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ARTICLE

Microglia-specific targeting by novel capsid-modified AAV6 vectors

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Recombinant adeno-associated viruses (rAAV) have been widely used in gene therapy applications for central nervous system diseases. Though rAAV can efficiently target neurons and astrocytes in mouse brains, microglia, the immune cells of the brain, are refractile to rAAV. To identify AAV capsids with microglia-specific transduction properties, we initially screened the most commonly used serotypes, AAV1–9 and rh10, on primary mouse microglia cultures. While these capsids were not permissive, we then tested the microglial targeting properties of a newly characterized set of modified rAAV6 capsid variants with high tropism for monocytes. Indeed, these newly characterized rAAV6 capsid variants, specially a triply mutated Y731F/Y705F/T492V form, carrying a self-complementary genome and microglia-specific promoters (F4/80 or CD68) could efficiently and selectively transduce microglia *in vitro*. Delivery of these constructs in mice brains resulted in microglia-specific expression of green fluorescent protein, albeit at modest levels. We further show that CD68 promoter–driven expression of the inflammatory cytokine, interleukin-6, using this capsid variant leads to increased astrogliosis in the brains of wild-type mice. Our study describes the first instance of AAV-targeted microglial gene expression leading to functional modulation of the innate immune system in mice brains. This provides the rationale for utilizing these unique capsid/promoter combinations for microglia-specific gene targeting for modeling or functional studies.

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INTRODUCTION

Recombinant adeno-associated viruses (rAAV) are safe and robust gene therapy vehicles for preclinical and clinical usage.[1–3] Due to its ability to efficiently transduce neurons and astrocytes,[4,5] AAV-mediated gene targeting has been used widely as disease-modifying paradigms in preclinical models of several neuropsychiatric disorders, such as Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, epilepsy, and malignant gliomas.[6,7] However, rAAV-mediated genetic targeting of microglia, and in general, cells of myeloid lineage, remains challenging. Aberrant activation of microglia, the resident immune cells of the brain, leads to pathological conditions underlying numerous neurologic disorders characterized by proteostasis and neurodegeneration.[8] Given that manipulating microglial function could result in disease modification in such intractable diseases, characterization of rAAVs that specifically transduce microglia would allow physiologically relevant disease modeling and development of glia-targeted immunobiotherapies.

Several factors are critical in improving rAAV-based gene expression: increased AAV trafficking into cells, limiting its vulnerability to intracellular clearance pathways, and overall, higher expression of transgenes from targeted cells. These parameters can be regulated by (i) modifying the capsid to achieve higher transduction efficiency and limiting proteasomal degradation, (ii) using self-complementary (sc) vectors for faster and efficient gene expression, and (iii) utilizing stronger or cell-type–specific promoters and regulatory elements for robust gene expression from physiologically relevant cell populations.[9–13] Incorporating these concepts, we demonstrate that a capsid-modified rAAV6 expressing the transgene from a sc vector under the control of microglia-specific promoters results in microglia-specific transgene expression *in vitro* and *in vivo*. Our study highlights the potential utility of such capsid-modified vectors in reprograming glial function in neuroinflammatory disorders.

RESULTS

Capsid-optimized AAV6 efficiently transduces primary murine microglia

To test whether the most widely used rAAV serotypes can transduce microglia, primary wild-type (WT) mouse neuroglia and microglia cultures were transduced with AAV capsids-1–9 and rh10 expressing the enhanced green fluorescent protein (EGFP) transgene. This EGFP transgene is driven by the hybrid chicken β-actin (hCBA) promoter

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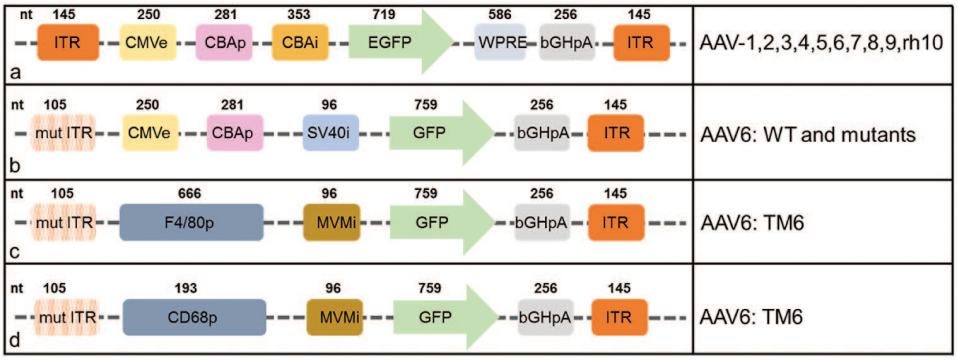
Table 1 Design of different AAV constructs



The single-stranded (ss) pAAV2 that contained chicken β-actin promoter (CBAp), cytomegalovirus enhancer (CMVe), CBA intron (CBAi), woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), and enhanced green fluorescent protein (EGFP) transgene was packaged in different AAV capsids-1–9 and rh10 (**a**). The self-complementary (sc) double-stranded vector expressing humanized GFP (GFP) and containing SV40-derived intron (SV40i) was constructed by mutating one inverted terminal repeat (mut ITR), such that the viral Rep protein cannot generate the ss DNA nick (**b**). Microglia-specific promoters (F4/80p or CD68p) containing a minute virus of mice intron (MVMi) was used to replace the hybrid CBA (hCBA) promoter and the SV40i (**c**, **d**). The length of different plasmid elements is depicted in nucleotides (nt) atop the corresponding elements. All constructs contain polyA element derived from bovine growth hormone (bGHpA). These constructs containing GFP or IL-6 transgenes were packaged in wild-type (WT) AAV6 and/or capsid-modified AAV6 (**b**–**d**). TM6 refers to the triple-mutant AAV6 capsid (Y731F/Y705F/T492V) (**c**, **d**). AAV, adeno-associated viruses; IL-6, interleukin-6.

