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Macrophage Delivery of Nanoformulated Antiretroviral Drug to the Brain in a Murine Model of NeuroAIDS

Huanyu Dou,* Cassi B. Grotepas,* JoEllyn M. McMillan,* Christopher J. Destache,‡ Mahesh Chaubal,§ Jane Werling,§ James Kipp,§ Barrett Rabinow,§ and Howard E. Gendelman2,*‡

Antiretroviral therapy (ART) shows variable blood-brain barrier penetration. This may affect the development of neurological complications of HIV infection. In attempts to attenuate viral growth for the nervous system, cell-based nanof ormulations were developed with the focus on improving drug pharmacokinetics. We reasoned that ART carriage could be facilitated within blood-borne macrophages traveling across the blood-brain barrier. To test this idea, an HIV-1 encephalitis (HIVE) rodent model was used where HIV-1-infected human monocyte-derived macrophages were stereotactically injected into the subcortex of severe combined immunodeficient mice. ART was prepared using indinavir (IDV) nanoparticles (NP, nanoART) loaded into murine bone marrow macrophages (BMM, IDV-NP-BMM) after ex vivo cultivation. IDV-NP-BMM was administered i.v. to mice resulting in continuous IDV release for 14 days. Rhodamine-labeled IDV-NP was readily observed in areas of HIVE and specifically in brain subregions with active astrogliosis, microgliosis, and neuronal loss. IDV-NP-BMM treatment led to robust IDV levels and reduced HIV-1 replication in HIVE brain regions. We conclude that nanoART targeting to diseased brain through macrophage carriage is possible and can be considered in developmental therapeutics for HIV-associated neurological disease. The Journal of Immunology, 2009, 183: 661–669.

In its most significant form, HIV-associated neurocognitive disorders, are defined as cognitive, motor, and/or behavioral impairments. These are linked to progressive viral infection and immune deterioration (1). A substantive pathogenic event for disease is the infiltration of blood-borne mononuclear phagocytes (MP; monocytes, tissue macrophages, and microglia) into affected brain tissue. This accelerates viral dissemination in brain precipitating productive HIV replication and the subsequent formation of macrophage-derived multinucleated giant cells (MGC) (2–4). We reasoned that as the vehicle for virus carriage into the nervous system, MP could also be harnessed as an antiretroviral drug carrier (5, 6). In this way, drug-loaded blood-borne macrophages would cross the blood-brain barrier (BBB) into diseased brain subregions and release antiretroviral drugs serving to improve its efficacy. The importance of this strategy is bolstered by antiretroviral therapy (ART) known to reduce HIV-associated neurocognitive disorder severity. Indeed, HIV patients on ART live longer and neurological dysfunctions are reduced, showing a mixture of more mild disease with reduced viral replication (7–11). All together, improving ART BBB penetration could positively affect disease outcomes and, as such, be an integral part of HIV treatments targeting the nervous system (8, 12–15).

Current ART limitations are due to its inability to combat viral mutation and achieve continuous, effective drug levels in virus target tissues (12, 16–18). Indeed, resistance to antiretroviral compounds can and often does develop and when present HIV-1 levels can rapidly rebound to pretreatment concentrations if ART is discontinued (19–22). Such effects might be attenuated if optimal ART transport across tissue barriers could be achieved. One impediment in reaching this goal is the BBB. This tissue barrier serves to restrict macromolecular drug transport and as such effective drug concentrations (23–26).

A means to facilitate ART passage through the BBB is by using circulating monocyte-derived macrophages (MDM) as drug depots. Previously, our laboratory used laboratory and animal systems to pursue this idea. The research demonstrated that macrophages can deliver drugs to sites of viral infection and show sustained antiretroviral activities (5, 27). Recently, we also showed that bone marrow macrophages (BMM) can cross the BBB into HIV-1-infected brain regions (6). Based on these findings, a BMM pharmacological nanoparticle (NP) delivery system (nanoART) was developed to test whether blood-borne macrophages could deliver ART directly to the brain. Our results demonstrate that BMM can serve as vehicles for indinavir (IDV) NP delivery, BMM showed consistent uptake and release of IDV-NP and free IDV while targeting areas of viral replication in a severe combined immunodeficient (SCID) model of HIV-1 encephalitis (HIVE). These data support the notion that nanoART brain penetration, drug distribution, and therapeutic responses can be achieved through cell-based nanof ormulation and as such lower drug-dosing intervals, adherence, and bioavailability.

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Abbreviations used in this paper: MP, mononuclear phagocyte; MGC, multinucleated giant cell; BBB, blood-brain barrier; ART, antiretroviral therapy; MDM, monocyte-derived macrophage; BMM, bone marrow macrophage; NP, nanoparticles; IDV, indinavir; HIVE, HIV-1 encephalitis; Vim, vimentin; GFAP, glial fibrillary acidic protein; NF, neurofilament; p-NF, phosphorylated NF; CSF, cerebrospinal fluid; RP-HPLC, reverse phase HPLC.
Materials and Methods
NP preparation and characterization

IDV-NP suspensions were prepared using high-pressure homogenization. The surfactant coating of the IDV crystals was made with 1.2% (w/v) Lipid E80, an egg phosphatide mixture of phosphatidylcholine, phosphatidylethanolamine, and the hydrolyzed lyso-forms (single aliphatic chain) of each phospholipid. Lipid E80 coated the actual particles. Cationic particles were washed twice with DMEM and each recipient mouse was injected i.v. through the tail vein.

