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Insulin-Like Growth Factor 2 Receptor Is an IFNγ-Inducible Microglial Protein that Facilitates Intracellular HIV Replication

Implications for HIV-Induced Neurocognitive Disorders

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Insulin-like growth factor 2 receptor (IGF2R), also known as cation-independent mannose 6-phosphate (M6P) receptor, is a transmembrane glycoprotein localized in the *trans*-Golgi region and is involved in targeting both M6P-bearing enzymes and IGF2 to the lysosomal compartment. During development, IGF2R plays a crucial role in removing excess growth factors from both tissue and blood. Due to the perinatal lethality of the global *Igf2r* knockout, the function of IGF2R in adults, particularly in the CNS, is not known. We made a novel observation that IGF2R is highly expressed in microglial nodules in human brains with HIV encephalitis. *In vitro*, microglial IGF2R expression was uniquely enhanced by IFNγ among the several cytokines and TLR ligands examined. Furthermore, in several *in vitro* models of HIV infection, including human and murine microglia, macrophages, and nonmacrophage cells, IGF2R is repeatedly shown to be a positive regulator of HIV infection. IGF2R RNAi also down-regulated the production of the IP-10 chemokine in HIV-infected human microglia. Injection of VSVg *env* HIV into mouse brain induced HIV p24 expression in neurons, the only cell type normally expressing IGF2R in the adult brain. Our results demonstrate a novel role for IGF2R as an inducible microglial protein involved in regulation of HIV and chemokine expression. Mice with the *Csf1r*- driven *Igf2r* knockout should be useful for the investigation of macrophage-specific IGF2R function. *(Am J Pathol 2010, 177:2446–2458; DOI: 10.2353/ajpath.2010.100399)*

The insulin-like growth factor (IGF) system consists of growth peptides (insulin, IGF1 and IGF2), their receptors (insulin receptor, IGF1R and IGF2R), and circulating binding proteins (IGFBPs 1-7).[1–3] Ligand binding activates the cytoplasmic protein tyrosine kinases of IGF-related receptors (IGF1R and insulin receptor) which then stimulates an intracellular cascade of signaling pathways, leading to the growth and metabolic effects.[2,4] In contrast, aside from rare reports,[5] IGF2R is not known to transduce signals.[6] IGF2R is a large molecule (~300 kDa) with a bulky extracellular domain with binding sites for IGF2 and mannose-6-phosphate (M6P), and a short cytoplasmic tail with amino acid sorting motifs.[6] The main physiological functions of IGF2R are i) regulation of circulating and tissue IGF2 levels[7,8] and ii) trafficking of newly synthesized M6P-containing lysosomal enzymes from the *trans*-Golgi network to lysosomes, hence the name cation-independent M6P receptor (MPRci).[6,9,10] This latter function is shared by another M6P receptor, the cation-dependent M6P receptor (MPRcd). In addition to the high affinity binding between the cognate receptors and the ligands, there is also a low affinity (~1/10) binding among other members of the receptors and li-

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gands of the IGF family. For example, IGF2 binds to IGF1R and insulin receptor, and IGF1 binds to IGF2R and insulin receptor.[4,11]

During development, IGF2R plays an essential role in degrading extracellular IGFs, preventing its binding and signaling through IGF1R and insulin receptor, as shown in global *Igf2r* KO mice that are perinatally lethal due to organ overgrowth.[7,12,13] However, due to the perinatal lethality of the KO mice, the postnatal function of this receptor is largely unknown. A cell type–specific or organ-specific deletion of IGF2R is necessary to determine the postnatal function of this receptor in physiological and pathological processes. For example, Wylie et al have investigated liver- and skeletal muscle–specific *Igf2r* functions by creating organ-specific KO mice using the Cre-lox technology.[14]

The importance of the IGF system in the maintenance of healthy brain function is widely recognized.[1,15] In normal CNS, neurons express abundant IGF2R but other cells are devoid of IGF2R expression.[16] IGF2R expression has been examined in a few human neurological conditions, and these studies reported various results ranging from a lack of expression in multiple sclerosis plaques[16] to expression in reactive astrocytes in senile plaques of Alzheimer's brains.[17] In the periphery, various viral infections have been associated with abnormal IGF systems. Specifically, reduced serum IGF1 and IGF2 levels have been found in individuals with HIV infection (and associated with disease progression), as well as infection with other viruses (herpes simplex virus, cytomegalovirus, rotavirus and adenovirus).[18–21] Furthermore, reduced IGF2 expression is reported in the brains of SHIV-infected macaques.[22] Despite many studies suggesting a link between the IGF system and the pathogenesis of CNS diseases including HIV infection,[23–25] there are no studies specifically examining the role of IGF2R in CNS infections.

Our study was inspired by an observation that IGF2R expression was robustly induced in microglial nodules in HIV encephalitis (HIVE). In the current study, we extended these findings in HIVE and further tested the hypothesis that IGF2R is an inducible microglial protein that plays a role in viral infection and inflammation. Our results indeed reveal a novel role for IGF2R as an inducible microglial protein involved in intracellular HIV replication. IGF2R might be considered as a new therapeutic target for HIV/AIDS.

Materials and Methods

Human Brain Tissues

Postmortem human brain tissue sections were obtained from the National NeuroAIDS Tissue Consortium (NNTC). They consisted of paraffin-embedded sections of frontal cortex and white matter from HIV^- individuals (HIV^-), HIV^+ individuals with or without HIV encephalitis (HIVE and HIV^+, respectively). The number of cases examined were HIV^- ($n = 5$), HIV^+ ($n = 5$), and HIVE ($n = 7$). Detailed clinical and postmortem pathological information on the cases are previously described.[26,27]

Tissue Immunohistochemistry

We used standard immunohistochemistry methods for paraffin-embedded tissue sections using antigen-retrieval by heat treatment in citrate buffer, as described.[27] The affinity purified rabbit anti-IGF2R/MPRci (HL5299) was obtained from Dr. Peter Lobel, Rutgers University, NJ. The antibody was raised against the extracellular domain of the bovine IGF2R. HIV infection was determined by anti-HIV gag p24 stain. Double labeling with cell-specific markers was performed for CD68 (lysosomal marker of microglia and macrophages), CD45 (membrane marker for macrophages, microglia, and lymphocytes), GFAP (astrocytes-specific intermediate filament protein), and neuN (neuronal nuclear protein). Mouse anti-neuN IgG1 (clone A60) was purchased from Millipore (Temecula, CA) and was used at 1:500. Procedures for other antibodies are previously published.[27] We combined labeled secondary (peroxidase-labeled or alkaline phosphatase-labeled) antibodies with various chromogens (diaminobenzidine, nitroblue tetrazolium and VIP) to develop brown, blue, or red color.

ImageJ Analysis

For quantitative analysis of IGF2R immunostain, single-labeled slides were analyzed for the percent positive area stained with the NIH ImageJ software, as previously described.[27] Because IGF2R is normally expressed in neurons but not in white matter, white matter areas were randomly photographed at ×400 magnification (15–20 per case) from a total of 14 cases. Three cases were excluded from analysis due to high background staining which interfered with image analysis.

MeWo Cells

MeWo cells (stable IGF2R knocked down melanoma cell lines: KD),[28] and control MeWo cells (WT) were generously provided by Dr. Michael Gershon, Columbia University, NY. Single cell suspension was plated at 1-10 × 10^6 cells per ml in DMEM supplemented with 5% FCS and penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml) (Gibco, now Invitrogen, Carlsbad, CA), in the presence of 2 μg/ml puromycin.

Human Fetal Microglial Culture

Human CNS cell cultures were prepared from human fetal abortuses as described.[29] All tissue collection was approved by the Albert Einstein College of Medicine Institutional Review Board. Primary mixed CNS cultures were prepared by enzymatic and mechanical dissociation of the cerebral tissue followed by filtration through nylon meshes of 230- and 130-μm pore sizes. Single

cell suspension was plated at 1-10 $\times$ 10^6 cells per ml in DMEM supplemented with 5% FCS and antibiotics for 2 weeks, and then microglial cells were collected by aspiration of the culture medium. Monolayers of microglia were prepared in 60-mm tissue culture dishes at 1-2 $\times$ 10^6 cells per 5 ml medium or in 96-well tissue culture plates at 1–2 $\times$ 10^4 cells per 0.1 ml medium per well. Two to four hours later, cultures were washed to remove nonadherent cells (neurons and astrocytes). Microglial cultures were highly pure consisting of >98% $CD68^+$ cells.

