cells (Deswal *et al*, 2013; Jean-Mairet *et al*, 2011; Riol-Blanco *et al*, 2004). Therefore, in order to characterize the role of ARMS in microglia, which are the tissue resident immune cells of the CNS and which significantly contribute to HIV-induced neuroinflammation, we first looked for ARMS expression via immunoblot analysis. These analyses revealed ARMS protein expression in both primary human microglia and in a mouse microglial cell line, BV-2 cells (Fig. 1a). The two samples were run in non-adjacent lanes on the same gel, and the total amount of protein and exposure

times were not equal between the two samples, therefore expression levels of ARMS should not be compared.

We then sought to deplete ARMS expression in BV-2 cells using the FG12 lentiviral vector system expressing ARMS-specific shRNA that was previously developed in our laboratory (Sniderhan *et al.*, 2008). Briefly, the ARMS shRNA cassette was placed under the U6 promoter derived from human RNA polymerase III, and an enhanced green fluorescent protein (eGFP) gene was controlled separately by the ubiquitin C promoter (Fig. 1b). Following production and concentration of both ARMS shRNA-expressing and GFP-expressing empty FG12 lentiviruses, BV-2 cells were transduced at MOI 100 for 72 h resulting in > 90 % transduction efficiency as visualized by GFP expression via fluorescence microscopy (Fig. 1c). Under these conditions, expression of ARMS-specific shRNA resulted in efficient depletion of ARMS levels in BV-2 cells, whereas α -Tubulin levels were unchanged (Fig. 1d). Altogether, these data suggest that microglial cells do express ARMS and that lentiviral delivery of ARMS-specific shRNA greatly reduces ARMS expression in BV-2 cells.

ARMS depletion inhibits HIV Tat-induced NF- κ B activation

As a transmembrane adaptor protein, ARMS facilitates the activation of many downstream signaling pathways, including mitogen activated protein kinase (MAPK) and NF- κ B (Neubrand *et al.*, 2012). Indeed, our laboratory was the first to show that ARMS plays a critical role in NF- κ B signaling in neurons and that this is necessary for BDNF-mediated neuronal survival (Sniderhan *et al.*, 2008). After showing that microglial cells do express ARMS, and considering that NF- κ B activation plays a large role in HIV Tat-induced microglial activation (Minghetti *et al.*, 2004; Nicolini *et al.*, 2001), we sought to determine the importance of ARMS in this process. To this end, a variety of methods to assess NF- κ B activation in BV-2 cells following Tat treatment were employed. First, using immunofluorescence analysis (IFA) we evaluated the cellular distribution of the NF- κ B protein, RelA, following 1 h Tat treatment of BV-2 cells. Upon stimulation, RelA is released from its complex with the inhibitory kappa B protein (I κ B α) in the cytoplasm, resulting in its nuclear localization. As shown in Fig. 2a, we observed nuclear accumulation of RelA as evidenced by its overlap with the nuclear dye Hoechst following Tat treatment in FG12 control lentivirus transduced cells. In contrast, the Tat-induced nuclear accumulation of RelA was considerably reduced in ARMS shRNA lentivirus transduced cells. As mentioned above, NF- κ B activation results in I κ B α proteasomal degradation followed by its re-synthesis as a negative regulatory feedback mechanism, therefore we next assessed I κ B α protein levels via immunoblot analysis following short-term Tat treatments. In FG12 lentivirus transduced BV-2 cells there was an 88 % reduction in I κ B α levels by 30 minutes of Tat treatment and complete recovery of I κ B α levels by 1 h of Tat treatment. However, in ARMS shRNA lentivirus transduced BV-2 cells there was only a 40 % reduction in I κ B α levels by 1 h of Tat treatment (Fig. 2b). We also confirmed that α -Tubulin levels were unchanged between samples and that ARMS was indeed depleted following 72 h ARMS shRNA lentivirus transduction (Fig. 2b).