Monocyte isolation, cultivation, and viral infection

Human monocytes were obtained from leukopaks of HIV-1, HIV-2, and hepatitis B-seronegative donors and purified by countergradient elutriation (28). The University of Nebraska Medical Center Institutional Review Board approved the procedure. Cells were cultured with DMEM with 10% heat-inactivated pooled human serum, 1% glutamine, 10 mg/ml ciprofloxacin (Sigma-Aldrich), and 1000 U/ml highly purified recombinant human M-CSF (a generous gift from Wyeth). Seven days after plating, MDM were infected with HIV-1ADA at a multiplicity of infection of 0.1 infectious viral particles per target cell. Culture medium was half-exchanged every 2–3 days. All viral stocks were tested and found to be free of Mycoplasma and endotoxin contamination.

Murine HIVE model

Four- to 6-wk-old male C.B.-17 SCID mice were purchased from The Charles River Laboratory, BALB/c-Rag2−/−γc−/− mice were bred at the University of Nebraska Medical Center for parallel studies of cell migration. Animal experiments were performed under strict observance of the National Institutes of Health and University of Nebraska guidelines for animal care. Animals were maintained in sterile microisolator cages. Briefly, all animals were anesthetized and placed in a stereotaxic apparatus for intracranial injection. The animal’s head was secured with Saran Wrap® for intracranial injection. The animal’s head was secured with Saran Wrap® for intracranial injection. The animal’s head was secured with Saran Wrap® for intracranial injection.

BMM isolation and cultivation

Male BALB/c mice (Charles River Laboratory), 4–5 wk of age were used as BMM donors. Briefly, the femur was removed, the bone marrow cells were dissociated into single-cell suspensions, and were cultured for 10 days supplemented with 1000 U/ml M-CSF (Wyeth). Cultured BMM proved to be 99% pure CD68+ and 98% CD11b- by flow cytometric analysis using a FACSCalibur flow cytometer (BD Biosciences).

Super paramagnetic iron oxide (SPIO)

HIVE SCID mice were injected with BMM containing SPIO particles (Feridx; Berlex). BMM were incubated at a SPIO concentration of 2 mg/107 cells/ml for 2 h. This resulted in >95% labeled cells as determined by Prussian blue staining. Cells were washed twice with DMEM and each recipient mouse was injected i.v. through the tail vein with 150 μl containing 1 × 107 BMM loaded with SPIO (SPIO-BMM).

Drug treatment

BMM were incubated with rhDFHE-IDV-NP at a concentration of 5 × 10−4 M for 12 h and IDV-NP packaged rhDFHE-IDV-NP (rhDFHE-IDV-NP-BMM) were washed twice with DMEM. A single dose of rhDFHE-IDV-NP-BMM or IDV-NP was injected into each mouse i.v. through the tail vein.

IDV measurements

IDV-NP-BMM-treated HIVE SCID mice (five in total per time point per group) were used to evaluate blood and brain tissue IDV levels at 1, 3, 7, and 14 days after treatment. Macroscopic sections of the injected brain regions (regions including HIV-1-infected MDM), control hemispheres and whole blood were homogenized by sonication in 95% methanol (1 ml of tissue and 1 ml 0.5 ml of blood). Prepared tissue lysates were maintained at 4°C overnight and clarified by centrifugation at 14,000 × g for 10 min at 4°C. Supernatants were collected and analyzed by reverse phase HPLC (RP-HPLC) (Waters) for determination of drug levels. Triplicate 20-μl aliquots of each sample were injected for RP-HPLC analysis. IDV was separated from other tissue components using a mobile phase of 60/40) 25 mM potassium phosphate (pH 4.15:acetonitrile at 0.4 ml/min and a Waters YMC Octyl C8 column (3.0 × 150 mm). IDV was quantified by comparison of peak area to that of a series of known IDV standards. Data are expressed as μg of IDV per 100 mg of tissue or μg/ml in blood. Processing and analyses were validated using known concentrations of IDV and spiking drug into homogenized tissue samples from naive animals.