from a single-stranded (ss) vector (Table 1a). We did not detect any EGFP fluorescence in primary microglia cultures transduced with this viral preparations (Supplementary Figure S1a), though we detected variable levels of neuronal and astrocytic expression of EGFP in primary mixed neuroglial cultures following transduction by these same viruses (Supplementary Figure S1b). This suggests that WT AAV capsids either (i) cannot transduce microglia efficiently or (ii) are efficiently cleared via their endoproteasomal machinery once internalized or (iii) does not have the transcriptional machinery available for efficient gene expression in microglia. A recent study demonstrated that using scAAV packaged in capsid-modified AAV6 can efficiently transduce dendritic cells of monocyte lineage.[9] This study identified several surface-exposed serine and tyrosine residues of AAV6, which when mutated to nonphosphorylatable valine residue increased transgene expression in dendritic cells by limiting proteasome degradation.[9] Since microglia are derived from a similar monocytic lineage,[14] we tested whether these mutant scAAV vectors expressing the GFP transgene under the hCBA promoter could transduce mouse primary microglia (Table 1b). For these studies, we compared the transduction efficiency of four different AAV6 capsids: WT, S663V, S663V/T492V, and Y731F/Y705F/T492V (abbreviated as triple-mutant AAV6 or TM6) in primary mixed neuroglial (Supplementary Figure S2) and primary microglial cultures (Figure 1). Transduction of primary mixed neuroglial cultures with these scCBA-GFP viruses revealed that in addition to neurons and astrocytes being efficiently transduced, there were GFP-expressing cells with typical microglia-like morphology in cultures transduced with the capsid-modified AAV6[15] (Supplementary Figure S2). These cells were neither microtubule-associated protein 2 (MAP2; neuronal specific cellular marker) or glial fibrillary acidic protein (GFAP; astrocyte-specific cellular marker) positive (Supplementary Figure S2a, arrows), suggesting that these GFP-expressing cells could potentially be microglia. We next tested whether these capsid variants could transduce primary murine microglia by incubating primary microglial cultures with these rAAV6 capsid variants for 5 days (Figure 1a). Quantitation of direct fluorescence (Figure 1b) and RNA (Figure 1c) shows that compared with WT AAV6-transduced microglia, the mutant AAV6 capsids, in particular Y731F/Y705F/T492V triple-mutant (TM6) AAV6 capsid, shows high levels of GFP expression. Immunocytochemical colocalization with the microglia-specific cellular marker, ionized calcium-binding adapter molecule 1(Iba-1), confirmed that the GFP-expressing cells are indeed microglia (Figure 1d). We were further curious whether the scCBA vector or the mutant capsid was key to increasing microglial transduction and transgene expression. Using flow sorting of microglia transduced with a conventional ss or the sc vector packaged in either WT AAV6 or TM6, we found that both the vector conformations are efficiently transduced only when packaged in TM6 capsid (Supplementary Figure S3a,b). This implies that the capsid variant indeed improves microglial targeting and gene expression. Overall, our data shows that the combination of scCBA construct in TM6 capsid resulted in the most efficient transduction efficiency (Supplementary Figure S3a,b). Notably, the morphology of the scCBA-GFP-TM6 transduced microglia assumed a large, round flat morphology with numerous microspikes all over the cell body resembling an activated state[15] (Figure 1d, arrows). We explored whether the TM6 capsid, by itself, or the high levels of GFP produced in the microglia could be responsible for such activation. Using major histocompatibility complex II (MHCII), a marker for microglial phenotypic activation,[15] we noticed that the TM6 capsid with an empty vector alone led to increased glial MHCII compared with naive glia or glia transduced with empty vector packaged in WT AAV6 capsid (Figure 1e, arrows). Microglia transduced with WT AAV6 expressing scGFP also showed increased MHCII immunoreactivity compared with empty vector transduced microglia (Figure 1e, arrows). Next, we used the inflammatory cytokine, interleukin (IL)-6, to further demonstrate the utility of scCBA-mediated expression of transgene in primary microglia cells. IL-6 is an inflammatory cytokine that plays a critical role in modulating neuroplasticity and neurodegeneration in aging and neuropsychiatric diseases.[16] Primary microglia were transduced with scCBA-IL-6 packaged in TM6 capsid and analyzed after 96 hours. Quantitative polymerase chain reaction (PCR) of microglial cells and enzyme-linked immunosorbent assay analyses of the culture media showed significantly increased IL-6 RNA and protein, respectively (RNA: 98.7-fold over control; protein: 241.4-fold over control; $P < 0.01$, Student's *t*-test).