As a transcription factor NF- κ B regulates the expression of a wide array of genes. One such gene is RelB (Bren *et al*, 2001), which is itself a member of the NF- κ B family. Therefore, to further assess the activation status of NF- κ B following 8 h Tat treatment we used immunoblot analysis to evaluate RelB expression levels. In FG12 lentivirus transduced cells there was a 2.5 fold Tat-induced increase in RelB expression as compared to non-treated cells, and ARMS depletion completely abolished this Tat-induced increase in RelB expression (Fig. 2c). Additionally, α -Tubulin levels were unchanged and ARMS depletion at 72 h post-transduction was confirmed (Fig. 2c). Based on multiple measures of NF- κ B activation including I κ B α degradation, RelA nuclear accumulation, and target gene (RelB) expression, all of which were greatly reduced following ARMS depletion, it appears that ARMS is important for Tat-induced NF- κ B activation in BV-2 cells.

ARMS physically interacts with IKK α/β to facilitate Tat-induced NF- κ B activation

Activation of the inhibitory kappa B kinase (IKK) complex is a key regulatory step within the NF- κ B signaling pathway (Oeckinghaus *et al*, 2011). This complex consists of IKK α and IKK β , both of which have kinase activity, and IKK γ (NEMO), which plays a critical structural/regulatory role. Upon activation, IKK α/β phosphorylate I κ B α leading to its ubiquitination and degradation, which frees NF- κ B heterodimers to enter the nucleus and regulate transcription of target genes. Our previous report in rat cortical neurons showed decreased IKK activity in response to BDNF treatment following ARMS depletion (Sniderhan *et al*, 2008). Likewise, herein, in BV-2 microglial cells in the absence of ARMS we detected a significant reduction in the level of phosphorylated RelA at serine 536, which is an IKK-sensitive site, while total RelA and α -Tubulin levels were unchanged (Fig. 3a). Interestingly, the activity of the IKK complex downstream of receptors including the tumor necrosis factor receptor (TNFR) as well as the TCR is facilitated through its interaction with a macromolecular complex termed the IKK signalosome (Ghosh and Karin, 2002; Kovalenko and Wallach, 2006). Based on this, together with the transmembrane/adaptor nature of ARMS, we tested whether IKK could interact with ARMS using co-immunoprecipitation. In HEK 293 T cells over-expressing IKK (either α , β , or γ) together with ARMS, there was a clear interaction between IKK α/β and ARMS (Fig. 3b). We also confirmed over-expression of ARMS and equal protein input (α -Tubulin blot) via immunoblot analysis (Fig. 3b). Finally, we wanted to confirm that this interaction was occurring in BV-2 cells in response to Tat. To do this we treated BV-2 cells over a short time course with Tat and subsequently pulled down with an IKK α/β antibody and looked for an interaction between ARMS and IKK via immunoblot analysis. Indeed, ARMS and IKK did interact, and this was increased following 30 minutes Tat treatment (Fig. 3c). In addition, we also saw increased ARMS/IKK interaction following 15 minutes treatment with LPS (Fig. 3c). Taken together, these data suggest that ARMS facilitates Tat-induced NF- κ B activation in microglial cells through physical interaction with IKK α/β , and that this interaction may also play a role in microglial activation induced by other pro-inflammatory stimuli, such as LPS.

Microglial function is impaired following ARMS depletion

As mentioned, microglia are the resident CNS immune cells, and their infection and activation by HIV virions and proteins play a large role in the pathogenesis of HAND.

Based on this, and on the apparent importance of ARMS in Tat-induced microglial activation, we next examined microglial function following ARMS depletion in BV-2 cells. We first measured TNF α levels via ELISA following 72 h transduction with either FG12 control or ARMS shRNA expressing lentivirus, and 8 h treatment with both Tat and LPS. With reduced ARMS expression, Tat- and LPS-induced TNF α release was significantly reduced as compared to the control vector (Fig. 4a). In addition to cytokine/chemokine release, microglia also up-regulate certain cell surface molecules upon activation. One such molecule is CD40, which is the receptor for CD40 ligand. Our lab has previously shown that CD40 expression is up-regulated in both BV-2 cells and primary human microglia following exposure to Tat (Sui *et al.*, 2007). Here, we also observed increased CD40 expression following Tat treatment in FG12 lentivirus transduced BV-2 cells; however, ARMS depletion abrogated this effect (Fig. 4b).