Confocal examination

For fluorescence evaluation of rhDFHE-IDV-NP-BMM-targeted migration to the regions of viral infection, brain tissue was collected on posttreatment day 3 after perfusion fixation with 4% paraformaldehyde in PBS. Immunofluorescent staining was performed on sucrose-processed 25-μm frozen brain sections. Abs to human specific vimentin (Vim)-intermediate filaments (clone 3B4; DakoCytomation) were used for detection of human microglia/macrophages in the mouse brain. Ab to HIV-1p24 Ag (DakoCytomation) was used to determine the number of HIV-1-infected MDM. Rabbit polyclonal Abs to ionized calcium-binding adaptor molecule 1 (Iba-1, 1/500; Wako) was used to identify both MDM and murine microglia. Astrocytes were detected with Abs against glial fibrillary acidic protein (GFAP; DakoCytomation). Abs to H chain (200-kDa) neurofilament (NF) Ags (DakoCytomation) were used to detect neurons. Fluorescent images were visualized with an LSM 410 confocal laser-scanning microscope (Zeiss) with argon/krypton at 488/568/647 nm. Quantification of rhDFHE-IDV-NP-BMM levels was analyzed by using Image-Pro Plus (version 4.0; Media Cybernetics). The red fluorescence area of rhDFHE-IDV-NP-BMM was determined as a percentage of the total image area per microscopy field and calculated for a 0.1-mm window of tissue immediately surrounding the injection site.

Immunohistochemistry and image analyses

Sham, MDM, and HIVE with or without IDV-NP-BMM-treated mice were sacrificed at 7 and 14 days after treatment. Each brain was paraffin processed and cut into 5-μm slices to identify the injection site. Immunohistochemistry was performed with the above Abs. For location of SPIO-BMM, paraffin brain sections were stained with Prussian blue. Quantification of GFAP, Iba-1, and NF-positive staining was achieved on serial coronal brain sections as a percentage of the total image area per microscopy field with a total of 30 fields (six sections per mouse, five mice in each group) using Image-Pro Plus (Media Cybernetics). The absolute number of Vim+ and HIV-1p24+ cells and MGC and MGC nuclei were counted under microscopy with six sections per mouse, five mice in each group.

Statistical analyses

The data were analyzed and comparisons were performed using five mice per time point per group by a two-tailed unpaired t test using Prism statistical software for Macintosh (version 4.0; GraphPad Software). Values of p < 0.05 were deemed significant.

Results

Uptake and cell release of IDV-NP

Our overarching idea is to use monocyte-macrophages as both carriers and extended depots for antiretroviral drugs for delivery to reservoirs for HIV and particularly the CNS. In a first step to test this idea, we analyzed BMM uptake and release of IDV-NP using confocal microscopy and RP-HPLC tests. We used successive washes of adherent >99% pure CD68+ BMM cultures to displace surface-bound NP and prove such displacement by confocal Z-scan analysis (27). NP visualized by fluorescence microscopy were seen within the cytoplasm of BMM and provided clear evidence...
that rDHPE-IDV-NP (red) were readily phagocytized within the macrophage (green, Fig. 1A). rDHPE-IDV-NP (red, Fig. 1A) were observed in >98% of BMM. This was supported by HPLC tests performed after rDHPE-IDV-NP treatment (Fig. 1B). Following sequential medium changes, drug was released continuously as shown by HPLC tests and demonstrated both intracellular and extracellular levels of IDV. These progressively diminished over 7 days (Fig. 1C).

**Tracking BMM migration to diseased brain subregions**

The next series of experiments examined the distributions of monocyte-macrophages after i.v. cell injections. To determine differences for BMM migration as a consequence of HIVE, we performed replicate experiments with virus-infected and uninfected human MDM and sham-operated injections into subcortical (caudate and putamen) brain regions. In these experiments, the subcortical injection of HIV-1-infected human MDM induced a focal HIVE reflective of human HIVE (30). This included astrogliosis and microgliosis, loss of neurons, and ongoing viral replication in affected brain regions as demonstrated by the presence of HIV-1p24\(^+\) cells (Fig. 2A). MDM and saline sham-operated mice were controls. Into these animals BMM-carrying IDV NP were administered through the tail vein 24 h after brain injections. Mice were sacrificed on days 1, 3, 7, and 14 for histopathological analyses and assay of IDV drug levels. We reasoned that the neuroinflammatory responses induced by viral infection and, in particular, HIVE, provided a biological system wherein blood-borne monocytes-macrophages carrying NP would ingress to diseased brain sites. Thus, BMM migration in diseased brain regions was measured. Initial experiments performed with BMM loaded with SPIO and administered i.v. to HIVE mice showed that the macrophages readily migrated to areas of HIVE as seen by Prussian blue staining (Fig. 2, A and C). This paralleled sites of reactive gliosis and HIV-1p24\(^+\) cells. No Prussian blue-stained BMM were obtained in the contralateral hemispheres of either HIVE or sham-operated animals (Fig. 2, B and D).

**HIV-1-infected macrophage neuroinflammatory responses elicit BMM brain transmigration**

HIV-1 infection of brain macrophages is associated with ongoing viral infection, astrogliosis, and microgliosis. This is seen where HIV-1p24\(^+\), GFAP\(^+\), and Iba-1\(^+\) stained cells are linked with each other (Fig. 3A–D). In regard to specificity of these responses, BMM levels were reduced in MDM mice when compared in HIV mice. More importantly, such neuroinflammatory responses were observed colocalized with rDHPE-IDV-NP-BMM (Fig. 3C). In regard to specificity of these responses, BMM levels were reduced in MDM mice when compared in HIV mice. Moreover, few red fluorescence cells were detected in sham-operated brains (Fig. 3, A and B). In all animal groups, no rDHPE-IDV-NP-BMM were found in the contralateral hemisphere. Few numbers of rDHPE-IDV-NP-BMM were seen in brains injected with uninfected MDM (supplemental Fig. 1A).