Generation of the Csf1r-Igf2r *KO Mice*

To create macrophage-specific *Igf2r* KO mice, a transgenic mouse line in which Cre recombinase is expressed under the promoter and the upstream enhancer of the CSF-1 receptor gene (*Csf1r*) to direct macrophage-specific expression, designated *Tg(Csf1r-icre)1jwp* (gift of Dr. Jeffrey W. Pollard, Albert Einstein College of Medicine) was used. Female mice of mixed genetic background (Sv129/C57Bl), heterozygous for the floxed *Igf2r* exon 10,[14] were obtained from Dr. Randy Jirtle, Duke University and were crossed with male *Csf1r Cre* mice to create litters in which 50% inherited a floxed allele and another 50% a wild-type (WT) allele maternally. Because the mouse (but not human) IGF2R paternal allele is normally silenced through imprinting, this strategy is expected to produce macrophage-specific KO mice and WT littermates at equal ratios. A PCR-based assay was used to determine the presence of the loxP site in IGF2R intron 9 as previously described.[14]

Isolation of Bone Marrow–Derived Macrophages (BMDM) from Csf1r-Igf2r *KO and WT Mice*

BMDM were isolated following a standard protocol. Briefly, femur and tibia were flushed and cell pellets were prepared in Hanks' balanced salt solution. Cells were resuspended and cultured in 60-mm plastic Petri dish in DMEM with 10% FCS and antibiotics, supplemented with 10,000 U/ml of recombinant human M-CSF (R&D, Minneapolis, MN) for 10–14 days. Real-time PCR was performed to confirm the presence or absence of *Igf2r* mRNA expression in the KO and WT macrophage cultures.

Preparation of Murine Microglia and Astrocyte Cultures

Murine microglia and astrocyte cultures were established from the neonatal (P1) brains using the standard protocol with minor modifications.[30,31] Briefly, brains were freed of meninges and then gently triturated using plastic pipettes. Cell suspensions were then incubated with trypsin/EDTA at 37°C for 25 minutes, washed, and filtered through the 100 μm nylon mesh. The mixed single cell suspensions were plated at a density of 10^5 cells per ml in complete medium (DMEM 5% FCS with antibiotics). Ten to 14 days later, microglia were pooled by gently tapping the dish and collecting the floating cells, as described for human microglia. They were resuspended and reseeded in fresh medium supplemented with M-CSF. Astrocyte cultures were generated by repeatedly trysinizing the remaining monolayer until the cultures were devoid of $CD11b^+$ microglia.

HIV Infection

MeWo cells, human microglia, murine BMDM, murine microglia, and astrocyte cultures were infected with single-cycle competent, vesicular stomatitis virus (VSVg) *env* pseudotyped HIV, as previously described.[32] VSVg *env* HIV was produced by cotransfecting 293T cells with $pHIV_{NL4.3}$ (Nef-intact, Vpr-intact, Env-deficient, gift of Dr. Maurizio Federico[33]) and pVSVg *env*. Cells were infected with ~40 ng/ml p24 viral input that resulted in 25% to 50% cell infection at three days post inoculation, unless otherwise indicated. For electron microscopy of progeny HIV virions in MeWo cells, chimeric *env* HIV were generated by cotransfecting 293T cells with $pHIV_{NL4.3}$ (Env-intact) and pVSVg *env*. The resulting virus has both HIV and VSVg *env* proteins.

Table 1. Human and Mouse Primers Used for Q-PCR

Gene	Primer (forward)	Primer (backward)
Human primers		
GAPDH	5′-CGCTCAATGACAATTTCGTG-3′	5′-ACCCGGTGACTGTAGCCATA-3′
PBDA	5′-ACGATCCCGAGACTCTGCTTC-3′	5′-GCACGGCTACTGGCACACT-3′
IGF2R	5′-GAAGGTGAAGGTCGGAGT-3′	5′-GAAGATGGTGATGGGATTTC-3′
HIV gag	5′-TGCTATGTCAGTTCCCCTTGGTTCTCT-3′ (40)	5′-AGTTGGAGGACATCAAGCAGCCATGCAAAT-3′
Mouse primers		
Gapdh	5′-TTGATGGCAACAATCTCCAC-3′	5′-CGTCCCGTAGACAAAATGGT-3′
b-actin	5′-TGCACCACCAACTGCTTAG-3′	5′-GGATGCAGGGATGATGTTC-3′
Igf2r	5′-GGGAAGCTGTTGACTCCAAAA-3′	5′-GCAGCCCATAGTGGTGTTGAA-3′
Igf1	5′-CTGGACCAGAGACCCTTTGC-3′	5′-GGACGGGGACTTCTGAGTCTT-3′
Igf2	5′-GTGCTGCATCGCTGCTTAC-3′	5′-ACGTCCCTCTCGGACTTGG-3′
Igf1r	5′-GTGGGGGCTCGTGTTTCTC-3′	5′-GATCACCGTGCAGTTTTCCA-3′
MPRcd	5′-CAAAGAACGAGGTGGCTCTC-3′	5′-CCCAACCACTGTCTCCTTGT-3′
Iba-1	5′-TGATGAGGATCTGCCGTCCAAACT-3′	5′-TCTCCAGCATTCGCTTCAAGGACA-3′
Gfap	5′-TTTCTCGGATCTGGAGGTTG-3′	5′-AGATCGCCACCTACAGGAAA-3′

Intracerebral Injection of HIV

To determine whether IGF2R has a similar HIV-enhancing role *in vivo*, we performed intracerebral injection of VSVg *env* HIV to WT and KO mice. Briefly, 10 μl of VSVg *env* HIV (~200 ng/ml p24) was injected manually to the right caudate-putamen of approximately 8-week-old WT (n = 5) and KO mice (n = 4). All mice recovered from anesthesia and appeared normal, except one which developed hemiparesis. This mouse was excluded from the analysis. The mice were sacrificed 7 days later, and the right cerebral hemispheres were processed for real time-PCR (Q-PCR) analysis. Brains from additional mice were also examined by H&E and p24 immunohistochemistry after fixation in formalin and paraffin embedding.

RNA Interference with IGF2R-Specific siRNA

Human microglia in 60-mm dishes were transfected with 10 nmol/L or 30 nmol/L human IGF2R-specific small interfering RNA (siRNA) or control nontargeting siRNA (Dharmacon, Chicago, IL) with *Trans*IT-TKO transfection reagents from Mirus following the manufacturer's instructions, as previously described.[34,35] After incubation for 48 to 72 hours, cells were washed with fresh medium and then infected with HIV or treated with stimulants for 6 hours to 48 hours. IGF2R knockdown was confirmed by Q-PCR.

Treatment of Cultures with Cell Stimulants

Cultures were treated with recombinant human or murine cytokines at 10 ng/ml or TLR ligands (LPS 100 ng/ml or poly IC 10 μg/ml), as previously described.[32,32,36] Soluble M6P was purchased from Sigma-Aldrich and was used at a concentration of 0.1–10 mmol/L.

Ultrastructural Analysis of HIV Virions

MeWo cells plated at 10^6 per 100-mm dish that reached ~50% confluence were infected with HIV for 3 days. Cells were washed with serum-free medium, fixed with the Trump solution, and then processed for transmission electron microscopy, as previously described.[37]

Western Blot Analysis

IGF2R is a high molecular weight (~300 kDa) protein and protein transfer can be susceptible to experimental conditions.[38] Therefore, Western blot analysis was performed under nonreducing conditions. Thirty to seventy micrograms of protein was separated by 6 or 8% SDS-PAGE and then transferred to polyvinylidene difluoride membrane. The blots were blocked in Tris-buffered saline-0.1% Tween-20 containing 5% nonfat milk and then incubated with antibodies at 4°C for 16 hours. Primary Antibodies included: rabbit anti-human IGF2R antibody 1/500 (gift of Carolyn Scott)[39]; anti-p24 gag, 1/500 (DakoCytomation, Carpinteria, CA); total Stat3, 1/1000 and β-actin, 1/3000 (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG and were used at 1:2000 to 1:10,000 for 1 hour at room temperature. Signals were developed using enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL). Densitometric analysis was performed using Scion NIH Image software (Scion, Frederick, MD).