In order to test the specificity of the role of ARMS in Tat-induced microglial activation, we employed a luciferase assay using an HIV LTR-luciferase reporter. Upon HIV infection, following production of the HIV Tat protein, Tat binds to the transactivation response element (TAR) in the HIV genome and thus initiates efficient transcription of HIV gene products. This process involves the recruitment of multiple host factors, including RNA polymerase II, as well as signaling complexes such as Sp1 and NF- κ B (Jeang *et al.*, 1999). Following ARMS depletion, we did not see a significant reduction in Tat-induced activation of the HIV LTR, as measured by HIV LTR-driven luciferase activity (Fig. 4c). This suggests that the role of ARMS in Tat-induced microglial activation is specific for NF- κ B, and that the reduction in NF- κ B activation following ARMS depletion is not sufficient to overcome the contribution of multiple other host factors involved in HIV LTR activation. It is also possible that because we do not see complete knock-down of ARMS, the residual amount of NF- κ B activity remaining is enough to contribute to efficient transcription of the HIV genome.

Finally, we assessed cell survival using the MTT assay in BV-2 cells with and without ARMS depletion. With increasing amounts of the ARMS shRNA expressing lentivirus at 72 h post-transduction, there was decreased cell survival compared to the FG12 transduced sample. At MOI 100, cell viability was reduced by approximately 30 % (Fig. 4d). This is not surprising, especially considering that ARMS is important for NF- κ B signaling, and NF- κ B is important for cell survival. In summary, ARMS is important for HIV Tat-induced microglial activation and also appears to play a role in the survival of these cells.

Discussion

Microglial cells are the principal component of the brain's intrinsic immune system and they respond to a variety of tissue insults, mainly through the release of an array of inflammatory cytokines and chemokines (Streit and Kincaid-Colton, 1995). HIV can productively infect microglia, and these infected cells then release viral proteins, including Tat, as well as various inflammatory cytokines and chemokines that can activate neighboring cells, thus causing an inflammatory cascade that generates a neurotoxic environment in the CNS (Gorry *et al.*, 2003; Streit and Kincaid-Colton, 1995). As such, microglia play a key role in HIV induced neuroinflammation and their contribution to CNS inflammation is a major

pathogenic factor in HAND. Indeed, markers of microglia/macrophage activation can be used as biomarkers for HAND (Marcotte *et al*, 2013).

The process of microglial activation involves multiple signaling pathways including MAPK, specifically ERK1/2 (D'Aversa *et al*, 2004), and NF- κ B (Nicolini *et al*, 2001). Importantly, NF- κ B signaling in microglia mediates the release of various cytokines and chemokines, such as TNF α (Minghetti *et al*, 2004), as well as the expression of NF- κ B proteins themselves, such as RelB (Kiebała *et al*, 2010), which helps to limit microglial over-activation. However, to date, the signaling mechanisms upstream of MAPK and NF- κ B activation have not been investigated in detail. It remains unclear as to which upstream receptors and adaptor proteins are involved in HIV Tat-induced activation of MAPK and subsequent transduction of NF- κ B signaling in microglia. In our earlier report we dissected neurotrophin signaling in neurons and identified the adaptor protein, ARMS, to play a critical role in mediating BDNF-induced NF- κ B signaling (Sniderhan *et al*, 2008). As an extension of this notion, in this study we investigated the role of ARMS in mediating HIV Tat-induced microglial activation via NF- κ B signaling.