**Cell-based NP delivery affect IDV brain levels**

To determine brain distribution of BMM loaded with nanoformulated IDV, the optical properties of red fluorescent rDHPE-IDV-NP were used. The images of brain sections reflect robust levels of BMM-rDHPE-IDV-NP (red) targeted in the areas of affected brain regions as demonstrated by the presence of HIV-1p24\(^+\) cells (Fig. 2). In HIV mice, significant GFAP\(^+\) astrogliosis (green, Fig. 3A) and Iba-1\(^+\) microglial responses (green, Fig. 3B) were observed. Importantly, such neuroinflammatory responses were observed colocalized with rDHPE-IDV-NP-BMM (Fig. 3C). In regard to specificity of these responses, BMM levels were reduced in MDM mice when compared in HIV mice. Moreover, few red fluorescence cells were detected in sham-operated brains (Fig. 3, A and B). In all animal groups, no rDHPE-IDV-NP-BMM were found in the contralateral hemisphere. Few numbers of rDHPE-IDV-NP-BMM were seen in brains injected with uninfected MDM (supplemental Fig. 1A).
HIV-1 infection. Mouse-specific BMM CD68+ cells (green, Fig. 4A) were vehicles for IDV-NP delivery to the brain. BMM migrated to sites of HIVE. The rDHPE-IDV-NP-BMM (red, Fig. 4B) were concentrated around the virus-infected sites (Fig. 4B) and colocalized with CD68+ immunostaining (Fig. 4A). Higher levels of CD68+ BMM and rDHPE-IDV-NP-BMM were in HIVE brains compared with sham-operated animals (A). Confocal imaging of Iba-1 (green) reflects microgliosis (B). The Vim (blue) staining was used to distinguish between the human MDM and murine microglia in B with composite of IDV, Vim, and Iba-1 immunostaining in C. The red rDHPE-IDV-NP-BMM differentiated microglia from migrated BMM. However, Iba-1 also labeled native circulating monocytes in the mice. Moreover, sham mice showed minimal glial reactions as visualized by GFAP and Iba-1 (green) in response to needle trauma with few rDHPE-IDV-NP-BMM. The photomicrograph shows BMM migration and brain immune responses in the infected animals. IDV* denotes rDHPE-IDV-NP-BMM. Original magnification, ×200.

FIGURE 3. Neuroinflammatory responses affect BMM migration. Stereotactic injection of HIV-1-infected MDM into the caudate/putamen of SCID mice was used to induce HIVE, resulting in neuroinflammation by astrocytes and microglia activation. Immunostaining was performed on frozen brain sections from sham-operated and HIVE mice with or without rDHPE-IDV-NP-BMM. Confocal imaging of Iba-1 (green, B) and GFAP (green, A) immunoreactivity reflect astrogliosis and microgliosis responses. The red fluorescence spots were around and within the site of injection in HIVE mice 3 days after rDHPE-IDV-NP-BMM treatment. rDHPE-IDV-NP-BMM are colocated with GFAP-reactive astrocytes in HIVE mice when compared with sham-operated animals (A). Confocal imaging of Iba-1 (green) reflects microgliosis (B). The Vim (blue) staining was used to distinguish between the human MDM and murine microglia in B with composite of IDV, Vim, and Iba-1 immunostaining in C. The red rDHPE-IDV-NP-BMM differentiated microglia from migrated BMM. However, Iba-1 also labeled native circulating monocytes in the mice. Moreover, sham mice showed minimal glial reactions as visualized by GFAP and Iba-1 (green) in response to needle trauma with few rDHPE-IDV-NP-BMM. The photomicrograph shows BMM migration and brain immune responses in the infected animals. IDV* denotes rDHPE-IDV-NP-BMM. Original magnification, ×200.