Real-Time PCR

Quantitative real-time reverse transcription-PCR was performed as described previously[32,32,34] Briefly, total RNA was extracted with TRIzol (Invitrogen Life Technologies, Carlsbad, CA), following the manufacturer's instructions. PCR was performed using a SYBR green PCR mix and conducted with the ABI Prism 7900HT (Applied Biosystems, Carlsbad, CA). All values were expressed as the increase relative to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or porphobilinogen deaminase (PBDA). The mean value of the replicates for each sample was calculated and ex-

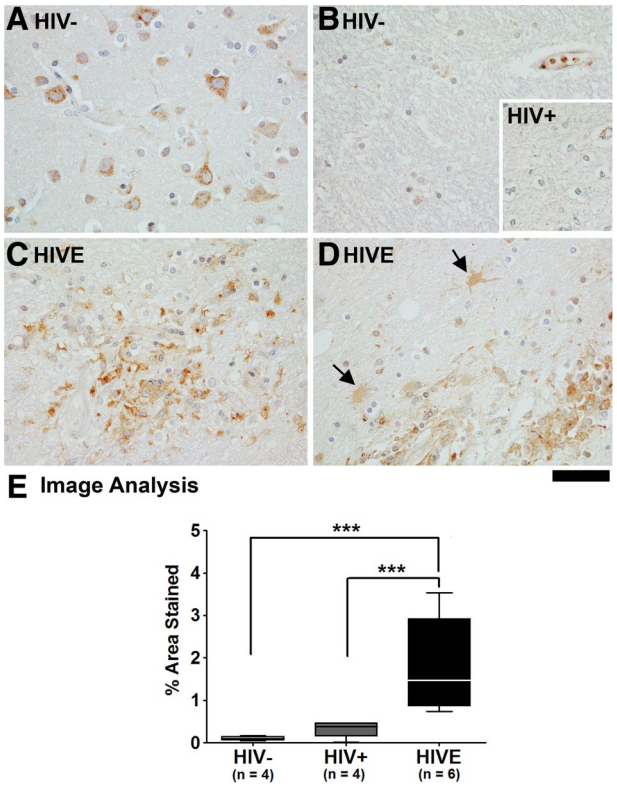

Figure 1. IGF2R expression is up-regulated in macrophages and microglia in HIVE. A rabbit anti-bovine IGF2R antibody was used to study the expression of IGF2R in postmortem human brain sections. **A:** All cases exhibited neuronal IGF2R expression regardless of their HIV status (shown for HIV$^-$). **B:** White matter of HIV$^-$ and HIV$^+$ (**inset**) brains showed little or no staining for IGF2R, while leukocytes within the blood vessels show positivity. **C:** HIVE cases displayed abundant IGF2R staining especially in areas of HIV infection (microglial nodules). The majority of IGF2R$^+$ cells were microglia and macrophages, based on morphology. **D:** Some reactive astrocytes in HIVE were also positive (**arrows**). **E:** A subset of cases were analyzed for the percent area immunoreactive for IGF2R using an image analysis computer program (NIH ImageJ). HIV$^+$ cases and HIV$^-$ had significantly lower levels of immunoreactivity ($P < 0.001$) compared to HIVE cases. While there was a trend for HIV$^+$ cases to have slightly higher levels of immunoreactivity than HIV$^-$, this did not reach significance. The line in the box indicates the median and the whiskers indicate range. The box indicates the quartiles (25th and 75th). Scale bar: 50 μm (**A–D**). ***$P < 0.001$.

pressed as the cycle threshold (Ct; cycle number at which each PCR reaches a predetermined fluorescence threshold, set within the linear range of all reactions). The amount of gene expression was then calculated as the difference between the Ct of the sample for the target gene and the mean Ct of that sample for the endogenous control. Each assay was performed in triplicate. The primer sequences for both human and mouse genes are listed in Table 1.[40]

Statistical Analysis

Results shown are representative of two to five separate experiments using cells from different brain cases. For certain experiments, results from multiple separate experiments were pooled after data were normalized to the baseline value. For statistical analysis, Student's *t*-test or one-way analysis of variance (analysis of variance) with Dennett's post test was performed to determine whether there were significance differences between the experimental and control groups.

Results

IGF2R Is Expressed Robustly in Microglial Nodules within HIV Encephalitic Brains

We examined the expression of IGF2R in human brain sections from individuals with HIVE or controls (HIV-seronegative and HIV-seropositive). As shown in Figure 1A, IGF2R was constitutively expressed in neurons in all brains. White matter of HIV^- and HIV^+ brains showed little or no staining for IGF2R (Figure 1B and data not shown); however, HIVE cases displayed abundant IGF2R staining in microglial nodules as well as in diffusely scattered glial cells which had no obvious relationships with microglial nodules (Figure 1C and data not shown). By morphology, the $IGF2R^+$ cells were neurons, macrophages, microglia, as well as occasional astrocytes (Figure 1D). In addition, ependymal cells were also positive (not shown). Quantitative analysis of IGF2R immunoreactivity was performed in a subset of cases singlely labeled for the receptor, using the NIH ImageJ software (Figure 1E). The results showed that HIVE cases had much larger percentage of the brain area immunoreactive for IGF2R

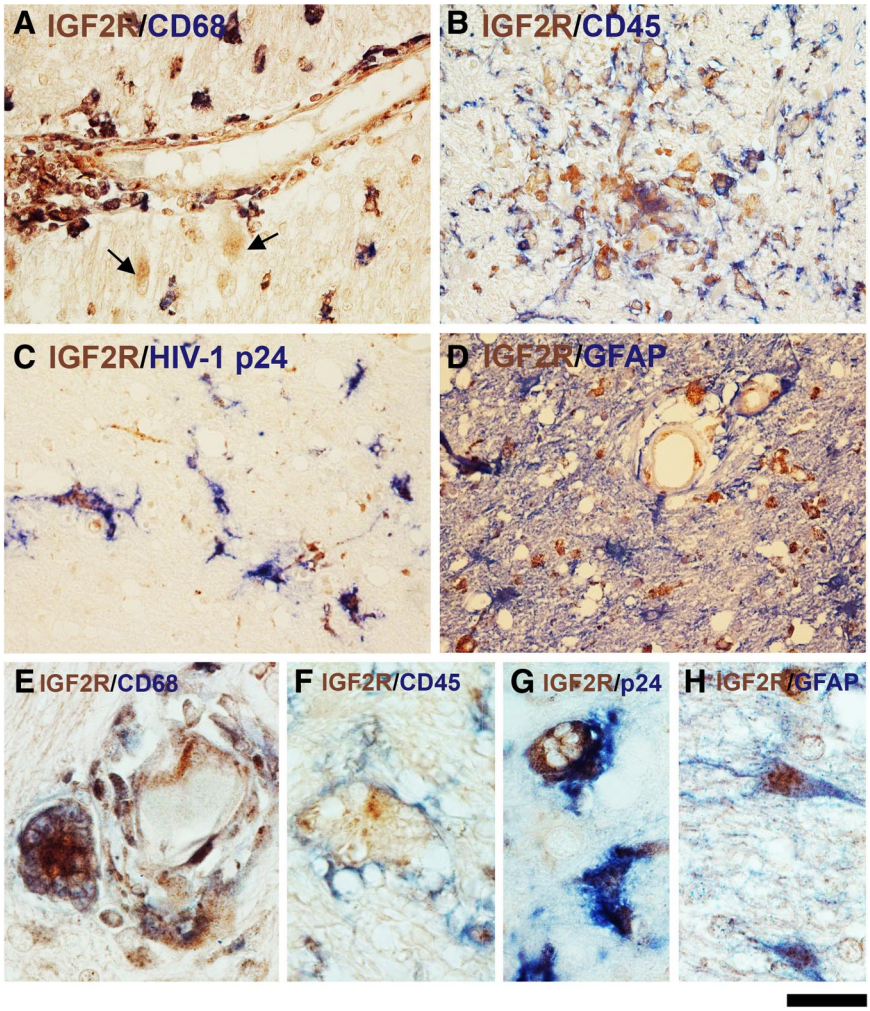

Figure 2. Double immunolabeling for IGF2R and cellular/viral markers in HIVE. To confirm the identity of $IGF2R^+$ cells, double immunohistochemistry for a series of glial markers was performed. Diaminobenzidine (brown) was used to mark IGF2R, and nitroblue tetrazolium (blue) was used for the cell-specific markers. **A:** Double label with CD68 shows many double positive cells outside the vessels. They have the shape of macrophages. Also note a cluster of smaller positive cells within the vessel to the left. CD68-/$IGF2R^+$ cells (**arrows**) in the vicinity have the shape of astrocytes. **B:** Anti-CD45RB was used to mark many process-bearing microglia as well as some round macrophages in this microglial nodule. A subset of $CD45^+$ cells are also $IGF2R^+$. **C:** Staining with an HIV-1 p24 antibody shows many double-labeled, process-bearing cells in this field. **D:** Double labeling with GFAP and IGF2R shows $GFAP^+$ astrocytes are not generally positive for IGF2R. (**E**, **F**, and **G**) High-power images of multinucleated giant cells coexpressing IGF2R (intracellular) and the markers CD68 (intracellular), CD45 (cell membrane), and HIV-1 p24 (intracellular), respectively. **H:** High-power view of rare $IGF2R^+$/$GFAP^+$ astrocytes showing different subcellular localization of the two chromogens. Scale bar: 50 μm (**A–D**); 20 μm (**E–H**).

compared to HIV$^-$ and HIV$^+$ control cases ($P < 0.001$, analysis of variance).