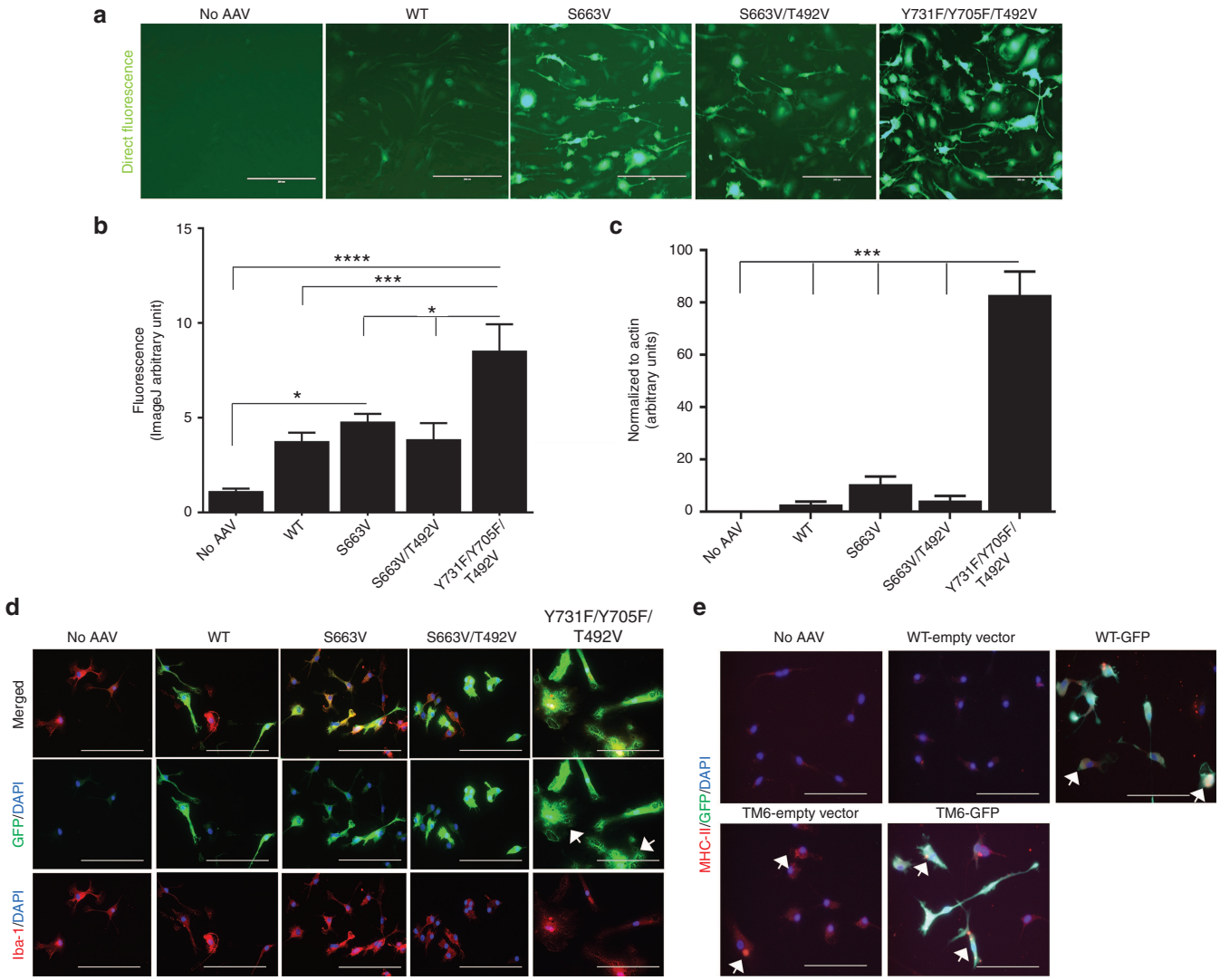


Figure 1 Capsid-modified scCBA-GFP transduces primary microglia cultures. Fluorescence micrograph of live primary murine microglia cultures transduced with wild-type (WT) AAV6 and capsid-modified AAV6 expressing hybrid chicken β-actin (hCBA) promoter–driven GFP (**a**). Intracellular fluorescence, indicative of GFP expression, was quantified using Image J (**b**). RNA levels of the transgene were quantified using custom-made Taqman probe against bGH sequence for each group (**c**). Immunocytochemical colocalization with microglia-specific marker (Iba-1 in red channel) confirms that GFP-expressing cells (immunostained in the green channel) are microglia (**d**). 4′,6-Diamidino-2-phenylindole (DAPI; blue) has been used as a nuclear counterstain. Arrows denote microglia that have an apparent altered morphology. MHCII immunostaining (red color) was performed to determine microglial activation induced by WT AAV6 and TM6 (Y731F + Y705 + T492V) capsids expressing empty vector or scCBA-GFP (arrows, **e**). Data are representative of three independent transduction experiments. Bar = 200 µm (**a**, **d**); bar = 100 µm (**e**). *$P < 0.05$; ***$P < 0.001$, ANOVA analysis. MHCII, major histocompatibility complex II.

F4/80 and CD68 promoter–driven gene expression in primary microglia by rAAV-TM6

We have demonstrated that capsid-modified TM6 can efficiently transduce microglia as well as neurons and astrocytes. To enable selective microglia targeting, we incorporated two different microglia-specific promoters, F4/80 and CD68, in the scTM6-GFP vector construct (Table 1c,d). F4/80 antigen is an adhesion G protein–coupled receptor present on murine mononuclear phagocyte surface, whereas CD68/macrosialin is an intracellular glycoprotein present in lysosomes.[17] Both F4/80 and CD68 are upregulated in macrophages and microglia following activation and are widely used as myeloid-specific promoters in transgenic mice.[15] We tested these promoter constructs on primary mouse microglial cultures and observed high levels of EGFP expression using the TM6 capsid variant (Figure 2a). We consistently observed >95% transduction of microglial cells by either promoter in different viral batches. Both the scF4/80 and scCD8 promoter–driven TM6 viruses resulted in robust and comparable GFP expression in primary microglia (Figure 2b). To demonstrate specificity of microglia-restricted GFP expression from scF4/80 and scCD68 constructs, we also tested these viruses in primary mixed neuroglial cultures. Both the promoter constructs showed extremely selective microglial expression (arrows in Iba-1 colocalization panel, Figure 2c) with no neuronal (arrowheads in MAP2 panel, Figure 2d) or astrocytic (arrowheads in GFAP panel, Figure 2c) expression.

F4/80 and CD68 promoters drive microglia-selective gene expression in mouse brain by rAAV-TM6

Next, we examined whether the TM6 capsid variant is able to transduce microglia in mouse brains. For these experiments, we injected