FIGURE 4. Brain tissue distributions of rDHPE-IDV-NP-BMM. Sham and HIVE mice were injected with rDHPE-IDV-NP-BMM for 3 days and frozen brain sections were stained with Ab to CD68, HIV-1p24, and GFAP (A and B). Confocal microscopy was used to assess the distribution of CD68+ cells (green) and around injection areas of the brain in both sham and HIVE animals 3 days after IDV-NP-BMM treatment. CD68+ cells were located in and surrounding the injection line in all animals seen in A. The intensity of CD68+ cells was associated with rDHPE-IDV-NP-BMM (red, A) in treated mice compared with untreated mice in all groups. A substantial increase of rDHPE-IDV-NP-BMM (red, B) was observed in viral infection areas in HIVE mice compared with sham and MDM animals. Confocal imaging of brain sections with double immunostaining to HIV-1p24 (green, B) and GFAP (blue, B) showed increased red fluorescence (rDHPE-IDV-NP-BMM) in brain areas where there was active viral infection (HIV-1p24+ MDM). Greater levels of rDHPE-IDV-NP-BMM were in HIVE compared with sham animals. GFAP (blue, B) and rDHPE-IDV-NP-BMM (red, B) were linked to HIV-1 infection and GFAP astrogliosis (blue, B and C); however, there was no correlation between rDHPE-IDV-NP-BMM levels and GFAP expression in treated animals compared with untreated mice in sham and HIVE mouse groups. Composite of rDHPE, HIV-1p24, and GFAP stainings are in C. IDV* denotes rDHPE-IDV-NP-BMM. Original magnification, ×200.
IDV levels were obtained in treated HIVE mice at experimental time points. Treatment, IDV levels were testable by HPLC until reaching day 14. Blood IDV levels were assayed by HPLC from blood in HIVE mice treated with IDV-NP-BMM compared with the contralateral hemisphere. SEM) percent area distribution of rDHPE-IDV-NP-BMM was determined for five mice per group.

Brain tissue IDV concentration. Quantitative image analysis of untreated (■) or treated (□) with rDHPE-IDV-NP-BMM (B). Mean (±SEM) percent area distribution of rDHPE-IDV-NP-BMM was determined for five mice per group. *p < 0.05 compared with untreated. IDV levels were assayed by HPLC from blood in HIVE mice treated with IDV-NP-BMM at days 1, 3, 7, and 14 (C and D). With a single dosage of treatment, IDV levels were testable by HPLC until reaching day 14. Blood IDV levels were obtained in treated HIVE mice at experimental time points after i.v. injection via the tail vein. Mean (±SEM) μg of IDV per ml of blood was determined for five mice per group. IDV were assayed by HPLC from lysates of brain tissues (D). The diseased (ipsilateral) brain tissue levels of IDV were analyzed and compared with the contralateral hemisphere at the experimental time points. Mean (±SEM) μg of IDV per 100 mg of brain tissue was determined for five mice per group. *p < 0.01 compared with the contralateral hemisphere.

Compared with uninfected MDM (supplemental Fig. 2B) and sham-injected animals. The area of rDHPE-IDV-NP-BMM, determined by digital image analysis, was increased in HIV-1 infection (p < 0.01) compared with both uninfected MDM and sham controls (Fig. 5A). Likewise, numbers of CD68+ cells were also increased in HIV-1 infection (p < 0.01) compared with untreated. IDV-NP-BMM were also increased in HIV-1 infection (p < 0.01) compared with sham-operated controls (Fig. 5B). Altogether, these findings support targeted migration of IDV-NP-BMM into areas of active HIV-1 infection and neuroinflammation.

To validate these findings of selective drug-carried BMM into brain-diseased areas, we administered by i.v. injection a single dose of IDV-NP-BMM to HIV-1 infected mice and determined IDV levels in tissues from the caudate and putamen on days 1, 3, 7, and 14 after treatment. Quantifiable amounts of IDV were obtained in blood (Fig. 5C) and comparable levels of IDV in diseased (ipsilateral) and control (contralateral) hemispheres (Fig. 5D) were assayed by RP-HPLC. One IDV-NP-BMM treatment elicited sustained drug levels in blood for up to 14 days (Fig. 5C). More importantly, IDV-NP-BMM delivery attained higher drug levels at day 14 in the ipsilateral than in the contralateral hemisphere of HIV-1 infected mice (Fig. 5D). These results confirmed that NP-IDV was successfully delivered into the brain by packaging into BMM, thus providing proof-of-concept for therapeutic drug delivery in animal models of human disease.