Our results clearly indicate that ARMS protein is expressed in microglial cells and that ARMS mediates Tat-induced NF- κ B activation, as depletion of ARMS protein by ARMS-specific shRNA abolished NF- κ B activation. However, the mechanism of how HIV Tat enters microglial cells is not yet clearly understood. Earlier studies have demonstrated multiple modes of entry for Tat, including receptor-mediated entry and direct entry by endocytosis. In neurons, HIV Tat can enter cells through interaction with the low-density lipoprotein receptor-related protein (LRP). Tat bound to LRP on the neuronal plasma membrane can be efficiently internalized and localized into the nucleus in its biologically active form (Kim *et al*, 2008; Liu *et al*, 2000). Additionally, endocytosis of HIV Tat can be mediated by clathrins, where low endosomal pH and cytosolic Hsp90 play key roles in regulating Tat's translocation (Vendeville *et al*, 2004). Furthermore, the highly charged, arginine-rich region of Tat can facilitate Tat's entry into cells independent of a specific receptor or endocytosis (Nakase *et al*, 2008). It is still unclear whether in microglial cells Tat interacts with ARMS protein directly or via any specific receptor, as additional experiments are required to address this question. However, in this study, we have successfully demonstrated that HIV Tat's interaction with microglial cells induced the interaction of ARMS with the IKK complex, which in turn caused the induction of NF- κ B activation. Knock down of ARMS reduced this effect. Thus, our findings clearly indicate that ARMS plays a key role in HIV Tat induced microglial activation. In addition, this study prompts the need for further investigations into the ARMS:IKK interaction in other CNS cell types, such as astrocytes and microvascular endothelial cells upon HIV infection, as to our knowledge no studies to date have examined the role of ARMS in the function of these cell types.

ARMS is an adaptor protein that has not been fully characterized, as NCBI PubMed only lists approximately 53 papers related to ARMS structure and function. Based on the conserved nature of ARMS together with its expression in many different tissue and cell types (Kong *et al*, 2001), ARMS is likely involved in many more cellular functions beyond what has been described. Interestingly, we observed that Tat-induced CD40 expression was abolished in the absence of ARMS. In this instance we used CD40 as a marker for

microglial activation as has been previously described (Sui *et al*, 2007). However, this data also suggests that CD40 signaling might involve ARMS, which is particularly of interest considering that CD40-CD40L signaling also plays a significant role in HIV Tat-induced neuroinflammation (Davidson *et al*, 2012; Sui *et al*, 2007).

ARMS is also important for cell survival. Our previous report implicated ARMS as essential for BDNF-mediated protection from HIV glycoprotein (gp120)-induced neuronal apoptosis (Sniderhan *et al*, 2008). In addition, ARMS expression was detected in neuroblastoma tumors and found to mediate stabilization of neurotrophin signaling providing survival advantages (Rogers and Schor, 2013). Earlier, Liao *et al*, 2007 demonstrated higher levels of ARMS in primary and metastatic melanoma as compared to benign melanoma cases. Furthermore, depletion of ARMS in a murine melanoma cell line (B16F0) resulted in inhibition of growth in soft agar as well as growth of melanoma in severe combined immunodeficient mice. This study suggested that ARMS expression supports melanoma formation by preventing apoptotic cell death mediated by the MEK/ERK signaling pathway (Liao *et al*, 2007). Similarly, we observed a significant decrease in the survival of BV-2 cells after depletion of ARMS. Thus, our findings strengthen the role of ARMS in providing survival advantages, perhaps via NF- κ B signaling.

Unfortunately, since ARMS merely works as an adaptor molecule that lacks kinetic activity, it is a poor therapeutic target. However, herein we have clearly established the importance of ARMS in Tat-induced microglial activation through its role in NF- κ B activation, and NF- κ B has multiple functions that can be targeted with therapeutic potential. Admittedly, NF- κ B is difficult to therapeutically target in humans because of its importance in vital processes such as proliferation, survival, and inflammation (Oeckinghaus and Ghosh, 2009). Furthermore, NF- κ B is involved in these processes in many different cell types, in addition to activated microglia. Nevertheless, recent reports from our group have indicated that mild inhibition of NF- κ B, both pharmacologically via Ibudilast (Kiebala and Maggirwar, 2011), and using cellular production of RelB (Kiebala *et al*, 2010), has proven to provide protection against Tat-induced over-activation of microglia.