Double labeling with cell-specific markers confirmed that the majority of IGF2R immunoreactive cells in the white matter of HIVE were microglia and macrophages (CD68$^+$, CD45$^+$) (Figure 2, A, B, E, F). In addition, some reactive astrocytes (GFAP$^+$) were also positive for IGF2R in some cases (Figure 2, D and H). Double labeling for HIVgag (p24) demonstrated that a subset of IGF2R$^+$ macrophages and microglia were also HIV$^+$ (Figure 2, C and G).

Role of IGF2R in HIV Replication in IGF2R kDa MeWo Cells

IGF2R is an endosomal protein that is expressed in all cells. The marked enhancement of IGF2R expression colocalizing with microglial nodules in HIVE led us to ask whether IGF2R plays a role in HIV infection. We examined the amount of HIV expression in cultures of human melanoma cells stably expressing IGF2R siRNA (MeWo cells), generously provided by Dr. Michael Gershon.[28] We also tested the effect of M6P (10 mmol/L) on WT MeWo cells. Results of Western blot analysis are shown in Figure 3 (A and B). A representative immunoblot (A) and pooled densitometric analysis from three separate experiments (B) demonstrate that IGF2R KD MeWo cells indeed showed reduced expression of IGF2R (albeit partial), and that these cells also showed reduced amount of HIV gag. M6P treatment of WT MeWo cells showed further increase of both IGF2R and HIV gag expression, although not statistically significant. These results suggested that IGF2R facilitated HIV replication.

We next asked whether disruption of IGF2R-mediated endosomal trafficking alters HIV virion morphogenesis. MeWo cells were infected with HIV bearing chimeric *env* (HIV and VSVg *env*, see *Materials and Methods*), and transmission electron microscopy was performed to compare virion morphology in WT and IGF2R KD cultures. As shown in Figure 3 (C and D), in MeWo cells (epithelial cells), HIV virions were found exclusively in the extracellular space. Although there was an impression that fewer virions were generated in KD culture compared to WT culture, no differences in virion morphology were observed between the two cultures.

IFNγ Up-Regulates IGF2R Expression in Microglia

Having established that IGF2R expression undergoes dramatic increase from barely detectable (in normal) to highly expressed (in HIVE) in glia, we next asked whether inflammatory mediators and/or HIV are responsible for IGF2R up-regulation, using well-established primary human fetal CNS cultures. We first determined the baseline IGF2R expression by immunofluorescence confocal microscopy. Figure 4A shows cultured human microglia

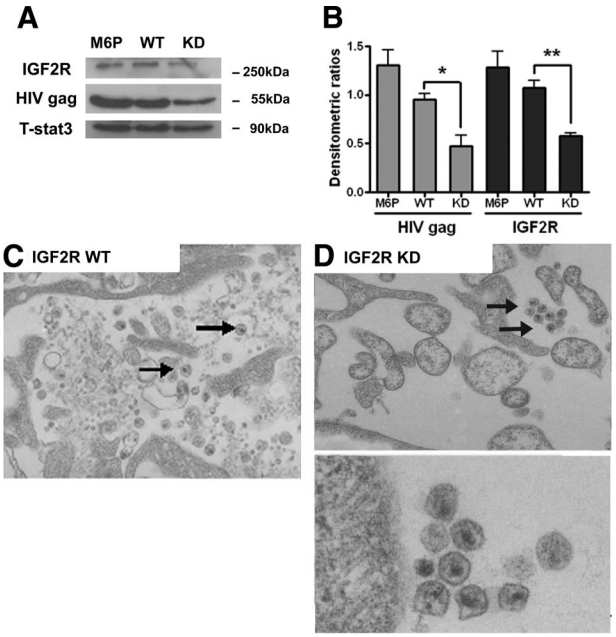

Figure 3. Role of IGF2R in HIV expression in MeWo cells. MeWo cells (human melanoma cell lines) stably expressing IGF2R siRNA (KD) and control (WT) are infected with HIV-bearing VSVg *env* (**A** and **B**) or chimeric env (both HIV$^-$ and VSVg *env*: **C** and **D**), as described in the *Materials and Methods*. **A** and **B:** The amount of HIV gag expression was examined by Western blot. Densitometric ratios from three separate cultures showed that HIV gag expression was significantly inhibited in MeWo cells with IGF2R siRNA (KD) compared to WT cells (mean ± SEM, $P < 0.05$ *t*-test). M6P (10 mmol/L) treatment of WT cells increased HIV expression (though not significant), probably through up-regulation of IGF2R. **C** and **D:** Electron microscopy of HIV-infected MeWo cells showed HIV particles (**arrows**) with characteristic morphology (eccentric cores, irregular shapes, and sizes) in extracellular spaces near cell processes. Although virions appear less frequent in kDa cultures compared to WT cultures, no difference in virion morphology was noted. Higher-power view is in the panel under **D**. $^*P < 0.05$, $^{**}P < 0.01$.

double labeled for CD45 (red) and IGF2R (green). Whereas CD45 was distributed along the plasma membrane, IGF2R was concentrated in the Golgi area and in endosomal vesicles, showing the typical *trans*-Golgi pattern of mannose phosphate receptor expression. In addition to microglia, neurons also expressed abundant IGF2R but astrocytes showed considerably less IGF2R immunoreactivity (not shown).

We next examined whether HIV can increase the expression of IGF2R in microglia, by infecting the cultures with VSVg *env* HIV for 3 days and then immunoblotting for IGF2R ($n = 3$). The results interestingly showed that there was a trend for HIV infection to *decrease* the IGF2R expression, but this effect was not significant (Figure 4B). We next asked whether treatment of microglia with cytokines or TLR ligands could change the level of IGF2R expression. Western blot analysis was performed using five different cultures derived from different donor brains. Given the limited number of microglial cells that can be obtained from each brain, we used various cell stimuli which consisted of the following: proinflammatory cytokines (interleukin [IL]-1β or tumor necrosis factor [TNF]α at 10 ng/ml), anti-inflammatory (Th2) cytokines (IL-4, IL-13, or IL-10 at 10 ng/ml), interferons (IFNγ or IFNβ at 10 ng/ml), and the TLR ligands (LPS, 100 ng/ml, poly IC: PIC