**Antiretroviral responses of IDV-NP-BMM**

We previously demonstrated that after IDV-NP-BMM administration long-term viral suppression and increased CD4+ T cell levels were achieved (5). A single administration of IDV-NP-BMM achieved drug concentrations 4- to 10-fold higher in plasma and lymph tissues and were more sustained than those attained with a single bolus of nonformulated IDV. To validate our results, we used BALB/c Rag2−/−γc−/− mice which provided long-term engraftment of human MDM in the murine environment (29). To reach a therapeutic IDV concentration and determine antiviral efficacy in brains, IDV-NP-BMM was administered to HIVE mice; replicate animals were untreated. After 7 and 14 days, the extent of HIV-1 infection in the brain was determined. Immunostaining of brain sections showed that human Vim+ MDM (Fig. 6A) colocated with activation of GFAP+ astrocytes in HIVE mice. Immunohistochemistry determined the levels of MDM reconstitution and viral infection by counting the absolute number of Vim+ and HIV-1p24+ MDM in brain sections. The absolute number of Vim+ and HIV-1p24+ MDM was counted as cells per section in six sections per mouse as shown in Table I. To determine the effects of IDV-NP-BMM administration on long-term antiviral responses, HIV-1p24+ cells were assessed as a percentage of total human MDM (Vim+). With a single treatment of IDV-NP-BMM, decreased numbers of HIV-1-infected cells were observed in IDV-NP-BMM-treated HIVE mice compared with untreated animals (Fig. 6B). This was significantly apparent in brain sections (p < 0.01) on days 7 and 14, reflecting a long-term robust antiretroviral response elicited by IDV-NP-BMM. Based on the observation of a reduction in HIV-1p24+ cells in the IDV-NP-BMM-treated HIVE mice, we studied MGC formation in brain sections found exclusively in injection sites where HIV-1p24+ cells were seen. Brain histopathology of untreated HIV-1 infected mice is shown (Fig. 6A). Visualization of MGC showed large numbers of nuclei within cells shown by arrowheads. MGC and nuclei were counted using absolute numbers in brain sections at days 7 and 14. The numbers of MGC were 11.9 ± 2.7 and 7.2 ± 4.08 (counts per section) on day 7 and 5.1 ± 1.6 and 1.7 ± 1.2 (counts per section) on day 14 in untreated and IDV-NP-BMM-treated HIV-1 infected mice, respectively. Mean numbers of nuclei within MGC were 13.8 ± 2.8 and 7.1 ± 2.0 on day 7 and 7.4 ± 0.7 and 4.9 ± 0.6 on day 14 in untreated and IDV-NP-BMM-treated HIV-1 infected mice, respectively. Significantly decreased numbers of MGC (both p < 0.01) and nuclei (p < 0.05 and p < 0.01) on days 7 and 14 were observed following IDV-NP-BMM treatment. Fig. 6A also demonstrated changes in the MDM phenotype (Vim+). A stalk containing nucleus became elongated in untreated HIV-1 infected mice (Fig. 6A). IDV-NP-BMM treatment limited MGC formation. Based on a significantly reduced HIV-1p24 expression in the treated group, we determined that MGC formation was linked to HIV-1 infection. The levels of MGC was significantly decreased when assayed by ratios of MGC:total Vim+ MDM (Fig. 6C) in IDV-NP-BMM-treated HIV-1 infected mice.

**Preliminary toxicology studies**

To investigate the potential toxicity of IDV-NP-BMM at the delivery site and more extensively throughout the brain, histopathological analysis of brain sections was used to examine neuronal integrity in SCID mice injected in the brain with human MDM (supplemental Fig. 2C) or HIV-1-infected MDM. Sham-operated animals served as controls. Neuronal injury induced as a consequence of viral infection and/or inflammation caused by xenogenic MDM was limited (supplemental Fig. 2). Three groups of mice (sham control, MDM, and HIV-1) were used to determine whether any neurotoxicity was induced by the nanoformulations themselves. MDM are known to induce inflammatory responses and are capable of promoting BMM migration into the brain. Immunostaining for NF was performed in brain sections to identify neuronal loss. Confocal microscopy
images demonstrated IDV-NP-BMM (red) migration into areas of MDM with or without HIV-1 infection (Figs. 3–7). HIV-1 infection and ongoing inflammatory responses are shown as HIV-1p24 expressions and GFAP/astrogliosis and Iba-1 microgliosis (Figs. 3, 4, and 6) were revealed in response to the needle track in sham-operated animals. Immunostaining for NF (green, Fig. 7A) was performed in brain sections to identify neuronal loss in HIVE-diseased areas with or without IDV-NP-BMM treatment (Fig. 7A). Indeed, abnormal accumulation of NF neuron bodies was located in the diseased areas where ongoing inflammatory and viral infection was occurring. Confocal microscopy images demonstrated IDV-NP-BMM (red) migration into diseased areas with ongoing HIV-1 infection (Fig. 7A) and/or inflammatory responses (supplemental Fig. 2C). Sham controls revealed few rDHE-positively stained spots. NF staining loss was seen in HIV-1-infected MDM-occupied sites and surrounding areas. Indeed, abnormal accumulation of phosphorylated NF (p-NF) in the neuron body was observed in both MDM and HIVE mice with or without IDV-NP-BMM treatment. NF expression (Fig. 7, A–C) and p-NF neuron bodies were evaluated. Image quantitation of neuronal damage (NF axons) and p-NF neuron bodies demonstrates that NF expression (Fig. 7B) was no different in the IDV-NP-BMM-treated HIVE mice compared with the untreated group. The

Table I. Antiretroviral activities of IDV-NP-BMM

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<th>Day 7</th>
<th>Day 14</th>
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<tr>
<td></td>
<td>MDM</td>
<td>HIV</td>
</tr>
<tr>
<td>Vim</td>
<td>368.88 ± 43.54</td>
<td>0</td>
</tr>
<tr>
<td>HIV-1p24</td>
<td>419.11 ± 45.64</td>
<td>128.5 ± 31.35</td>
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<tr>
<td>Vim</td>
<td>342.26 ± 18.88</td>
<td>52.67 ± 20.12*</td>
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*Absolute numbers of Vim and HIV-1p24 MDM were determined from serial immunohistological sections. These were used to assess levels of HIV-1 infection. Means (±SEM) are absolute numbers of Vim and HIV-1p24 MDM determined for five animals per group. IDV-NP-BMM i.v. administration significantly reduced levels of HIV-1p24 MDM when compared to untreated HIVE mice. Uninfected MDM injected into the subcortex of immunodeficient mice served as a control.