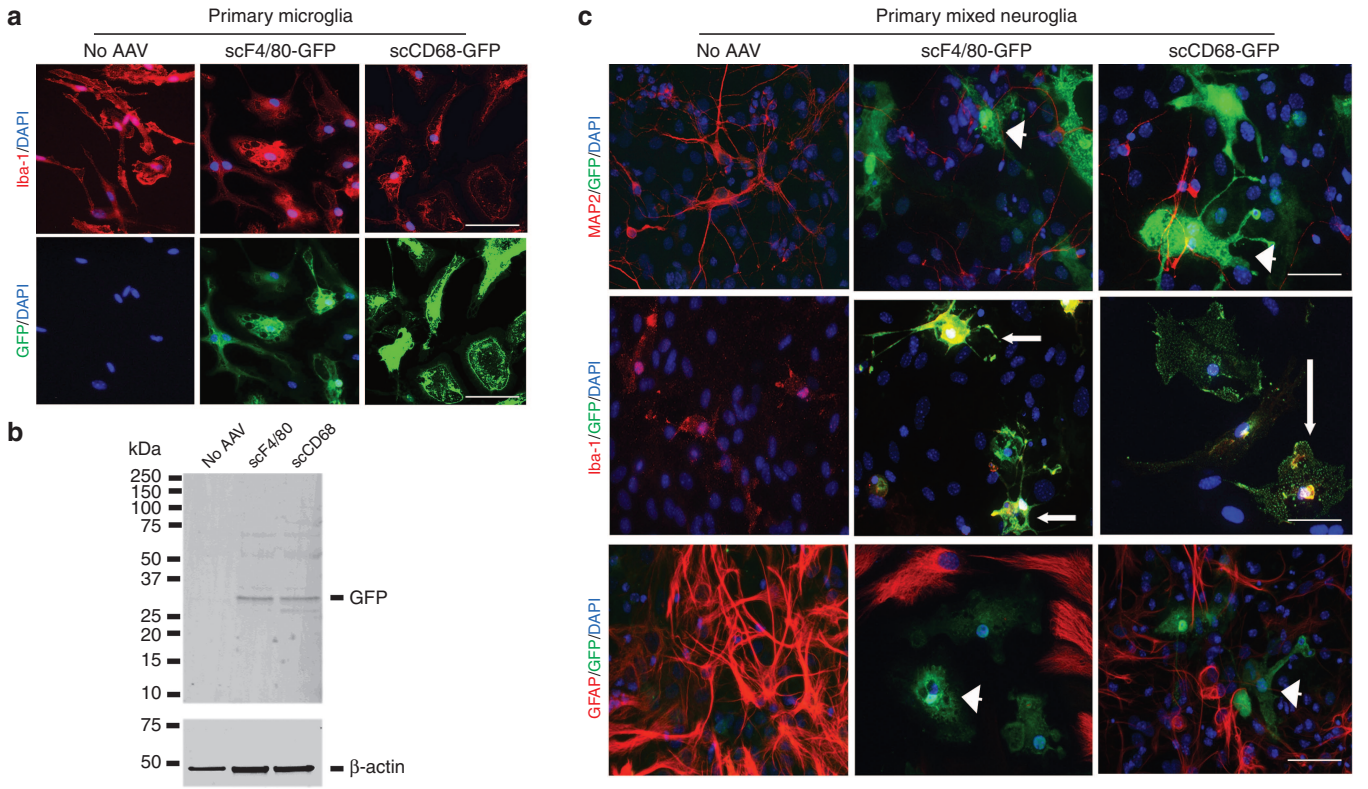


Figure 2 Robust and selective transduction of microglia by TM6 capsid–expressing scF4/80-GFP and scCD68-GFP in primary cultures. scF4/80-GFP and scCD68-GFP viruses packaged in TM6 capsid were used to transduce primary microglia (**a**, **b**) or primary mixed neuroglia (**c**). Immunohistochemical analysis demonstrates robust F4/80- and CD68-driven GFP expression (green) in Iba-1 (ionized calcium-binding adapter molecule 1) immunopositive microglia (red) in primary microglia (**a**). Western blot depicting GFP expression in microglia transduced with scF4/80 and scCD68 viruses (**b**). β-Actin depicts total protein loaded in each lane, and kDa refers to molecular weight standards. (**c**) Microglia-selective targeting by these promoter/capsid combinations is shown by colocalization of GFP (green) immunofluorescence in Iba-1-positive microglia only (red color, arrows, Iba-1 panel) in primary neuroglial cultures; GFP does not colocalize with MAP2 (microtubule-associated protein 2; neuronal marker, red) or glial fibrillary acidic protein (GFAP; astrocyte marker, red) immunoreactivity (arrowheads, **c**) in these cultures. 4′,6-Diamidino-2-phenylindole (DAPI; blue) has been used as a nuclear counterstain. Data are representative of two independent replicate experiments. Bar = 100 µm (**a**); bar = 50 µm (**c**).

TM6 virus expressing scCBA-GFP, scF4/80-GFP, and scCD68-GFP in the cerebral ventricles of WT mouse pups on neonatal day P0 or in the hippocampus of 2- to 3-month-old WT adult female mice. Intracerebroventricular injections of rAAV in neonatal day P0 mice lead to extensive brain transduction and transgene expression.[4] On the other hand, hippocampal stereotaxic injections of AAVs in adult mice lead to restricted transgene expression in the hippocampus and overlying cortex.[18] Brains were analyzed by immunohistochemistry to detect cells with microglial morphology (Figure 3a) and confirmed by immunofluorescence showing colocalization with Iba-1 (microglia-specific marker) (Figure 3b). Injection of scCBA-GFP in neonatal P0 pups resulted in widespread GFP expression in the forebrain after 15- and 30-day postinjection (Figure 3a and Supplementary Figure S4). The majority of cells transduced were neurons (Figure 3a, arrowhead and Supplementary Figure S4b), though few microglia expressing GFP were also seen (Supplementary Figure S4c). GFP was detected from neurons as well as microglia in adult mice injected in the hippocampus with scCBA-GFP and analyzed after 15 days (Figure 3, arrow). Injection of scF4/80-GFP and scCD68-GFP in neonatal pups resulted in microglia-restricted expression of GFP when analyzed after 15 and 30 days (Figure 3a,b, arrow). F4/80 and CD68 promoter–driven GFP–expressing microglia were observed to be clustered around the ventricles and overlying cortex in the P0-injected cohorts (~50–60 microglia per 10-µm cryosection). In the hippocampus-injected adult cohort, few microglia-expressing GFP from these promoter-specific constructs were noticed in the hippocampal area or white matter tracts overlaying the hippocampus (Figure 3a,b, arrow). Quantitative analysis of cortical microglia shows that P0 delivery of scCBA and scF4/80 promoter constructs result in higher transduction rates compared with the scCD68 promoter construct (Figure 3c), suggesting that overall CBA and F4/80 promoters are more efficient than CD68 under these conditions. Using the scCBA promoter resulted in transduction of both microglia (Iba+ GFP+) and non-microglia cells (Iba− GFP+), and under certain conditions, such as P0 →30 days and adult delivery paradigms, most of the AAV-transduced cells were not microglia (Figure 3c). On the other hand, both scF4/80 and scCD68 constructs resulted in GFP expression from exclusively Iba+ microglia cells, showing that these promoters drive microglia-selective gene expression *in vivo* (Figure 3c). Since we observed MHCII upregulation in primary microglia transduced with these constructs, we tested for MHCII immunoreactivity in scCBA-TM6 injected brains to explore whether there are any similar changes in microglial activation following viral delivery. Overall, MHCII immunostaining was rarely observed in these mice brains, including naive and injected brains; we did not observe any MHCII immunostaining in TM6 transduced microglia *in vivo* (Supplementary Figure S5).

In order to test whether TM6-targeted microglia–specific transgene expression can be used to alter immune activation state in the brain, we injected scCD68-IL6 in neonatal WT mice. In primary

