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ORIGINAL ARTICLE

Enhanced Human Immunodeficiency Virus Type 1 Expression and Neuropathogenesis in Knockout Mice Lacking Type I Interferon Responses

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Abstract

The roles of Type I interferon (IFN) in human immunodeficiency virus Type 1 (HIV-1) neuropathogenesis are poorly understood; both protective and deleterious effects of IFN signaling have been described. We used genetically modified mice deficient in the Type I IFN receptor (IFNRKO) to analyze the progress of HIV-1 brain infection and neuropathogenesis in the absence of IFN signaling. IFNRKO and wild-type (WT) mice on the 129xSv/Ev or C57BL/6 strain backgrounds were infected systemically with EcoHIV, a chimeric HIV-1 that productively infects mice. IFNRKO mice showed higher HIV-1 expression in spleen and peritoneal macrophages and greater virus infiltration into the brain compared to WT mice. Neuropathogenesis was studied by histopathological, immunohistochemical, immunofluorescence, and polymerase chain reaction analyses of brain tissues after the virus was inoculated into the brain by stereotaxic intracerebral injection. Both IFNRKO and WT mice showed readily detectable HIV-1 and brain lesions, including microglial activation, astrocytosis, and increased expression of genes coding for inflammatory cytokines and chemokines typical of human HIV-1 brain disease. Parameters of HIV-1 neuropathogenesis, including HIV-1 expression in microglia/macrophages, were significantly greater in IFNRKO than in WT mice. Our results show unequivocally that Type I IFN signaling and responses limit HIV-1 infection and pathogenesis in the brains of mice.

Key Words: HIV-1, Interferon α/β receptor, IFN signaling, Neuropathogenesis, Receptor knockout mice, Stereotaxic injection, Type I interferon.

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INTRODUCTION

Human immunodeficiency virus Type 1 (HIV-1) infection causes a range of nervous system impairments known in aggregate as HIV-1–associated neurocognitive disorders or HAND (1). The prevalence of individual diagnostic categories encompassed in HAND reflects the cumulative effect of combination antiretroviral therapy (cART) in populations with access to treatment (1). HIV-1–associated dementia, which before the advent of cART was a frequent occurrence, has been responsive to treatment, with its prevalence diminishing to approximately 1% (2). In contrast, mild HAND manifestations including mild neurocognitive disorder and asymptomatic neurocognitive impairment have been largely refractory to cART, even in people with sustained virus suppression and immunological improvement (3–6). These CNS manifestations have emerged as major complications of HIV-1 infection in populations on cART, with a combined prevalence of approximately 50% (3, 6, 7).

We are interested in the mechanism of mild HIV-1 neuropathogenesis seen in many individuals on cART. Recent clinical studies indicate that nadir CD4 is the strongest predictor of HAND in such individuals (8–10), suggesting that the pathophysiological processes underlying HAND originate early after infection (9). These processes likely include early HIV-1 entry into the CNS (11–14) and persistent infection of infiltrating macrophages and glial cells, the main HIV-1 reservoirs in the brain (15, 16). Indeed, some infected individuals tested during the early/acute stage of infection show mild metabolic changes in the brain that indicate cell activation and inflammation and some have symptoms of neurocognitive disease (13, 17–20); however, the majority develop clinically apparent HAND years after primary infection (8–10). These findings point to a substantial period of disease latency between HAND initiation at the molecular level and the appearance of clinical HAND. Understanding the mechanisms controlling this latency may help to prevent or mitigate HAND.

Type I interferons (IFNs) may contribute to the imposition of HIV latency. The innate immune responses mediated by Type I IFNs serve as the first line of defense against viral infections including HIV-1 (21–23). These responses are triggered by pattern-specific viral ''sensors,'' including Toll-like and RIG-1-like receptors, which in turn lead to activation of

interferon regulatory factors IRF3 and IRF7 and induction of IFNs (24). Type I IFNs, including IFN-α and IFN-β, act through a common cell surface receptor, IFN-α/β receptor or IFNAR1 (21, 25, 26), to initiate a cascade of cellular signaling pathways that culminates in induction of interferon-stimulated genes with antiviral, proapoptotic, and ubiquitination-modifying properties (27).