Overall, in this study we have shown that ARMS is expressed in microglia and that it mediates HIV Tat-induced activation of NF- κ B signaling through interaction with the IKK complex. To our knowledge, this is the first report demonstrating a physical interaction between ARMS and the IKK complex. ARMS depletion in BV-2 cells resulted in inhibition of HIV Tat-induced NF- κ B activation, as shown by inhibition of I κ B α degradation as well as restriction of NF- κ B subunits within the cytoplasmic compartment. ARMS expression was found to be indispensable for HIV Tat-mediated microglial activation, TNF α release, CD40 expression, and cell survival. These findings, together with additional studies in the future, may potentially establish ARMS, and the downstream signals that it facilitates, as functional therapeutic targets for the treatment of HAND, which is particularly important considering that successful adjunctive therapies for HAND are currently lacking.

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Fig. 1. ARMS expression and lentivirus-mediated knock-down in microglial cells

(a) Whole cell lysates prepared from BV-2 cells or primary human microglia were subjected to immunoblot analysis using either ARMS-specific (top panel) or α -Tubulin-specific (bottom panel) antibodies. The two samples were run in non-adjacent lanes on the same gel, and the total amount of protein was not equal in the two samples, therefore expression levels of ARMS should not be compared. (b) The diagram depicts the method for generation of a lentiviral vector containing ARMS shRNA-GFP. The ARMS shRNA cassette was sub-cloned into a plasmid (pBluescript) under the control of the human U6 RNA polymerase III promoter. The plasmid region containing the cassette and the promoter was subsequently sub-cloned into the FG12 lentiviral vector, which also encodes for GFP under the control of the Ubiquitin C (UbiC) promoter. The nucleotide sequence within the rat ARMS mRNA sequence that is targeted by the shRNA is indicated. (c) Analysis of the efficiency of lentiviral transduction of BV-2 cells. Cells were transduced with FG12 control (top panels) or ARMS shRNA-GFP lentivirus (bottom panels) (MOI 100). The panels on the left show the bright field, whereas the panels on the right indicate GFP-positive cells in the same field of view following fluorescence microscopy at 72 h post-transduction. (d) Whole cell lysates prepared from BV-2 cells transduced for 72 h with FG12 control or ARMS shRNA lentivirus at the indicated MOI were subjected to immunoblot analysis using either ARMS-specific (top panel) or α -Tubulin-specific (bottom panel) antibodies.

Fig. 2. ARMS is necessary for Tat-induced NF- κ B activation

(a) BV-2 cells were transduced with either FG12 control lentivirus (upper two panels) or ARMS shRNA expressing lentivirus (lower two panels) at MOI 100 for 48 h. The cells were then either left untreated (NT) or were treated with Tat (100 nM) for 1 h. Subsequently, cells were fixed with 2% paraformaldehyde and were subjected to indirect immunofluorescence analysis using a primary antibody specific to RelA. Cells were incubated with the appropriate Alexa Fluor® secondary antibody for 60 min. Hoechst dye was used to visualize nuclei. GFP expression indicates cells that were transduced with lentivirus.

Immunofluorescence was observed via fluorescent microscopy. The magnified zoom field shows the highlighted region with the RelA and Hoechst images overlayed. (b) BV-2 cells were transduced with either FG12 control lentivirus or ARMS shRNA expressing lentivirus at MOI 100 for 72 h. Cells were then either left untreated (NT) or were treated with Tat (100 nM) for the indicated amount of time. Whole cell lysates were subjected to immunoblot analysis using either ARMS-specific (top panel), I κ B α -specific (center panel), or α -Tubulin-specific (bottom panel) antibodies. (c) Whole cell lysates prepared from BV-2 cells transduced for 72 h with FG12 control or ARMS shRNA lentivirus (MOI 100), and subsequently left untreated (NT) or treated with Tat (100 nM) for 8 h, were subjected to immunoblot analysis using either ARMS-specific (top panel), RelB-specific (center panel), or α -Tubulin-specific (bottom panel) antibodies. The graph indicates quantification of RelB/Tubulin bands from three independent experiments. Statistical significance (***, $p < 0.001$) is denoted as compared to FG12 NT samples.