* p < 0.05 compared with IDV-NP-BMM-treated to untreated HIVE mice.
infection of brain MP including blood-borne perivascular macrophages and microglia, culminating in neuronal injury and death (39–43). Interestingly, these same MP cells carry the virus from the periphery into the brain and serve as sources of neuroinflammatory mediators. Such an inflammatory response generates chemokine gradients, encouraging additional monocyte-macrophages to enter the brain as well as providing a rich source of neurotoxins (42–46). Cognitive, motor, and behavioral abnormalities occur as a consequence of such pathogenic events and are fueled by continuous viral growth in the face of damaged or lost adaptive immunity (47–49). We reasoned that improving brain penetration of ART would affect the tempo and progression of disease by controlling viral growth. To accomplish this, we took advantage of the ingress of monocytes-macrophages from the blood to the brain operative in disease. Such cell ingress correlates with disease severity (50–52) and could be harnessed for therapeutic gain. By using BMM as ART carriers, the actual entry of disease-causing cells could be used to improve disease outcomes. BMM loaded with IDV-NP readily penetrate the BBB, enter brain subregions, and migrate to disease sites of continuous viral replication and neuroinflammation. These results provide further validation for the use of macrophage-drug delivery systems to combat HIV infection (5, 27, 53, 54).

ART can restore cognitive function while limiting neural damage in HIV-1-infected individuals (11, 55, 56). Indeed, the HIV load present in cerebrospinal fluid (CSF) and the number of immune-competent macrophages correlate with the degree of cognitive deficits and most notably, the numbers of CD4+ T lymphocytes (57–61). This supports the idea that sustained penetration of ART across the BBB improves clinical neurological outcomes (41, 62). Indeed, ART can prolong life expectancy and restore immune activities, resulting in improved surveillance of virus and reductions of opportunistic infections and primary CNS lymphomas (63–66). In contrast, ineffective use of ART or its reduced brain penetration could contribute to viral mutation and sustained HIV replication within the brain sanctuary (67). Significant evidence shows that viral resistance patterns within the CSF compartment are distinct from that found in plasma (68, 69). Moreover, virological CSF suppression is associated with ART brain penetration (70). Nonetheless, the BBB limits the numbers of drugs that readily enter the CNS. Therefore, drugs that enter the CNS and suppress ongoing viral replication are believed to provide the best clinical outcomes. These observations, taken together, indicated that the development of a novel antiretroviral drug delivery system to improve the CNS penetration and ART efficacy is important.

Our laboratory and those of others developed macrophage-based nanoformulations to treat neuroAIDS and other neurodegenerative diseases (5, 27, 53, 54, 71). Such macrophage drug carriage was shown to enhance local drug concentration, elicit limited systemic side effects, and affect ART efficacy in rodent models of HIV infection (5). Our previous works with the HIV-1 protease inhibitor IDV showed that IDV-NP carried in BMM could positively affect pharmacokinetic drug delivery and improve tissue distribution in laboratory and animal models of HIV disease (5, 27). The current results extend these observations significantly by demonstrating the biodistribution and antiretroviral activity of IDV-NP-BMM within CNS tissue compartments exhibiting active HIV-1-induced disease. Levels of IDV in HIV-1-infected brain areas were significantly increased and extended to 14 days with a single dosage of IDV-NP-BMM treatment in comparison to i.v. administered IDV. Compared with control hemispheres, a significantly high level of IDV was obtained in diseased hemispheres on day 14.

Nanotechnology has revolutionized modern-day pharmacology (72–76). The ability to alter carrier size, shape, and composition

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**FIGURE 7.** IDV-NP-BMM does not affect neural morphology. Neuronal immunostaining for NF, which included both the NF and p-NF forms, was performed in brain tissue sections of SCID mice 3 days after a single i.v. injection of rDHPE-IDV-NP-BMM (A). SCID mice were intracranially injected with saline, MDM, or HIV-1- infected MDM. Serial 25-μm frozen brain tissue sections were stained with Abs to NF (green). The local rDHPE-IDV-NP-BMM (red) distribution showed no changes in NF (B). Spatial relationships between NF+ axon loss and p-NF neuronal body accumulation in viral infection were determined by confocal image analysis. The local rDHPE-IDV-NP-BMM (red) distribution showed no changes in NF+ axon loss and p-NF accumulation compared with three groups of animals that did not receive treatment. Original magnification, ×200. Quantitative image analyses was used to assess immunostaining of NF (B), p-NF (C), GFAP (D), and Iba-1 (E). Absolute number of p-NF+ neuronal bodies was counted in HIVE mice with or without IDV-NP-BMM treatment. These results showed that there was no relationship between IDV-NP-BMM levels to either neuronal injury or neuroinflammatory responses. IDV* represents rDHPE-IDV-NP-BMM.