Both HIV-1 and the closely related simian immunodeficiency virus (SIV) elicit strong Type I IFN responses in their respective hosts as indicated by broad activation of IFN-related genes in peripheral tissues and brain (28–33). These responses might be particularly important in the imposition of latency upon HIV-1 in the brain because Type I IFN has been shown to reduce HIV DNA transcription in monocytes and macrophages (34, 35) and persistent expression of HIV-1 and neuroinflammatory products by these cells is believed to mediate HAND pathogenesis (15, 16). Indeed, work in SIV-infected macaques (36) suggested the role of IFN-β in the establishment of transient SIV transcriptional latency in the brain during the early stages of disease in these animals (37, 38). Of interest, recent analysis of brain tissues from HIV-1–positive pre-symptomatic patients found a preponderance of HIV-1 DNA compared to viral RNA in glial cells, although the role of Type I IFN in this process was not determined (39). In longer-term infection, native Type I IFN responses are insufficient for the stable suppression of SIV or HIV-1 after the onset of immunodeficiency (36, 39, 40). There is some evidence that they contribute to disease through chronic immune activation and IFN-α neurotoxicity (41–43). The antiviral efficacy of IFN in vivo might be reduced by the capacity of HIV-1 to antagonize innate immune effectors (44–48). The balance of beneficial versus harmful effects of Type I IFN responses in early HIV brain disease has not been conclusively determined.

The present study was designed to investigate directly the potential role of IFN responses in the control of both early HIV-1 infection in the brain and of neuropathogenesis. To that end, we took advantage of 2 experimental systems, i.e. genetically modified IFN-α/β receptor-negative (IFNRKO) mice lacking the IFN receptor and thus rendering the mice incapable of mounting IFN responses (21, 22), and chimeric HIV-1 (EcoHIV) that expresses gp80 of ecotropic murine leukemia virus in place of gp120 to permit infection of mice (49–53). Similar to the acute phase of HIV-1 infection in humans (14, 40), conventional mice infected with EcoHIV mount adaptive immune responses that limit virus replication but do not prevent HIV-1 transmission (50) or HIV-1 neuroinvasion and induction of pathogenic events in the brain (52, 53). We report that IFNRKO mice permit EcoHIV expression at higher levels, with more extensive changes in host cell gene expression in the brain and more florid brain abnormalities than are found in WT mice. These findings show directly that Type I IFN responses can exert significant control over HIV-1 expression and pathological effects in the brain.

MATERIALS AND METHODS

Mice

Breeding pairs of IFN-α/βR$^{-/-}$ (IFNRKO) mice on the 129Sv/Ev background (22) were kindly provided by Dr Joan Durbin, Ohio State University, Columbus, OH, and were bred in our colony. To generate IFNRKO mice on the background of the better-breeding C57BL/6 strain, the region containing the insertionally mutated *IFN-α/βR* gene (22) was bred into C57BL/6 mice by crossing 129Sv/Ev/IFNRKO with C57BL/6; progeny mice were screened for mutated IFN-α/β receptor and homozygotes were back-crossed to C57BL/6 with sequential progeny screening for 6 generations. The WT *IFN-α/β* receptor gene was distinguished from the interrupted gene containing a *neo* gene insert (22) by a custom-designed reverse transcription–polymerase chain reaction (RT-PCR) amplification protocol yielding a 571-bp RNA product for intact and a 689-bp RNA product for mutant receptor gene allele. Total cellular RNA was isolated from peritoneal cells obtained by lavage and reverse transcribed to complementary DNA (cDNA) using the Super-Script RT-PCR kit (Life Technologies, Grand Island, NY). The cDNAs were subjected to PCR using GoTaq DNA polymerase (Promega, Madison, WI) and custom-designed primers IF5 (IFNARI forward primer) 5′-AAT ATC GAA CAA AAG ACG AGG CGA AG-3′, IF3 (IFNARI reverse primer) 5′-GAC GCA ATG TAG TCC CAT TTC AGG AC-3′, and Neo3 (Neo reverse primer) 5′-TCG GCA GGA GCA AGG TGA GAT GAC-3′. The expected mouse screening results: +/+ 571 bp; +/− 571 bp/689 bp; and −/− 689 bp. The C57BL/6 and 129x1/Sv mouse strains used as controls were obtained from Jackson Laboratory (Bar Harbor, ME). All studies using mice were conducted in full compliance with the National Institutes of Health guidelines and St. Luke's-Roosevelt IACUC approval.