Fig. 3. ARMS facilitates Tat-induced NF- κ B activation through physical interaction with IKK
(a) BV-2 cells were transduced with either FG12 control lentivirus or ARMS shRNA expressing lentivirus at MOI 100 for 72 h. Whole cell lysates were subjected to immunoblot analysis using either phospho-RelA Ser536-specific (top panel), total RelA-specific (second panel), ARMS-specific (third panel), or α -Tubulin-specific (bottom panel) antibodies. The graph indicates quantification of p-RelA S536/RelA/Tubulin bands from four independent experiments. Statistical significance (***, $p < 0.001$) is denoted as compared to FG12 samples. **(b)** HEK 293 T cells were transfected with plasmids expressing IKK α , β , or γ either alone or together with a plasmid expressing full-length ARMS for 48 h. Whole cell lysates were subjected to immunoprecipitation (IP) using an antibody that recognizes both IKK α/β . The immunoprecipitated complexes were then subjected to immunoblot (IB) analysis with an ARMS-specific antibody (top panel). The input whole cell lysates were subjected to immunoblot analysis with ARMS-specific (center panel) or α -Tubulin-specific (lower panel) antibodies. **(c)** BV-2 cells were treated with either Tat or LPS at the indicated concentrations for the indicated periods of time. Whole cell lysates were subjected to

immunoprecipitation (IP) using an antibody that recognizes both IKK α / β . The immunoprecipitated complexes were then subjected to immunoblot (IB) analysis with an ARMS-specific antibody.

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Fig. 4. Knock-down of ARMS results in inhibition of microglial function

(a) BV-2 cells were transduced with FG12 control lentivirus or ARMS shRNA expressing lentivirus at MOI 100 for 72 h. The cells were then left untreated (NT) or were treated with Tat or LPS at the indicated concentrations for 8 h. Subsequently, culture supernatants were collected and TNFα levels were measured via ELISA. Results are representative of three replicates from a single experiment that was repeated three times. Statistical significance (***, $p < 0.001$) is denoted as compared to FG12 samples. (b) BV-2 cells were transduced with either FG12 or ARMS shRNA expressing lentivirus as described above. Cells were then left untreated (NT) or were treated with Tat (100 nM) for 8 h. Whole cell lysates were subjected to immunoblot analysis using CD40-specific (top panel), or α-Tubulin-specific (bottom panel) antibodies. (c) BV-2 cells were transfected with a plasmid encoding the HIV LTR upstream of a luciferase reporter (HIV LTR-Luc) alone or together with a plasmid expressing ARMS-specific shRNA or the empty FG12 plasmid as a control. Twenty-four hours post-transfection cells were left untreated (NT) or were treated with Tat (100nM) for 8 h. Whole cell lysates were subjected to luciferase assay analysis. Data are presented as fold change in relative light units (RLU) as compared to the HIV LTR-Luc NT sample. Results are combined from three replicates from a single experiment that was repeated twice. Statistical significance (*, $p < 0.05$) is denoted as compared to the HIV LTR-Luc NT sample.

(d) BV-2 cells were transduced as described above at the indicated MOI for 72 h. Following this, cell viability was assessed using the MTT assay. Data are presented as % viability as compared to non-transduced (NT) cells. Results are representative of three replicates from a single experiment that was repeated two times. Statistical significance (***, $p < 0.001$) is denoted as compared to NT cells, or as compared to the equivalent FG12 sample (##, $p < 0.01$).

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