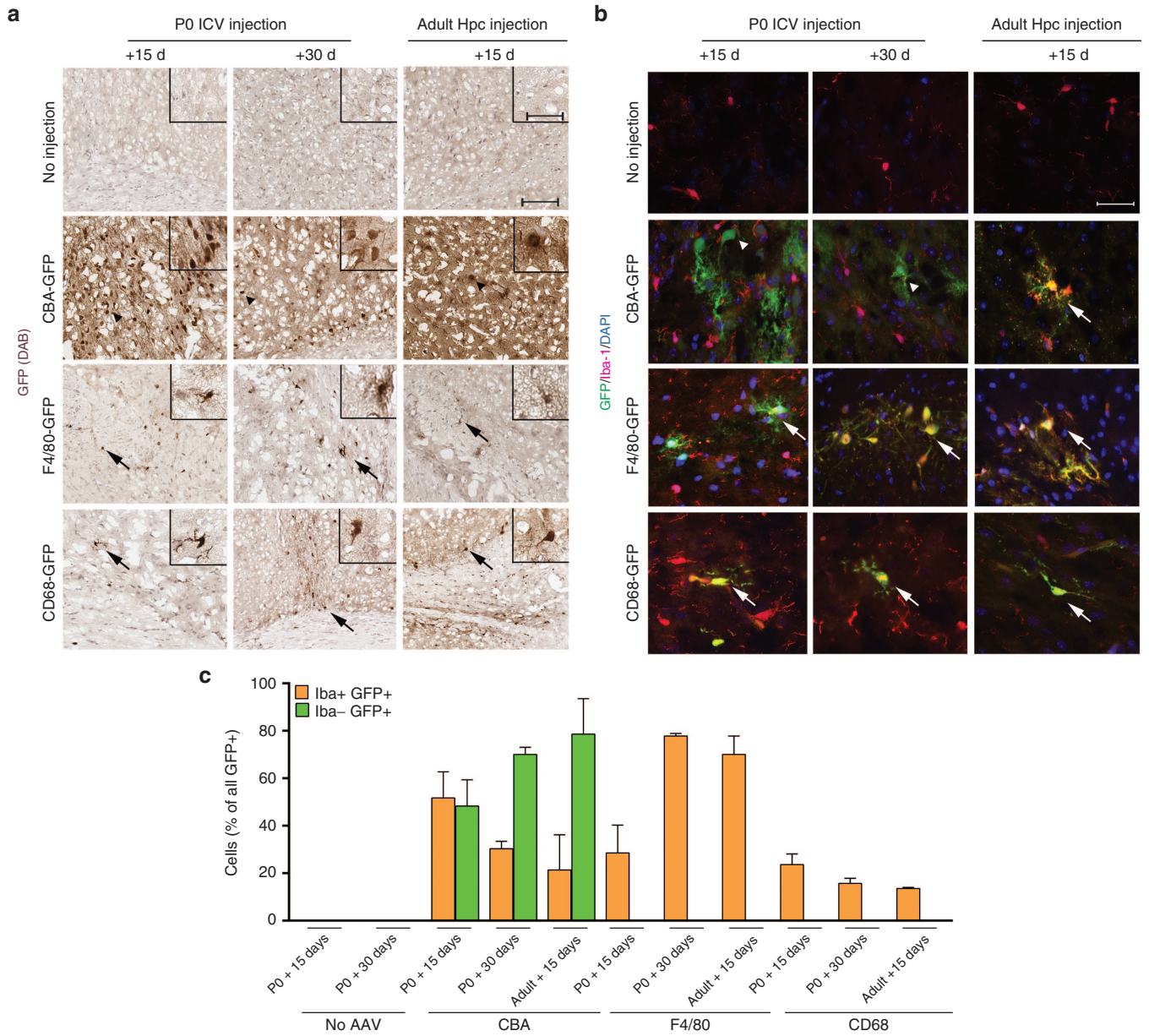


Figure 3 *In vivo* transduction of scF4/80-GFP and scCD68-GFP packaged in TM6 results in exclusive microglial expression of GFP. Wild-type (WT) mice were injected in the cerebral ventricles on neonatal day P0 with TM6 viruses expressing GFP transgene from different promoters (chicken β actin (CBA), F4/80, or CD68) and analyzed at P15 and P30 (P0 intracerebroventricular (ICV) cohort, +15 days and +30 days, respectively). An additional cohort of 2- to 3-month-old WT mice were injected in the hippocampus (Hpc) with these same viruses and analyzed after 15 days (adult Hpc cohort, +15 days). Immunohistochemical analysis with DAB (brown color) for GFP shows cells with typical microglia-like morphology in the cohorts injected with F4/80 and CD68 promoter constructs (arrows, **a**), but mostly neuronal transduction in the CBA promoter construct (arrowhead, **a**). Representative immunofluorescent pictures shows colocalization of GFP (green) with microglia-specific Iba-1 (red) epitope (arrows, **b**). Arrowheads depict GFP expression in non-microglia cells in CBA promoter–driven viruses (**b**). 4′,6-Diamidino-2-phenylindole (DAPI; blue) has been used as a nuclear counterstain (**b**). Total number of transduced microglia (Iba-1+ and GFP+) and transduced non-microglia cells (Iba-1− and GFP+) were counted in mice brains following coimmunostaining with Iba-1 and GFP (**c**) and presented as fraction of all AAV-transduced cells (GFP+, mean ± standard error of mean). At least, five independent fields of view from each mouse cortex in each injection group were averaged. Three groups were analyzed for the injected cohorts—P0-injected mice euthanized at 15 and 30 days, and adult mice injected intrahippocampally and euthanized after 15 days. No AAV represents uninjected control mice. Bar = 150 µm (**a**, main panel); bar = 15 µm (**a**, inset, and **b**, main panel). $n = 3$ mice/group.

microglia culture, transduction with scCD68-IL6 results in increased IL-6 levels (RNA: 214.99-fold over control; protein: 250.82 ± 5.1 pg/ml; $P < 0.01$, Student's *t*-test). Neonatal day P0 mouse pups were injected in the cerebral ventricles with scCD68-IL6 vector and analyzed after 15 days. This led to increased IL-6 levels in the brain (RNA: 7.5× over uninjected mice; protein: 121% over uninjected mice; $P = 0.05$, Student's *t*-test), increased microgliosis (lectin staining, Figure 4a), and increased astrocytosis (GFAP immunostaining, Figure 4b). The astrogliosis was restricted to the hippocampus, ventricles, and white matter tract (corpus callosum) overlying the hippocampus, whereas the cortex remained unaffected. This is consistent with P0 injections targeting the area around the cerebral ventricles.[4] GFAP protein levels were significantly increased in IL-6 expressing mice brains, demonstrating that TM6-scCD68–mediated IL-6 expression leads to substantial immune modification *in vivo* (Figure 4c).