Responses to IFN-α in Tissue Culture

Bone marrow cells were isolated and differentiated to macrophages as described (54). Adherent macrophages were or were not activated with recombinant murine IFN-α2 (1,000 U/ml) at 37°C for 24 hours, and cells were harvested for amplification of cellular transcripts by quantitative real-time PCR (qPCR). Responses were reported as fold change in the number of copies of a transcript in IFN-α activated compared to control cells.

EcoHIV Plasmids and Infectious Virus Stocks

We used chimeric EcoHIV clones on the background of either HIV-1/NDK (EcoHIV/NDK) or HIV-1/NL4-3 (EcoHIV/NL4-3) (49, 53). The infectious viruses generated from these clones are referred to as EcoHIV, and specific clones used are indicated in legends or as appropriate. Chimeric HIV-1 expressing indicator enhanced green fluorescence protein (EGFP) (EcoHIV/NL4-3-GFP) was constructed in 2 steps. First, the EGFP coding region and the internal ribosome entry site (IRES) region were inserted into the HIV-1 clone NL4-3 immediately after the termination codon in gp41 and immediately preceding the initiation codon of Nef, generating NL4-3-GFP. The insert consisted of EGFP, GenBank number U-55761 nt 97-816, a linker region containing the *Not*I restriction site (5′-AGCGG CCGCACTAGTGATAATTCCG-3′), and an IRES region, GenBank number U-89673 nt 1299-1873. EGFP and IRES clones were obtained from Clontech (Mountain View, CA). In the second step, EcoHIV/NL4-3-EGFP was constructed by replacing the gp120 coding region in NL4-3-EGFP with the gp80 coding region of MuLV, exactly as described for EcoHIV/NL4-3

(53). Infectious EcoHIV stocks were prepared and titered for their p24 HIV-1 core antigen by p24 Elisa (PerkinElmer, Waltham, MA), as described (49, 52, 53).

Infection of Mice

To initiate systemic infection, groups of mice were injected intraperitoneally with 10^6 pg of p24 each of sterile suspension of virus stock, as described (49). To deliver virus directly to the brain, stereotaxic intracranial inoculation was conducted essentially as described (52, 55). Briefly, heavily anesthetized mice were immobilized in a stereotaxic chamber (Stoetling Co., Wood Dale, IL), and a small hole was drilled into the exposed skull above the right hemisphere at a distance of 2.5 mm to the right of bregma. Ten microliters of virus stock was injected by a micropump into the caudate putamen at a rate of 10 μL/20 min, and the wound was sutured (52). At the designated times, groups of animals were killed by asphyxiation with 100% CO_2, and tissues were collected and processed for isolation of total cellular DNA and RNA, as described (49, 52, 53).