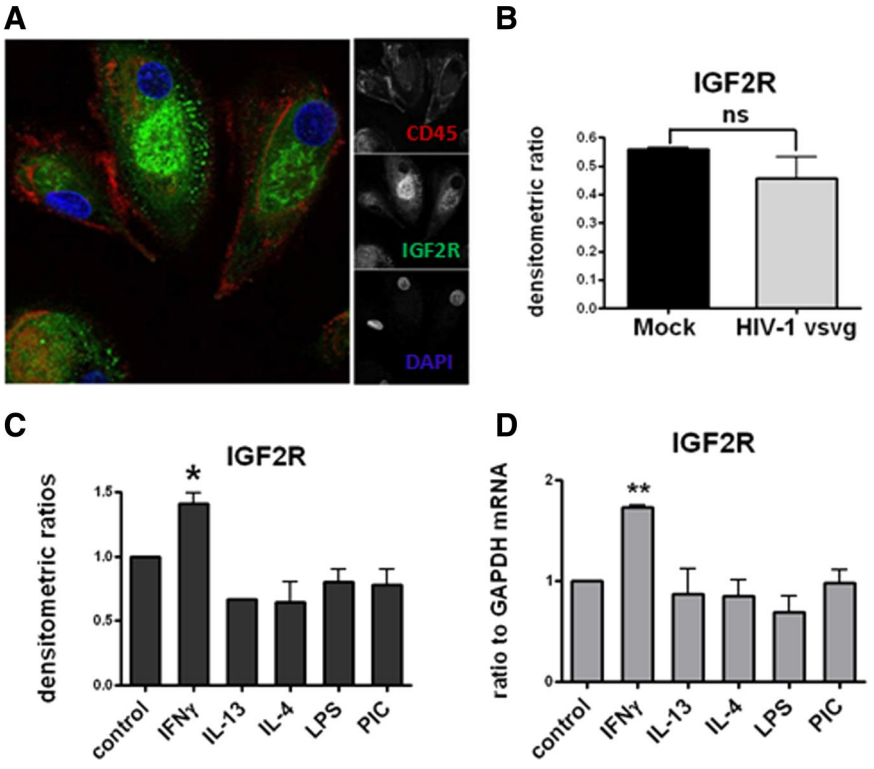

Figure 4. IFNγ up-regulates IGF2R expression in microglia. **A:** Primary human fetal microglia in culture were examined for IGF2R expression by double immunofluorescence microscopy (CD45 red; IGF2R green). They show typical *trans*-Golgi distribution of IGF2R expression. **B:** IGF2R expression was compared in mock or HIV-infected microglial cultures by Western blot and densitometric analysis, as described in the text. There was slight decrease in IGF2R levels in HIV-infected microglia, but this difference was not significant ($P > 0.05$, $n = 3$). **C:** Microglia were stimulated with various cytokines (10 ng/ml) or TLR ligands (LPS at 100 ng/ml; PIC = poly IC at 20 μg/ml) for 24 hours then Western blot analysis was performed for IGF2R as described in the *Materials and Methods*. Densitometric ratios to housekeeping protein (vinculin or β-actin) were compared after normalizing to the IGF2R level in control microglial culture (n ≥3). Mean ± SEM. $^{*}P < 0.05$, analysis of variance with Dunnett's posttest. **D:** Microglial cultures were subjected to a similar treatment as in **C** except that IGF2R expression was examined by Q-PCR. Mean ± SEM. $^{**}P < 0.01$.

20 μg/ml). We observed that although individually many of these factors altered microglial IGF2R protein levels, the changes were minor and variable. We therefore performed statistical analysis on pooled normalized densitometric data for the treatment conditions tested at least three times. The results showed that IFNγ alone significantly increased the expression of IGF2R ($P < 0.05$, analysis of variance with Dennett's post test, Figure 4C), while others (IL-4, IL-13, LPS, or poly IC:PIC) did not. Additional microglial cases were subjected to IGF2R mRNA analysis by Q-PCR. These experiments again showed that IFNγ (Th1 cytokine) but not the Th2 cytokines or TLR ligands increased IGF2R expression in microglia ($P < 0.01$, Figure 4D).

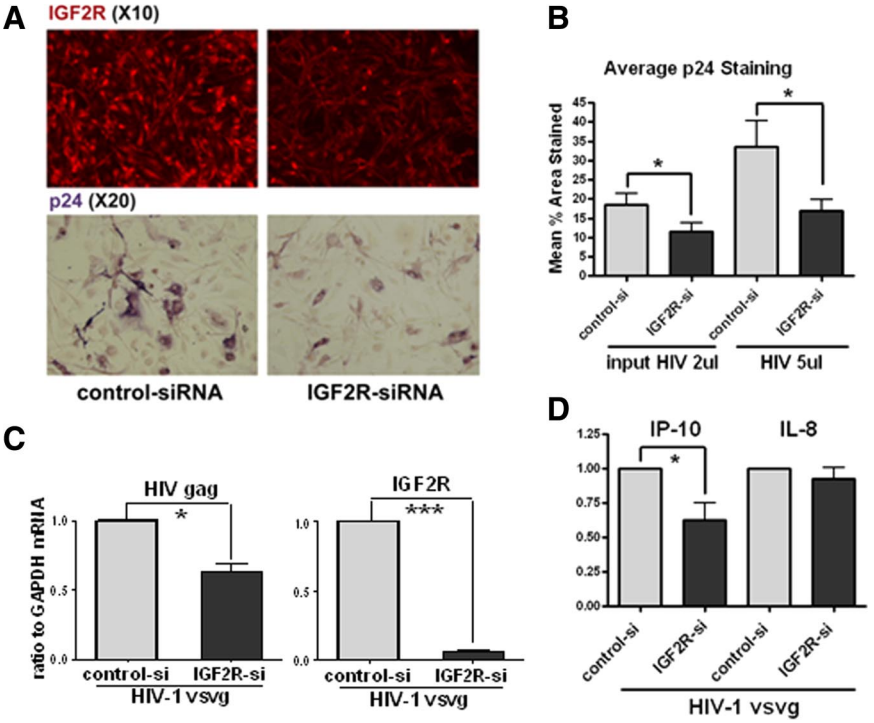

Figure 5. IGF2R siRNA decreases HIV infection and IP-10 production in human microglia. **A:** Primary human microglial cultures were transfected with IGF2R siRNA or control siRNA at 10 nmol/L for 5 days then infected with VSVg *env* HIV for an additional 3–4 days. Immunofluorescence microscopy showed decrease in IGF2R expression (red) in specific siRNA treated cultures compared to control. In these cultures, HIV gag expression was also decreased as determined by immunostain for p24 (immunoperoxidase method, purple). **B:** Quantitative ImageJ analysis showed significant inhibition of p24 in IGF2R siRNA-treated microglial cultures (mean ± SEM, $P < 0.05$ *t*-test) infected with two different input viral doses. **C:** Q-PCR analysis of HIVgag and IGF2R mRNA in microglial cultures treated with IGF2R siRNA and infected with HIV as in **A**, showing marked knockdown of IGF2R expression and inhibition of HIV expression by IGF2R RNAi. $^{*}P < 0.05$, $^{***}P < 0.001$ (*t*-test) **D:** The production of chemokines IP-10 and IL-8 was examined by ELISA in microglial cultures treated with IGF2R siRNA and infected with HIV as in **A**. The production of IP-10 but not IL-8 was significantly reduced by IGF2R RNAi ($^{*}P < 0.05$, $n = 3$, *t*-test).

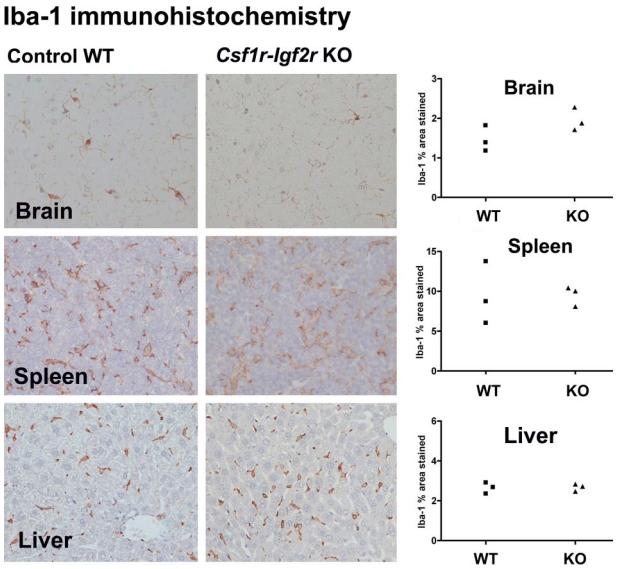

Figure 6. Characterization of *Csf1r-Igf2r* KO mice (Iba-1 immunohistochemistry). Iba-1 (a macrophage marker) immunohistochemistry was used to examine tissue macrophage populations in the brain, spleen, and liver of the WT (**left column**) and KO (**right column**) mice. In the brain, Iba-1 stained delicate ramified microglial cells, while in the spleen, numerous positive cells were present both in the white and red pulp. Kupffer cells were positive in the liver. Morphometric analyses by ImageJ from three mice each show no differences in the amount of Iba-1 stain. These results indicate that *Csf1r*-driven *Igf2r* deletion did not alter tissue macrophages in an appreciable way.

Role of IGF2R in HIV Infection of Human Microglia

Having obtained the results that IGF2R KD epithelial cell lines have reduced HIV gag expression and HIV virion production, we next asked whether IGF2R plays a similar role in HIV production in human microglia, a cell type that is physiologically relevant. Microglia were transfected with siRNA specific to IGF2R or control siRNA (Smart Pool, Dharmacon) for five days then infected with VSVg *env* NL4-3 for additional 3–4 days. HIV gag expression was examined by p24 immunostain or by Q-PCR. IGF2R siRNA-transfected microglial cultures showed markedly reduced IGF2R expression by immunofluorescence (Figure 5A) and by Q-PCR (Figure 5C). HIV gag expression was significantly reduced in these cultures, as determined by HIV p24 stain (Figure 5B: ImageJ analysis, $n = 6$) or by Q-PCR (Figure 5C: $n = 3$), although the amount of reduction was less than IGF2R reduction. As we have previously determined that the Th1 chemokine IP-10 (CXCL10) was consistently induced in HIV-infected microglia,[32,32] we tested the effect of IGF2R siRNA on IP-10 expression in these cultures. The results showed that IP-10 release from these cultures was significantly reduced by IGF2R KD as measured by ELISA, whereas no such change was observed for another α-chemokine IL-8 (CXCL8) (Figure 5D). These results together demonstrate that IGF2R plays a significant role in microglia as a positive regulator of HIV replication and IP-10 production.