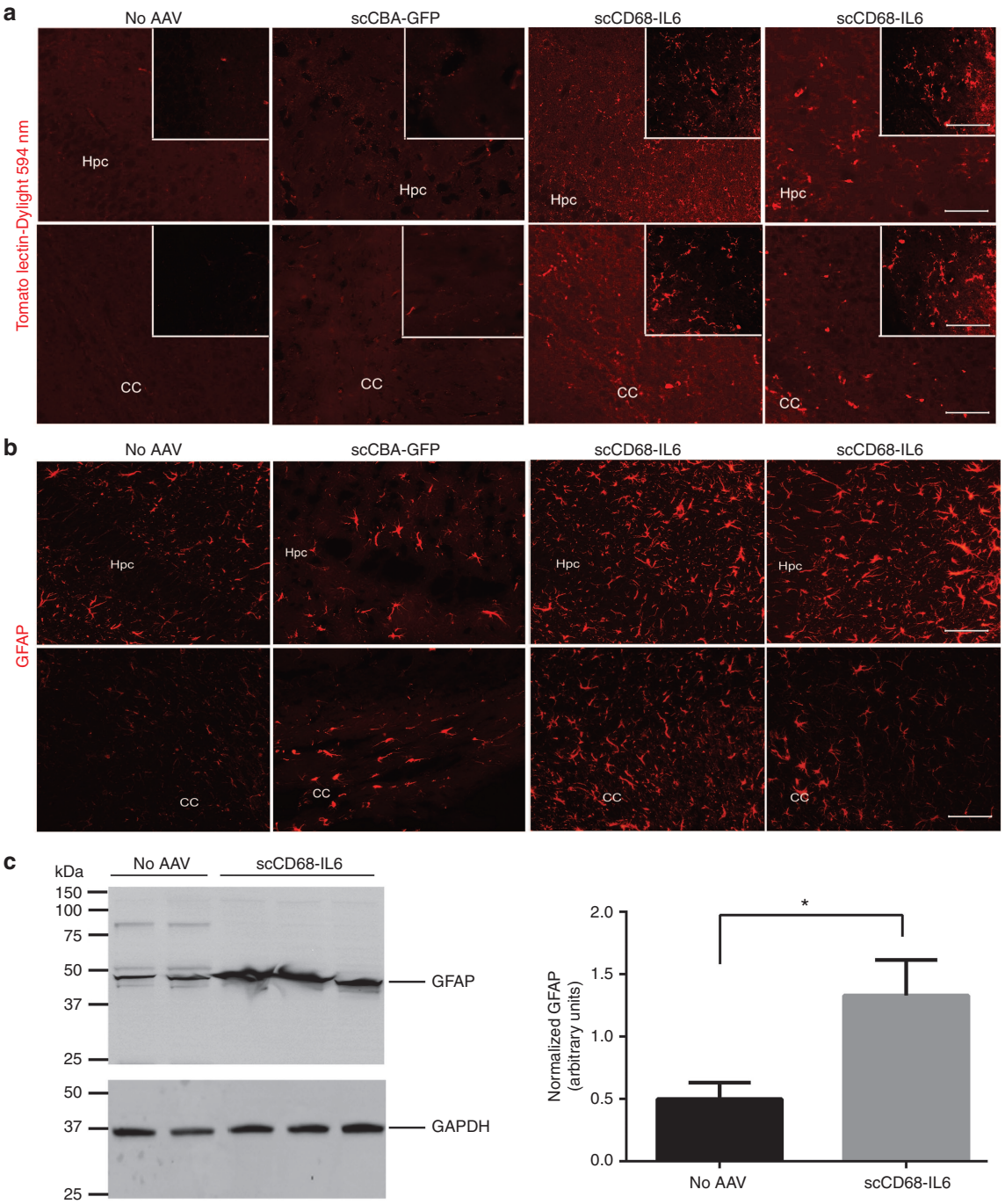


Figure 4 scCD68-IL6 transduction induces gliosis *in vivo*. Intracerebroventricular injection of scCD68-IL6 into neonatal wild-type mice leads to increased microgliosis (tomato lectin staining, red, **a**) and astrocytosis (glial fibrillary acidic protein (GFAP) immunostaining, red, **b**) in the hippocampus (Hpc) and overlying white matter tracts. Two independent controls have been used—naive uninjected mice and adult mice injected in the Hpc with scCBA-GFP packaged in TM6 capsid. Immunoblotting depicts increased GFAP protein levels (normalized to the housekeeping protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) in the interleukin-6 (IL-6) expressing mice compared with control uninjected mice (**c**). Bar = 300 µm (**a**, main panel); bar = 150 µm (**a**, inset, and **b**, main panel). CC, corpus callosum; Hpc (**a**, **b**). $n = 3$ mice/group. kDa refers to the molecular weight of protein standards. *$P < 0.05$, *t*-test.

DISCUSSION

rAAVs are being increasingly used as ideal gene therapy vehicles in preclinical studies and clinical trials in central nervous system diseases.[2] Multiple rAAV serotypes lead to efficient gene expression from neurons, oligodendrocytes, and astrocytes when injected into the brain, spinal cord, tail vein, or even hindlimb muscles of rodents (reviewed in ref. 2) but robust microglial gene targeting has not been reported. Inefficient targeting of microglia is a major roadblock in devising accurate models of microgliopathy and design of therapies against neurologic diseases marked by microglial activation. As more AAV serotypes are being naturally isolated or invented by *in vitro* rational design in recent years,[19] it is now possible to test these newer variants for microglia-restricted transduction properties. Here, we show that (i) the capsid-mutated TM6 serotype with

a sc vector sequence efficiently transduces neurons, astrocytes, and microglia in primary murine cultures and (ii) using F4/80 and CD68 promoters enables selective targeting of microglia by TM6 viruses *in vitro* and *in vivo*. This is the first study that demonstrates unique microglia tropism by TM6 and related capsid variants derived from AAV6. Our study establishes new technology platforms for microglial targeting by novel capsid-optimized rAAVs and opens up the possibility of designing more improved rAAVs with specific microglial biotransduction properties.

In spite of the advent of new generations of engineered AAVs with improved stability and transduction properties, there have been only two previous reports that have shown some degree of microglial targeting.[20,21] The first of these reports showed that WT AAV2 can transduce microglia in culture, albeit at low kinetic rates compared with neurons.[21] The other report demonstrated selective microglial targeting by AAV5 serotype using the F4/80 and to a lesser extent, the CD68 promoter.[20] However, we were unable to detect microglial transduction by either rAAV2 or rAAV5 in primary microglial cultures in this study. In addition, following intracerebroventricular delivery in neonatal mice, we have shown earlier that though both rAAV2 and rAAV5 can efficiently transduce neurons and astrocytes, they do not transduce microglia in the brain.[4]

Transgenic approaches using myeloid-specific promoters have been widely used to genetically model immune diseases or to test therapies designed against immunological diseases.[22] Since most of these promoters are active in all cells of hematopoietic lineage, exclusive targeting microglia by transgenic technology is challenging. In addition, presently available viral and nonviral methodologies display widely variable transduction efficiency for cells of myeloid lineage. Standard transfection methods achieve modest efficiency (~20%) of transgene expression *in vitro*,[23] though HIV-1–derived lentivirus–mediated gene delivery can, however, achieve high transduction efficiency *in vitro* and *in vivo*.[1] However, lentiviruses can potentially engage with the immune system, and because of their propensity to integrate randomly into the chromosome, this otherwise powerful gene targeting technology can result in variable gene expression in preclinical models and adverse outcomes such as leukemia in human patients.[24] On the other hand, AAV, especially the capsid variants, display widespread tolerability and relatively low immunogenicity.[2] Using a series of *in vitro* and *in vivo* experiments, Martino *et al*.[25] showed that by minimizing proteasomal trafficking in antigen-presenting cells, the triple capsid variant AAV2 (Y444F/Y500F/Y730F) potentially has lower antigen-presenting capabilities that, in turn, can prevent induction of innate immune responses and cytotoxicity. Similar studies have confirmed that though there is an initial and transient induction of immune response following virus transduction into monocytes, capsid-modified AAVs do not induce phenotypic alterations in the transduced cells.[26,27] More importantly, AAV-driven hepatic expression of transgenes can even drive immune tolerance by inducing hepatic Treg production.[28–30] Given such data, we expect that the triple-mutant AAVs used in our study would potentially share similar nonpathogenic and nonimmunogenic properties. Such a rAAV platform offers an attractive transgenesis paradigm for microglial targeting applications in neurodegenerative disorders without confounding side effects of peripheral immune alterations.

In our study, we have used three relatively novel strategies to ensure increased transgene expression in the microglia: the use of surface tyrosine–mutated capsids, sc vectors, and microglia-specific promoters. Recent studies exploring different aspects of molecular trafficking of AAVs have shown that phosphorylation of surface-exposed serine and tyrosine residues on AAV capsids results in rapid ubiquitination and proteasomal degradation of AAVs.[11,31] In addition, oxidation of surface tyrosine can impair externalization of the N-terminal portion of the capsid proteins.[32] Mutation of surface-exposed serine and tyrosines to phenylalanines can reduce post-translational modifications of the capsid proteins and thereby reduce viral clearance by intracellular pathways.[33] This can potentially improve nuclear transport of the viral particles and increase transgene expression.[27] For example, S663V/T492V variant of AAV6 was shown to be 5× more efficient in transducing mouse monocyte derived dendritic cells than WT AAV6.[9] Similar results were observed for other AAV capsid serotypes—a triple-mutant AAV2—Y444F/Y500F/Y730F vector led to robust higher levels of transgene expression *in vitro*[34] and *in vivo*.[33] Intriguingly, several reports have demonstrated that the proteasomal system appears to be more active in glia than in neurons,[35] which may partially explain the inability of native WT capsids to result in high levels of microglial gene expression in our study.