qPCR and Conventional PCR

The procedures for harvesting spleen cells, peritoneal macrophages (PMs), and brain tissues; the preparation of cellular DNA and RNA; and the detection of EcoHIV/NDK *gag* DNA or spliced *vif* message by qPCR were previously described (49, 52). Samples were run in duplicate in the AB7300 real-time thermal cycler (Life Technologies). Data analysis was performed with the 7300 System software according to the manufacturer's instructions. For the detection of EcoHIV/NDK 2LTR circular DNA by qPCR, the reaction mixture contained custom-designed forward primer 2LTRF3 (5′-CTAGAGATCC CTCAGATCCGTTTAGT-3′), reverse primer 2LTRR1 (5′-T GGTGTGTAGTTCTGCCAATCG-3′), and probe 2LTRP (5′-(FAM)-TTTGGGTCTACAACACACAAGGCATCT TCC-(MGBNFQ)-3′). A standard curve for the quantitation of 2LTR copy number was constructed using graded numbers of plasmid TA-NDK2LTR containing the NDK U5 (3′-LTR)-U3 (5′-LTR) segment. Sample DNA content was normalized by murine β-globin DNA amplified by qPCR using forward primer MGBF (5′-CTGCCTCTGCTATCATGGGTAAT-3′), reverse primer MGBR (5′-TCACTGAGGCTGGCAAAGGT-3′), and probe MGBP (5′-FAM-TTAACGATGGCCTGAATC-MGBN FQ-3′). For the detection of double-spliced HIV/NDK *nef* transcript, RNA was isolated from brain tissue and reverse transcribed to cDNA as described above, and cDNA was amplified by conventional PCR using primers ND160 (5′-GAGTGAAA AATCTCTAGCAGTGGCGC-3′) and ND8002 (5′-GCTGAA GAGGCACAGGTTCCTCAGGTCG-3′). The PCR products were resolved on agarose gels and visualized by Southern blotting using a (^{32}P)-labeled probe NDS3 (5′-GAAGAAGCGGAG ACAGCGACGAAAACCTCC-3′) and standard methods. Murine β-actin was amplified in parallel to standardize RNA input. The predominant product of this PCR is a 342 bp *nef* transcript; minor products representing *rev* (364 bp) and *tat* (541 bp) can be seen upon film overexposure.

Cellular Gene Expression in Brain Tissues

The changes in the expression of genes from mouse brain samples were analyzed by qPCR using TaqMan chemistry and probes from the Universal Probe Library (Roche, Indianapolis, IN), as previously described (28). Data were normalized using the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Relative quantification used the comparative threshold cycle method (Applied Biosystems Technical Bulletin no. 2).

Histopathology and Immunohistochemistry

The procedures were conducted essentially as described (52, 53). Brains from dissected mice were fixed in 4% paraformaldehyde, then the right cerebral hemisphere was marked with ink, and the brain was sectioned in the coronal plane. Whole coronal sections approximately 3 mm in thickness were dehydrated overnight in a VIP Tissue-Tek tissue processor and embedded in paraffin in a Leica EG1160 embedding center. Six-micrometer-thick sections were cut on a Leica microtome and stained with hematoxylin and eosin for histopathological assessment. Selected paraffin blocks were cut at 6 μm for immunohistochemistry, which was performed using the Dako EnVision system (Dako, Carpinteria, CA), using diaminobenzidine as chromogen with light counterstaining with hematoxylin. The antibodies used were a rabbit polyclonal antibody to glial fibrillary acidic protein (GFAP) (Dako) for labeling astrocytes, a rabbit polyclonal antibody to ionized calcium binding adaptor molecule-1 (Iba-1) (Wako, Osaka, Japan) for labeling macrophages/microglia, and a mouse monoclonal antibody 183-H12-5C (National Institutes of Health AIDS Reagent Program, Germantown, MD; catalog no. 3537) for the detection of HIV-1 p24 antigen. Photomicrographs were taken on a Zeiss Photomicroscope III equipped with a Nikon DN100 digital camera.

Quantification of immunostaining was performed using Volocity 5.5 image analysis software (PerkinElmer) using 10 to 20 images from multiple coronal brain sections (5–8 images/section from 2 to 3 mice per group). Each evaluated protein was quantified by determining the positive stain area as a percentage of the total stained area per microscope field or count of stained cells or lesion per field normalized by image area.