Generation and Characterization of Csf1r-Igf2r *KO Mice*

Mice with global deletion of *Igf2r* die perinatally due to organomegaly. Therefore, investigation of the role of IGF2R in mature animals requires a generation of conditional knockout. To examine the role of microglial IGF2R, we created macrophage-specific *Igf2r* knockout mice by crossing the *Igf2r* loxP mice[14] with the *Csf1r* Cre mice (see *Materials and Methods*). The resulting *Csf1r-Igf2r* KO mice were normal in phenotype. Necropsy and routine histochemistry were performed on mice at 2–3 months of age. The brain, lungs, heart, thymus, spleen, liver, and kidneys were normal grossly and microscopically (H&E: not shown). Iba-1 (a macrophage marker) immunohistochemistry was used to examine tissue macrophage populations and they showed no appreciable difference between KO and WT animals (Figure 6). These results indicate that *Csf1r*-driven *Igf2r* deletion did not alter tissue macrophages in an appreciable way. The expression of IGF2R was also examined by immunohistochemistry (Figure 7). In the brain, IGF2R immunoreactivity was noted exclusively in neurons, and the neuronal IGF2R was comparable between WT and Csf1r-Igf2r KO (KO) mice. In the lungs, IGF2R expression was extensive and was present in the bronchial epithelial cells, alveolar septae, as well as macrophages. IGF2R-immunoreactive cells were very few in the spleen (Figure 7), kidney, or heart (not shown). The expression of IGF2R in WT and KO mice was examined quantitatively in the brain and spleen by Q-PCR (Figure 8, A and B), and this showed a statistically significant reduction in IGF2R mRNA in the spleen but not the brain of KO mice. Analysis of additional IGF family and related proteins revealed that IGF1 was significantly reduced in the spleen of KO mice (not shown). None of the changes in the brain, including GFAP, MPRcd, and Iba-1, were significant.

HIV Replication in Bone Marrow–Derived Macrophages from Csf1r-Igf2r *KO Mice*

Certain primary murine cells have been shown to be permissive to infection with HIV when pseudotyped with VSVg *env*.[41] Therefore, to determine the effect of *Igf2r* KO in peripheral macrophages, we examined BMDM from *Csf1r-Igf2r* KO and WT mice. BMDM grown in the presence of M-CSF were infected with VSVg *env* HIV, then IGF2R and HIV gag expression were determined by Q-PCR. As shown in Figure 9, IGF2R expression was virtually abolished in macrophages from *Csf1r-Igf2r* KO mice, while the expression of IGF1, IGF1R, MPRcd, and Iba-1 mRNA were not altered. IGF2 mRNA was undetectable in either KO or WT culture (not shown). Importantly, HIV gag was highly expressed in murine macrophage cultures, with significant inhibition observed in *Igf2r* KO macrophages ($P < 0.01$) (Figure 9).

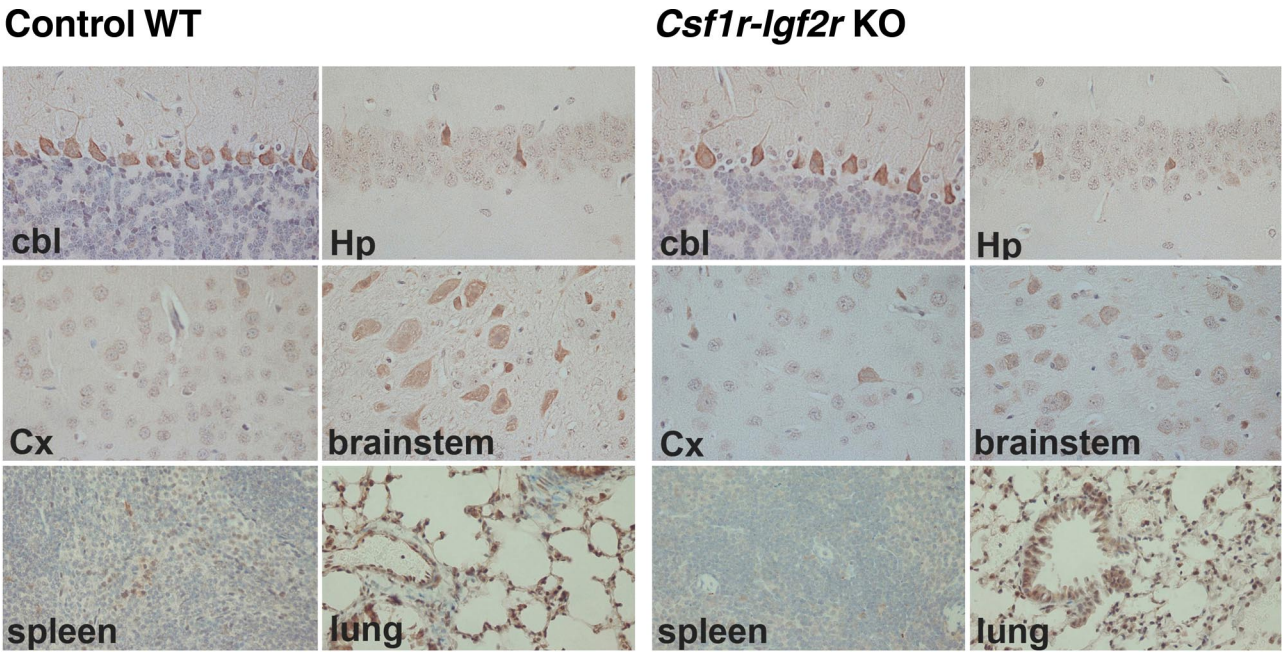

Figure 7. Characterization of *Csf1r-Igf2r* KO mice (IGF2R immunohistochemistry). In the brain, IGF2R immunoreactivity was noted exclusively in neurons with no appreciable difference between WT and KO (cbl: cerebellum, Hp: hippocampus, Cx: cortex and brainstem). Spleen had very few IGF2R-immunoreactive cells, while lungs showed strong IGF2R-immunoreactivity in bronchial epithelial cells, alveolar septae, as well as macrophages. Together, these results show that under normal conditions, IGF2R is expressed mostly in nonmacrophage populations. There are no appreciable differences in the IGF2R immunoreactivity between WT and *Csf1r*-driven *Igf2r* KO mice.

HIV Replication in Microglia and Astrocytes from Csf1r-Igf2r *KO Mice*

Murine microglia and astrocyte cultures were also examined in a manner similar to BMDM. Cultures were established from neonates and were exposed to VSVg *env* HIV as described in the *Materials and Methods*, and then subjected to Q-PCR analysis (Figure 10, A and B). The results demonstrate that similar to macrophages, primary murine microglia and astrocytes sustained high levels of HIV replication and that microglia but not astrocytes from the *Csf1r-Igf2r* KO mice showed reduced IGF2R and HIV gag expression (***$P < 0.001$, **$P < 0.01$).

Together, the mouse experiments demonstrated that i) primary murine macrophages, microglia, and astrocytes in culture are permissive to HIV infection when exposed to VSVg *env* HIV; ii) IGF2R expression is selectively reduced in macrophages and microglia derived from the *Csf1r-Igf2r* KO mice; and iii) HIV expression is suppressed in cells deficient in IGF2R. These results confirm the notion that IGF2R is a positive regulator of HIV expression.

Intracerebral Injection of HIV to WT and Csf1r-Igf2r *KO Mice*

To determine whether IGF2R has a similar HIV-enhancing role *in vivo*, we performed intracerebral injection of VSVg *env* HIV to WT and KO mice. When the brain tissues were examined by Q-PCR at 7 days post inoculation, neither WT nor KO brains showed detectable HIVgag (data not shown). However, HIV gag immunohistochemistry on tissue sections demonstrated focal p24 expression along the injection track in both WT and KO brains (Figure 11). Interestingly, in both of these brains, HIV p24 expression was limited to neurons, the only cell type with detectable IGF2R expression by immunohistochemistry (see Figure 7). Because mouse microglia *in vivo* (but not *in vitro*) lacked IGF2R expression, the lack of microglial infection might simply be a reflection of absent IGF2R expression. By contrast, the *neuronal* expression of both IGF2R and HIV gag *in vivo* strengthens the notion that IGF2R is a positive regulator of intracellular HIV replication.