Discussion

Invasion of HIV into the CNS occurs early after viral exposure and during the development of a seroconversion reaction (37, 38). Disease, however, occurs years later as a consequence of chronic viral

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numbers of accumulated p-NF+ neuron bodies along with GFAP+ and Iba-1+ astrocytes and microglia showed (Fig. 7, C–E) no changes among treated and untreated mice.
allows incorporation of drugs with a broad range of physical and biochemical properties (77). Nanoformulations have a number of advantages over conventional oral or i.v. drug systems in their capacity to increase systemic bioavailability and solubility and to slow drug degradation. Our macrophage-based system expands these observations even further in a number of divergent ways. First, monocyte-macrophages can carry drugs across the BBB to target disease areas and improve local drug distribution. Second, the macrophage delivery system relies on natural pathogenic processes elicited during inflammatory responses. These responses serve to target disease sites of active HIV-1 replication. In this way, there is a natural control for drug penetration that is based on disease severity. Third, monthly dosing positively affects therapeutic outcomes by prolonging the presence of local drug and, in so doing, reducing opportunities for viral mutation and disease (5).

Macrophages have received significant attention for their role as drug carriers (78, 79). However, relatively few in vivo studies have assessed the ability of the macrophage-drug delivery system to target migration to disease sites. We developed a novel method using macrophages for delivery of IDV-NP across the BBB to improve antiviral efficacy and enhance brain drug distribution. The advantages of BMM as a carrier of NP for antiretroviral drugs include an effective and systemic delivery system in vivo to track cell migration and to use therapeutic activities. The significance of this work is reflected by its interdisciplinary approaches to strategizing crossing of the BBB, targeting migration, improving brain drug levels, and assessing antiretroviral responses. Based on the numbers of blood-borne macrophages that have entered affected brain regions and taking into account that >98% of the cells carry drugs (5), the IDV levels observed in brain were lower than would be expected. Although measures of the drug in wedge brain sections provide proof-of-concept, absolute drug levels are diluted by the necessary inclusion of surrounding unaffected tissues in drug analysis. Thus, the precise amount of drug delivered into areas of active disease will require microdissection of encephalitic brain subregions. This remains a major and ongoing focus of our own research efforts. Improvements of CNS drug penetration, targeted delivery, single dosage administration, economy, sustained release, and drug bioavailability can assuredly make nanoART attractive for human use. This study is certainly important because it represents a new direction for effective treatment of one of the most debilitating complications of HIV-1 infection, namely, cognitive impairment.

Acknowledgments


Supplemental Figure 1

Stereotactic injection of human MDM into the caudate/putamen of SCID mice show limited BMM brain migration. Human MDM injections generate a xenogenic reaction resulting in limited astro- and microgliosis. Immunostaining was performed on frozen brain sections from MDM mice with or without rDHPE-IDV-NP-BMM. Confocal imaging of Iba-1 (green, A) and GFAP (green, B) immunoreactivity reflects astrogliosis and microgliosis responses. The red fluorescence spots were around and within the xenogenic MDM site 3 days after rDHPE-IDV-NP-BMM treatment (A and B). The rDHPE-IDV-NP-BMM co-located with levels of GFAP and Iba-1 protein expression and reflect glial responses. Original magnification x 200.

Supplemental Figure 2

Human MDM injected into the caudate/putamen of SCID mice induce mild neuroinflammatory responses. MDM were treated with rDHPE-NP-IDV-BMM for 3 days and frozen brain sections stained with antibody to mCD68, HIV-1p24, GFAP and NF. Untreated MDM mice served as controls. Confocal microscopy for distribution of mCD68^+ cells (green, A) in and around the MDM sites at 3 days post-treatment. mCD68^+ cells were at the injection site in both untreated and NP-IDV-BMM treated MDM mice co-localized with rDHPE-IDV-NP-BMM (red, A arrowheads). IDV^* represents rDHPE-IDV-NP-BMM. Double immunostaining to HIV-1p24 (green, B) and GFAP (blue, B) showed red fluorescence stain (rDHPE-IDV-NP-BMM) in areas located in or around MDM (arrowhead). GFAP^+ cells (blue, B) showed co-localization with rDHPE-IDV-NP-BMM (red); however, few red^+ areas were observed. Neuronal immunostaining for NF, included both the NF and phosphorylated NF (p-NF) forms, was performed in brain tissue sections of MDM SCID mice 3 days after a single intravenous injection of rDHPE-IDV-
NP-BMM (C). Spatial relationships between NF\(^+\) axon loss and p-NF neuronal body accumulation were determined by confocal image analysis in MDM inoculated sites. The local rDHPE-IDV-NP-BMM (red) migrated areas showed no changes in NF\(^+\) axon loss and p-NF accumulation when compared to untreated animals. IDV* represents rDHPE-IDV-NP-BMM. Original magnification, x 200.
A

B

C

rDHPE  mCD68  merge

rDHPE  HIV-1 p24  GFAP  merge

rDHPE  NF  merge

IDV*