Confocal Microscopy

Mice were perfused first with phospate-buffered saline (PBS) followed by 4% paraformaldehyde, tissues were then dissected, flash-frozen, and stored at −80°C. Thirty-micrometer-thick coronal sections were cut on a Leica cryostat and stored in cryoprotectant at −20°C until staining. For immunofluorescence staining, sections were rinsed in PBS, treated with 10% fetal bovine serum (FBS) and 0.2% Triton X-100 in PBS for 1 hour, washed 3 times in PBS–0.2% Triton X-100, and incubated with chicken anti-GFP (1:500; Life Technologies) (for the detection of marker protein in EcoHIV/NL4-3-GFP) and with rabbit anti–Iba-1 (1:400; Wako) (to identify macrophages/microglia) in PBS–10% FBS + 0.2% Triton X-100, at 4°C overnight. Sections were then washed and incubated with goat anti-chicken Alexa 488 or goat anti-rabbit Alexa 555 conjugated antibodies (1:100; Life Technologies) in PBS–10% FBS + 0.2% Triton X-100, at 37°C for 2 hours, washed, and mounted onto slides with PROLONG GOLD ANTIFADE Reagent. Images including Z-stacking were captured with fully motorized Leica TCS FP5 confocal microscope and analyzed using Improvision Volocity software (PerkinElmer).

Statistical Analysis

Differences in HIV-1 burdens and other parameters between WT and IFNRKO mice were tested by paired Student *t*-test.Changes in cellular gene expression in brain tissues of infected mice were first normalized to respective uninfected controls then compared in *t*-test between WT and IFNRKO.

RESULTS

The studies used 2 IFNRKO mouse strains, based on strains 129Sv/Ev (22) and C57BL/6. The C57BL/6/IFNRKO strain was derived as described in Methods. Figure 1A shows an example of confirmatory screening of 7 C57BL/IFNRKO mice (lanes 1–7) versus WT control (lane 8). KO mice yielded a 689-bp product designed to span part of the *neo* gene transcript and IFNR flanking sequences; WT controls showed a smaller 571-bp PCR product representing part of uninterrupted IFNR transcript from the same region (Fig. 1A). Because each mouse sampled in lanes 1 to 7 showed a single 689-bp band, we conclude that these animals are homozygous for the IFNRKO allele. To determine the capacity of WT and IFNRKO mice for signaling through the IFN receptor, bone marrow–derived macrophages from mice on either background were activated by treatment with recombinant mouse IFN-α and tested for induction of 2 interferon-stimulated genes, i.e. IRF-7 and IFIT1, by qPCR 24 hours later. Both interferon-stimulated genes were induced in WT 129x1/Sv and C57BL/6 but not in their IFNRKO counterparts (Fig. 1B), confirming lack of Type I IFN responses in cells from KO mouse lines used in this study.

Next, we investigated the progress of HIV-1 infection and neuroinvasion in mice as a function of the integrity of IFN responses. 129 WT or IFNRKO mice were infected with EcoHIV by intraperitoneal injection and PMs were harvested at the times indicated after infection to measure spliced *vif* HIV-1 RNA, a form absent from the virus inoculum (Fig. 2A). Viral RNA was detected in both WT and IFNRKO mice up to 4 weeks after infection but the viral RNA burden was significantly greater in IFNRKO mice. To determine whether the inhibitory effect of IFN extends to HIV-1 replication in the brain, the experiment was repeated, and PMs and brain were harvested 4 weeks after infection and tested for *vif* RNA burden by qPCR (Fig. 2B). Results from PMs were similar to those shown in Figure 2A with viral RNA burdens significantly higher in PMs from IFNRKO mice compared to WT mice; however, we were unable to detect viral RNA by qPCR in brain tissue from either mouse strain (Fig. 2B). We then tested these brain tissues for HIV-1 DNA using the sensitive traditional PCR amplification in combination with Southern blotting (Fig. 2C), as previously described (53). HIV-1 DNA was detected with weak signals in 2 of 3 WT mice and with stronger signals in 3 of 3 IFNRKO mice (Fig. 2C). Similar results were obtained in IFNRKO mice on the C57BL/6 background infected by peripheral inoculation of EcoHIV (not shown). Overall, these results indicate that absence of functional Type I IFN responses increases HIV-1 expression in peripheral tissues and may result in increased virus burdens in the brain. Because traditional PCR is semiquantitative, the effect of higher HIV-1 expression in macrophages on HIV-1 neuroinvasion in peripherally infected IFNRKO animals could not be precisely determined.