Discussion

In the current study, we undertook a systematic investigation of IGF2R expression and function in HIV encephalitis and in the tissue culture and mouse models of HIVE. One of the most surprising findings of this study was that in human CNS, IGF2R was an inducible microglial protein whose expression increased from normally undetectable to robust expression (comparable to neuronal expression) at sites of microglial activation and HIV replication (microglial nodules). Neurons expressed high levels of IGF2R but the neuronal IGF2R immunoreactivity did not show an appreciable difference between normal and HIVE, indicating that IGF2R in neurons and microglia are under different regulatory control.

Despite low to undetectable levels of IGF2R in (normal) microglia *in vivo*, cultured microglia showed high basal level IGF2R expression. This may be due to an ongoing microglial lysosomal phagocytic activity in tissue culture, as well as high concentrations of IGF1 and IGF2 in fetal bovine serum to which cultured microglia are exposed.

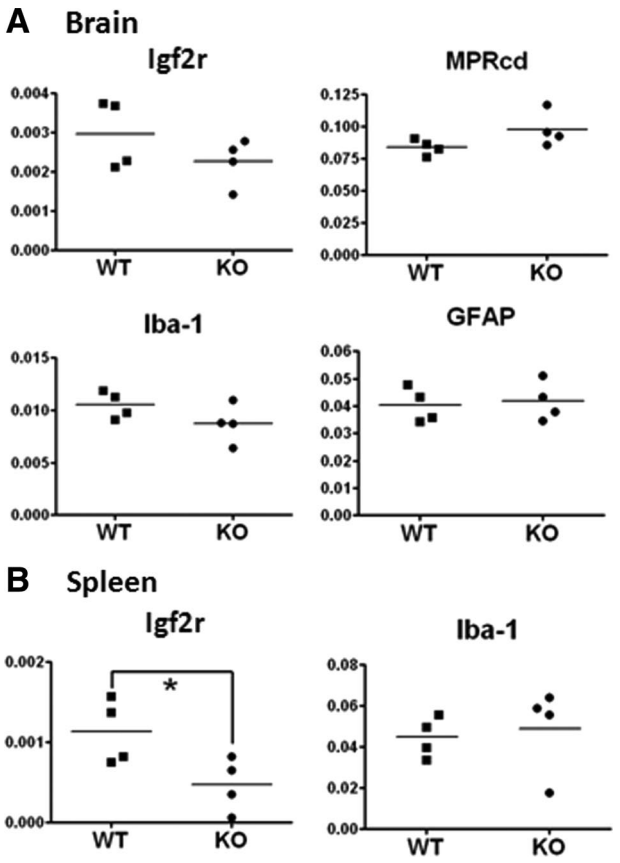

Figure 8. Characterization of *Csf1r-Igf2r* KO mice (Q-PCR of brain and spleen tissue). Further quantitative gene expression analysis was performed by Q-PCR of the brain (**A**) and spleen (**B**) tissue. IGF2R mRNA was reduced significantly in the spleen (but not brain) of KO mice ($^{*}P < 0.05$, *t*-test). Iba-1 levels were not different in either organ (bars = mean values). Analysis of additional IGF family and related proteins revealed that IGF1 was significantly reduced in the spleen of KO mice (not shown). None of the changes in the brain including GFAP and MPRcd were significant.

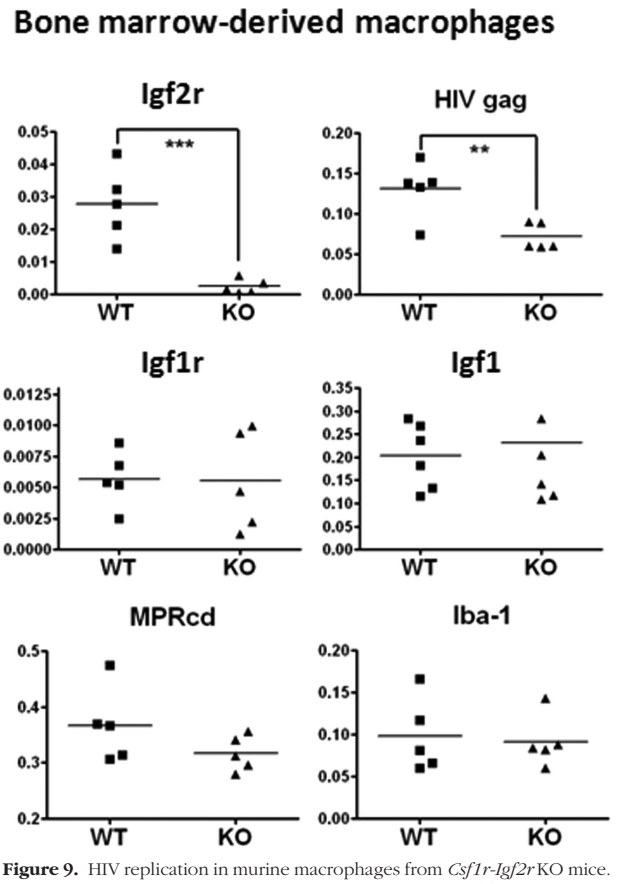

Figure 9. HIV replication in murine macrophages from *Csf1r-Igf2r* KO mice. Bone marrow–derived macrophages (BMDM) were isolated from the WT or KO mice and cultured in the presence of M-CSF as described in the *Materials and Methods*. Macrophages were infected with VSVg *env* HIV for 4 days and then analyzed for HIV gag and IGF family and related proteins by Q-PCR. Transcript levels for IGF2R and HIV gag were significantly reduced in KO macrophages ($^{**}P < 0.01$, $^{***}P < 0.001$). IGF1R, IGF1, MPRcd, and Iba-1 were not significantly altered. IGF2 was not detected (not shown).

Even with high basal level expression, human microglia *in vitro* stimulated with IFNγ showed further increase in IGF2R mRNA and protein, demonstrating that inflammatory cytokines can indeed increase microglial IGF2R. However, it is possible that our tissue culture study had missed other potentially important inflammatory mediators that may induce IGF2R expression *in vivo*, due to high baseline expression of IGF2R in tissue culture.

As we observe that IGF2R is induced by mannose-6-phosphate itself (this study and data not shown), it is possible that IGF2R expression will increase under the conditions of increased lysosomal activity. In this context, it is of interest that recent proteomics studies have identified lysosomal enzymes (cathepsins) and related proteinases (cystatins) to be the ones most significantly altered in the CSF of cognitively impaired HIV^{+} individuals.[42] *In vitro*, HIV-infected mononuclear phagocytes display similar changes in cathepsins, cystatins, and other lysosomal enzymes,[43,44] suggesting that the altered metabolism of HIV-infected macrophages and microglia is central to the pathogenesis of HIV-associated neurocognitive disorder (HAND). Significant elevations of lysosomal hydrolases and cathepsins have been found in earlier tissue-based studies HIVE and SIV encephalitis.[45–47] It is therefore of great significance that IGF2R, a protein critically involved in the lysosomal function, is increased in microglia and macrophages in HIVE (this study). In addition to strengthening the notion that microglial lysosomal integrity might be important in the pathogenesis of HAND, these findings also indicate that increased endosomal and lysosomal activity (in addition to inflammatory cytokines such as IFNγ) may also contribute to the enhanced expression of IGF2R in the CNS.