A second, rate-limiting step in rAAV-mediated gene expression is the conversion of the AAV genome into double-stranded DNA, once the viral genome enters the nucleus.[13] This can be circumvented by using a scAAV genome that consists of inverted repeats arranged in a way that facilitates the folding of the AAV genome into a double-stranded form without the requirement for DNA synthesis. Though packaging constraints impose size limits on the genes that can be delivered using this approach, scAAV vectors typically result in 20× to 100× more expression than conventional ss AAV vectors.[36]

Using either the F4/80 or CD68 promoter in combination with the surface-mutated AAV6 capsid, we could demonstrate >95% transduction of primary microglial cells in culture and selective microglial transduction *in vivo*. Whereas we could achieve high transduction rates with TM6 capsids in culture, the biodistribution of transduced microglia was restricted *in vivo*. Since microglial turnover rates in the normal brain are slow (in order of months or more[8,37]), it is difficult to explain the modest levels of microglial transduction *in vivo* compared with the excellent transduction properties of the TM6 viruses *in vitro*. One factor may be that the relative strength of the promoters used (F4/80 and CD68) is inherently low in the mouse brain and that our current detection methods are unable to detect such low levels of transgene expression. Though F4/80 and CD68 promoters are commonly used promoters,[17] recent RNA-sequencing studies have shown that neither of these promoters are highly active in the resting central nervous system.[38] This may have contributed to the modest levels of transgene expression following scGFP and scCD68-IL6 transduction of mouse brains in our studies, compared with our earlier observations using hCBA promoter.[4,18] Future manipulations of mouse microglia using other highly active microglial promoters could enhance transgene expression following transduction by capsid-modified rAAV6. In addition, understanding the exact mechanism that allows for high microglial transduction by the capsid variants and potential pathologic effects of long-term transgene expression affecting microglial homeostasis need to be characterized further *in vivo*. In summary, our studies will pave the way toward the development of improved engineered AAV vectors for selective microglial targeting as a means to explore microglial function in health and disease.

MATERIALS AND METHODS

Cloning and rAAV preparation

A pAAV vector containing a hybrid cytomegalovirus enhancer/CBA promoter, a CBA intron (first intron of CBA gene plus the splice acceptor of the rabbit β-globin gene),[39] woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), and the EGFP transgene was used as a template for

further subcloning (Table 1). This vector was modified by swapping in a modified SV40 late 16S rRNA intron in place of the CBA intron and also deleting the terminal resolution site sequence from the inverted terminal repeats to produce a mutant inverted terminal repeats, such that the viral Rep protein cannot generate the ss DNA nick. This resulted in a double-stranded sc pAAV vector.[40] To facilitate subcloning in this sc vector, a multiple cloning site was introduced[41], and the SV40 intron was replaced by the minute virus of mouse intron (MVMi).[36] This scAAV vector expressing humanized GFP formed the template for expression vectors driven by the F4/80 or CD68 promoters. The F4/80 promoter was released from pAAV-F4/80-RFP (kind gift from EF Terwilliger[20]) by XbaI (blunt ended) and SalI digestions and used to replace the hCBA promoter region of the scAAV vector digested with KpnI (blunt ended) and SalI to produce the scF4/80-GFP construct. To produce the scCD68-GFP construct, the hCBA promoter was released from the scAAV vector and the free ends modified by PCR to yield 5′-Kpn I and 3′-Sal I sites, into which the CD68 promoter region was ligated from pAAV-CD68-RFP (kind gift from EF Terwilliger[20]). Mouse IL-6 cDNA[18] was swapped with the GFP sequence in the scCD68-GFP vector using EcoRV and NheI restriction sites producing scCD68-IL6. The scAAV plasmids were cotransfected along with rep and cap plasmids (modified AAV6 capsid plasmids encoding S663V, S663V/T492V, and Y731F/Y705F/T492V mutations George Aslanidi and Arun Srivastava, University of Florida) into HEK293T cells. rAAV was prepared under sterile endotoxin–free conditions by methods described earlier using cell stacks.[42] Briefly, 180 µg of endotoxin-free vector DNA (Qiagen, Valencia, CA) was used to transfect HEK293T cells in two-chambered cell stack (Costar Product #3269, Thermo Fisher Scientific, Waltham, MA) and purified using iodixanol gradient into a final volume of ~200-µl sterile endotoxin–free phosphate-buffered saline (PBS) buffer. Genomic titers were determined by quantitative PCR as described earlier[4,43] (Table 2). The following custom primers corresponding to GFP was used: forward primer, 5′GAAACATTCTCGGCCACAAG; reverse primer, 5′TTGTCGGCCATGATGTACAC. For IL-6 quantitative reverse transcription–PCR, the following probe/primer combinations were used: Probe #6 (catalog # 04685032001, Roche Universal Probe Library, Roche, Indianapolis IN), forward primer, 5′GCTACCAAACTGGATATAATCAGGA; reverse primer, 5′CCAGGTAGCTATGGTACTCCAGAA. Quantitative PCR for bGH was carried out using custom TaqMan probe (5′TGCCAGCCATCTGTTGTTTGCC) and primers (forward: 5′CCTCGACTGTGCCTTCTAG and reverse: 5′TGCGAT GCAATTTCCTCAT). All the primers were ordered from Integrated DNA Technologies (Coralville, IA). The purity of the AAVs were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis for detecting bands corresponding to the viral structural proteins, VP1, VP2, and VP3. Typical genomic titers of purified viruses are presented in Table 2.

Mixed neuroglia and microglia primary cultures

All animal husbandry procedures were performed following approval by University of Florida Institutional Animal Care and Use Committee. Primary neuroglia and microglia cultures were prepared by isolating mouse cortices from B6/C3H newborn mice (neonatal day P0–P2) as described earlier.[18] Mixed neuroglia were maintained in Neurobasal media (Life Technologies, Carlsbad, CA) supplemented with 0.02% NeuroCult SM1 (STEMCELL Technologies, Vancouver, British Columbia, Canada), 0.5 mmol/l GlutaMax (GIBCO, Life Technologies, Carlsbad, CA), 0.5% fetal bovine serum (Hyclone, GE Life Sciences, Pittsburgh, PA), and 0.01% Pen-strep (GIBCO, Life Technologies). Neuroglial cultures were plated in CC2 Lab Tek II 8-chamber slide (Fisher Scientific, Waltham, MA). The mixed microglia–astrocyte cultures were maintained in Tripleflasks (Thermo Fisher Scientific) with 200 ml of 5% Dulbecco's modified Eagle's medium–containing serum. After 10–14 days of incubation, the flasks were shaken for 30 minutes at 37°C at 150 rpm to dislodge the microglia from the astrocyte layer and plated in Nunc Lab-Tek II CC2 chamber slides (Thermo Fisher Scientific). All cells were maintained at 37°C in a humidified incubator with 5% CO_2. Microglia or neuronal cultures were transduced with purified rAAVs (1×10^8 vector genomes total for sc and ss viruses, Table 2) for 5 days. Direct fluorescence pictures were captured using Evos FL Cell Imaging System (Life Technologies, New York, NY) and analyzed using ImageJ 1.47b (NIH, Bethesda, MD).