To increase the resolution of virological studies in mouse brain, we used our recently reported approach to initiate robust EcoHIV infection in mouse brain by stereotaxic intracerebral inoculation of virus into the basal ganglia region as described (52) (Fig. 3). 129x1 WT and IFNRKO mice were infected with EcoHIV by intracerebral injection, and brains were harvested at the indicated times after infection to measure HIV-1 RNA either by qPCR (Fig. 3A) or by conventional RT-PCR combined with Southern blot hybridization (Fig. 3B). To ensure that only de novo synthesized viral products are detected, we amplified spliced HIV-1 RNA species *vif* and *nef*, which are not packaged in virions. At 1 week after infection, *vif* RNA was clearly

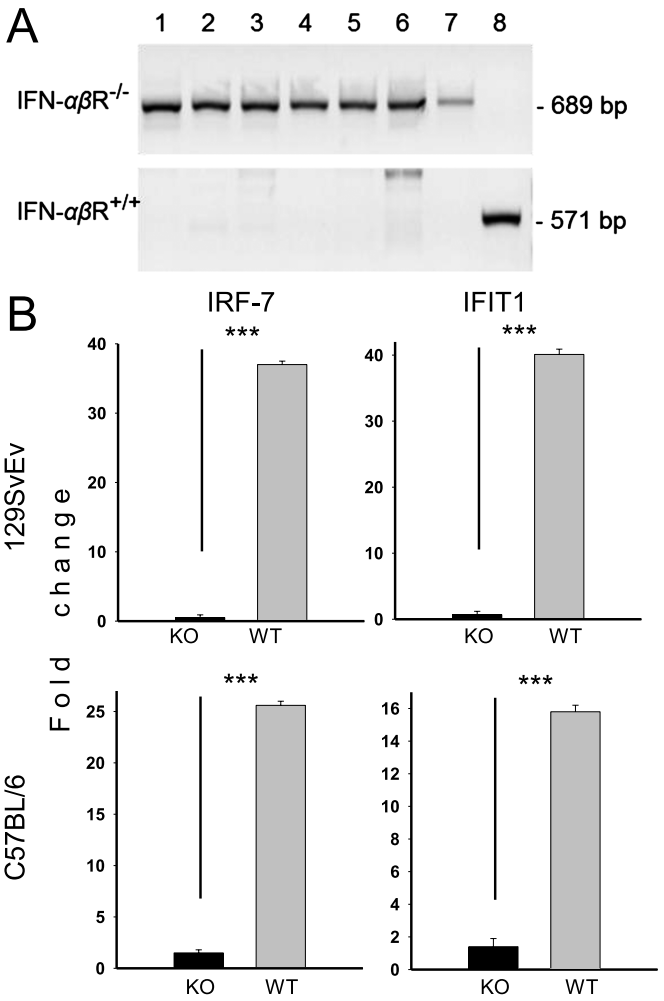

FIGURE 1. Confirmation of the interferon-α/β receptor–negative (*IFN-αβR*$^{-/-}$) phenotype of mouse strains used. **(A)** Southern blot hybridization within the *IFN-αβR* gene showing the mobility change after insertion of the targeting vector. Data from 7 C57BL/IFNRKO mice are compared to a wild-type (WT) control. A 571-bp RNA product for intact (WT, lane 8) and 689-bp RNA products for mutant receptor gene alleles (lanes 1–7) are demonstrated. **(B)** Detection of responses to Type I interferon (IFN-α) in culture by cells from WT or *IFN-αβR*$^{-/-}$ mice. Bone marrow–derived macrophages were stimulated with recombinant mouse IFN-α and tested for induction of the IFN-stimulated genes *IRF-7* and *IFIT1* by qPCR after 24 hours. ***, p = 0.001. KO, knockout.