In an effort to elucidate possible functions of increased IGF2R in microglia observed in HIVE, we investigated its role in HIV replication. We used several experimental systems ranging from MeWo cell lines with stable IGF2R knockdown, to primary human microglia transiently expressing IGF2R siRNA, to mice with *Csf1r* promoter-driven macrophage-specific *Igf2r* knockout. For HIV infection, we have used a single-cycle competent, VSVg *env*-pseudotyped NL4–3 construct that has intact HIV Nef and Vpr genes,[33] to obtain fast and efficient replication in microglia. The use of the modified HIV constructs also enabled efficient replication in murine cells. The results of these experiments demonstrated that IGF2R enhances the level of HIV expression, a novel function for IGF2R. Specifically, in four separate accounts (MeWo,

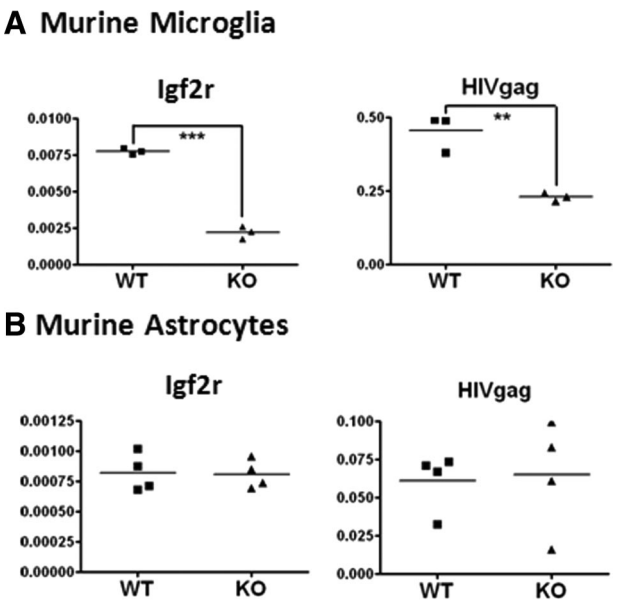

Figure 10. HIV replication in murine microglia and astrocytes from *Csf1r-Igf2r* KO mice. Microglia (**A**) and astrocytes (**B**) were cultured from neonates as described in the *Materials and Methods* and then subjected to HIV infection and Q-PCR analysis as in Figure 9 legend. The results demonstrate that microglia but not astrocytes from the *Csf1r-Igf2r* KO mice show reduction of IGF2R expression and HIV gag expression compared to WT cells. $^{**}P < 0.01$, $^{***}P < 0.001$.

human and murine microglia, and murine macrophages), suppression of IGF2R expression also led to significant and consistent suppression of HIV gag expression. These results together show that once HIV entry is achieved, the intracellular HIV life cycle critically depends on the cellular endosomal and lysosomal machinery and that IGF2R positively contributes to the productive infection of HIV. The specific molecular mechanism(s) underlying this observation is unknown. Because HIV proteins do not bear M6P residues, increased HIV production by IGF2R must be through indirect mechanisms of facilitating intracellular HIV protein trafficking. Indeed, recent studies have demonstrated the importance of endosomes and vesicular sorting proteins in HIV biogenesis in various cell models including primary macrophages.[48,49] In macrophages, HIV has been shown to bud into the late endosomal compartment called "multivesicular bodies," although the types and the specific details of the intracellular compartments involved in HIV biogenesis are still debated. Additionally, it is curious that IGF2R was not identified in recent genome-wide surveys of host genes involved in HIV replication.[50–52] Four screens have been performed to date and the results of these studies are surprisingly different from each other, suggesting that conditions and readouts of the screens affect the identities of the hits recovered.

We have generated mice with *Csf-1r* promoter-driven deletion of *Igf2r* to study macrophage and microglia-specific functions of this receptor *in vivo*. The *Csf1r* promoter, similar to other macrophage-specific promoters such as CD11b and lysozyme promoters, are common to all myeloid cells. Our *Csf1r-Igf2r* KO mice showed normal development and normal phenotype, suggesting that myeloid IGF2R (at least that which is *Csf1r*-driven) was not essential for fetal development. There was no obvious change in the systemic and brain macrophage populations, as examined by Iba-1 immunohistochemistry. *Csf1r-Igf2r* KO mice should be useful for future investigation of macrophage IGF2R function in innate immunity and inflammation.

We had anticipated the usefulness of the *Csf1r-Igf2r* KO mice as an *in vivo* model of HIVE and that the use of VSVg *env* HIV would enable quantification of replicating HIV in all cell types of the mouse brain. Interestingly, while VSVg *env* HIV replicated efficiently in cultures of

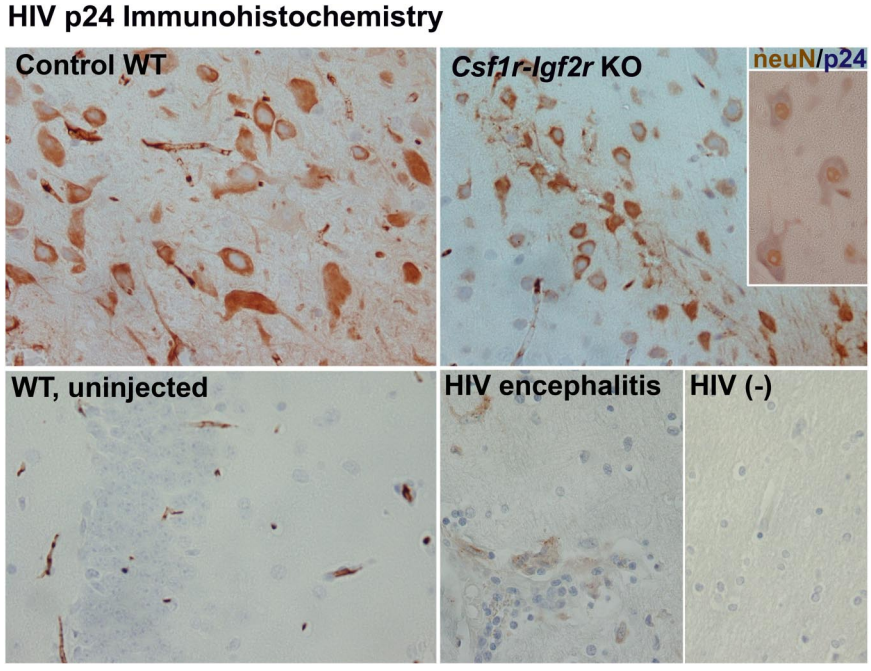

Figure 11. HIV p24 in WT and *Csf1r-Igf2r* KO mouse brains inoculated with VSVg *env* HIV. Brain tissues of the mice injected with VSVg *env* HIV are immunostained for HIV gag (p24) 7 days later. In both WT and KO mouse brains, only the neurons along the needle track are positive for p24 (**top panels**). Note the linear needle track (**top right**). The neuronal identity of HIV-infected cells is confirmed by double label immunohistochemistry for neuN (neuronal nuclei: brown) and HIV p24 (cytoplasm: blue) (**inset**, **top right**). Neurons away from the injection site and in uninjected brains are negative for p24 (**bottom left**). Vessels in mouse brains are positive due to the presence of mouse IgG reactive to the secondary antibody. Controls for HIV p24 consisted of human brain with HIV encephalitis (microglia and multinucleated giant cells positive) and a normal HIV (−) brain (no reactivity) (**bottom right panels**). All singly labeled sections were lightly counterstained with hematoxylin.

primary mouse macrophages, microglia, and astrocytes, as reported,[41] the only cell type expressing HIV gag after intracerebral injection of VSVg *env* HIV *in vivo* was neurons. These results indicate that murine glial cells *in vivo* may have additional barriers (possibly including the lack of IGF2R expression) to HIV replication. Because neurons are the only cell type normally expressing abundant IGF2R in these mice, these results are an affirmation of our hypothesis that intracellular HIV replication is critically dependent on IGF2R expression. These results also bring out the (generally recognized) difficulties of adopting mouse models for HIV studies. They highlight that the most significant block for HIV infection in mammalian cells (including human) might be the entry step due to lack of specific HIV receptor/coreceptor expression because once the entry step is bypassed virtually all cells are capable of supporting HIV replication (that is, at least *in vitro*). The parallel between IGF2R expression and the ability to support intracellular HIV replication found in neurons in the mouse brain is interesting and suggests that microglial infection by HIV in human brain may require their prior activation to acquire the expression of IGF2R.

Our results have implications for the pathogenesis of HAND. The dramatic up-regulation of IGF2R in activated microglia *in vivo* may have several undesirable consequences. First, IGF2R, as shown repeatedly in this study, could facilitate HIV expression in microglia, contributing to viral persistence and spread within the CNS. Second, IGF2R may also control induction of microglial inflammatory genes. For example, we show that IP-10 is acutely induced in VSVg *env* HIV-infected microglia[32] and that IP-10 production in these cultures is suppressed by IGF2R siRNA (this study). IP-10 or signaling through its cognate receptor CXCR3 has been shown to contribute to HIV-induced neurodegenerative mechanisms,[53,54] suggesting that IGF2R might contribute to HIV-mediated neurodegeneration. Third, up-regulation of IGF2R in activated glia could potentially serve as a growth factor "sink" mechanism by which IGF family peptides are internalized and degraded, further serving as an agent of neural damage. Future studies aiming at validating these hypotheses might be of significant value, as they might enable identification of a novel biomarker or a therapeutic target.

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