Table 2 Typical genomic titers (expressed in vg/ml of purified viruses) of AAV constructs used in the study

Virus	*Capsid*	*Titer (vg/ml)*	*Experiment*
scCD68-IL6	AAV6-TM6	2.07 E12	*In vivo*, *in vitro*
scCD68-GFP	AAV6-TM6	1.42 E11	*In vivo*, *in vitro*
scF4/80-GFP	AAV6-TM6	1.86 E11	*In vivo*, *in vitro*
scCBA-GFP	AAV6-TM6	1.1 E11	*In vivo*, *in vitro*
scCBA-GFP	AAV6-WT	1.5 E11	*In vitro*
scCBA-GFP	AAV6-S663V	1.0 E11	*In vitro*
scCBA-GFP	AAV6-S663V/T492V	1.0 E11	*In vitro*
ssCBA-EGFP	AAV1–9, -rh10	1.0 E13	*In vitro*

The different constructs containing GFP or IL-6 transgenes were packaged in different AAV capsids. TM6 refers to a triple-mutant AAV6 capsid (Y731F/Y705F/T492V). All the constructs were used at 1E11/ml following dilution in sterile PBS. AAV, adeno-associated viruses; EGFP, enhanced green fluorescent protein; IL-6, interleukin-6; PBS, phosphate-buffered saline; vg, vector genomes.

AAV injections in mice

Intracerebroventricular injections of rAAVs were carried out in neonatal mice (B6/C3HNhsd, Envigo, Indianapolis, IN) on day P0 as described earlier.[4] Two-month-old naive B6/C3HNhsd mice were also stereotaxically in the hippocampus with rAAV as described earlier.[18] In both cases, purified 2×10^8 viruses (2 µl in PBS) were administered bilaterally. After 15 or 30 days, mice were euthanized by transcardiac perfusion, brains harvested, and fixed in 10% normal buffered formalin (Thermo Fisher Scientific) and then transferred to 30% sucrose solution in PBS for cryosectioning.

Quantitative PCR analysis

Total RNA from mouse brain was purified using Trizol and RNeasy kit (Qiagen, Gaithersburg, MD) and reverse transcribed using Superscript III (Invitrogen, New York, NY). Quantitative reverse transcription–PCR was performed using custom primer/probe mix. Probes (from Roche Universal Probe library: #6 (cat. no. 04685032001) for IL-6) were labeled at 5′ end with Fluorescein (FAM) and at the 3′ end with dark blue quencher. Target-specific primer sequences were ordered from Integrated DNA Technologies (IL-6: forward primer: GCTACCAAACTGGATATAATCAGGA; reverse primer: CCAGGTAGCTATGGTACTCCAGAA). The reaction utilized the SSoFast EvaGreen supermix (Bio-Rad, Hercules, CA) and used the following steps: initial denaturation cycle 95 °C/30 seconds, followed by 39 amplification cycle of 95 °C/5 seconds and 60 °C/5 seconds.

Immunocytochemistry, immunohistochemistry, and immunoblotting analyses

For immunocytochemical analysis, fixed primary cultures were incubated in primary antibody overnight at 4 °C (Iba-1 (1:1,000; Abcam, San Francisco, CA), MHCII (1:500; Abcam), GFAP-Cy3 (1:1,000; Sigma–Aldrich, St Louis, MO), MAP2 (1:1,000; Cell Signaling, Beverly, MA), cd11b (1:500; Novus, Littleton, CO), MHCII (IBL-5/22) (1:250; Santa Cruz, Dallas, TX), and EGFP (1:1,000; Life Technologies, Carlsbad, CA)). Cells were washed three times with PBS, and fluorescence-conjugated secondary antibody (Life Technologies) was added and incubated for 1 hour at room temperature. Nuclei were stained with 4′,6-Diamidino-2-phenylindole (Southern Biotech, Birmingham, AL).

Formalin-fixed, sucrose-saturated mouse brains were frozen in optimum cutting temperature compound (Tissue Tek, Torrance, CA) and sliced into 10-µm coronal sections. Sections were blocked in 2.5% horse serum (Vector Biolabs, Burlingame, CA) for 1 hour at room temperature and incubated in primary antibody to Iba-1 (1:1,000; Abcam), GFAP-Cy3 (1:1,000; Sigma–Aldrich), and EGFP (1:1,000; Life Technologies) overnight at 4 °C. Bound antibodies were detected with either fluorescent-conjugated secondary antibodies (1:500; Life Technologies) or ImmPress reagents (Vector Labs) for visualization with 3,3′-diaminobenzidine (DAB) colorimetric assays. DyLight-594 labeled *Lycopersicon esculentum* (tomato) lectin (10 µg/ml, Vector Biolabs) staining was carried out using manufacturer's instructions. Images were captured using Olympus Microscope or Aperio whole-slide imager. Brightness and contrast alterations were applied identically using Photoshop CS5.

GFP-immunopositive microglia were manually counted to determine the extent of transduction *in vivo*. For each cohort, at least five independent fields of view (20× field of view, Olympus DP71 microscope) from the cortex of injected mice were counted, averaged over all the mice in the cohort (n = 3), and presented. Iba-1 immunostaining was used to determine the

total microglia count in the same sections using double immunofluorescence staining.

For immunoblotting, anti-GFAP (1:1,000; Sigma–Aldrich), anti-EGFP (1:1,000; Life Technologies, NY), anti-β-actin (1:1,000, Sigma–Aldrich), and anti-glyceraldehyde 3-phosphate dehydrogenase (1:2,000; Abcam) were used as primary antibodies and signals detected by fluorescent secondary antibodies using Li-Cor Biosciences Odyssey system (Lincoln, NB).

Flow-sorting analysis

Primary microglia were transduced in a six-well plate with viruses for 5 days, fixed in 10% normal buffered formalin (Fisher Scientific, Pittsburgh, PA) containing 0.05 M sucrose, washed in sterile PBS, and collected using a cell scraper. Fixed microglia were resuspended in PBS containing 1% bovine serum albumin (Sigma–Aldrich). Flow sorting was carried out using BD Accuri C6 Cytometer (BD Biosciences, San Jose, CA) at the University of Florida Interdisciplinary Center for Biotechnology Research.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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