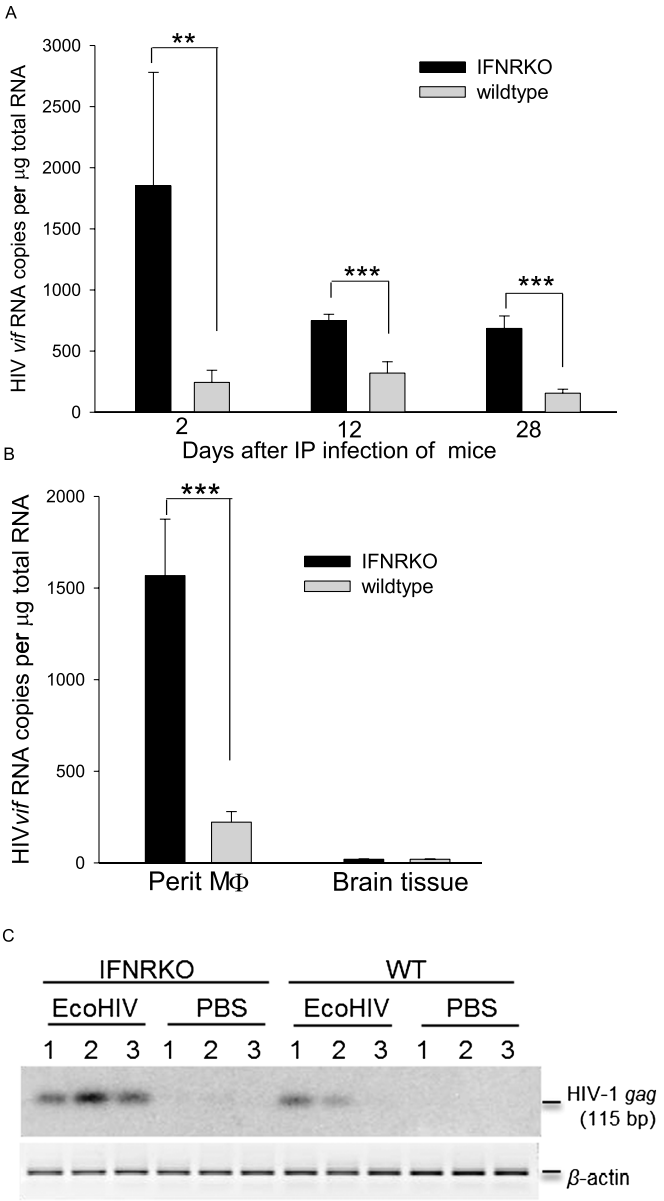

FIGURE 2. Absence of Type I interferon (IFN) receptors in mice permits increased systemic human immunodeficiency virus Type 1 (HIV-1) replication and viral neuroinvasion after intraperitoneal (IP) EcoHIV inoculation. **(A)** Interferon-α/β receptor–negative (IFNRKO) and wild-type (WT) 129x1 mice were infected by IP injection of 10^6 pg of p24 of EcoHIV/NDK, and at the indicated time points, peritoneal macrophages isolated from groups of infected mice (n = 3) were tested for expression of spliced *vif* mRNA by RT-qPCR. **, p = 0.01; ***, p = 0.001. **(B, C)** In a repeat experiment, mice infected as in **(A)** were tested 4 weeks after IP infection for *vif* mRNA expression by qPCR in peripheral macrophages (Perit M Φ) and brain tissue **(B)** and for HIV-1 *gag* DNA in brain tissue by Southern blot hybridization **(C)**.

detected by qPCR in brain tissues of both WT and IFNRKO mice (Fig. 3A); although HIV-1 RNA burdens were lower in WT mice, the difference was not significant at this time point. By 2 and 4 weeks after infection, *vif* RNA was significantly lower in brains of WT mice than in IFNRKO brains (Fig. 3A), suggesting a marked effect of IFN signaling on HIV-1 replication. Similar results were obtained by a semiquantitative analysis of brain RNAs from this experiment by conventional RT-PCR amplifying spliced HIV-1 RNAs that share a splice acceptor site at the 3′ end of the viral genome (56), of which a 342-bp *nef* message is a predominant product (Fig. 3B). To determine whether Type I IFN acts at the level of HIV-1 DNA or RNA synthesis in the brain, we repeated the experiment in the same format twice, using either the 129x1 or the C57BL mouse strain. At 4 weeks after infection, we sampled viral RNA as well as DNA in brain tissues, amplifying 2-LTR circular DNA and *vif* RNA (Fig. 3C, D). We chose to amplify circular HIV-1 DNA because, unlike full-length DNA, this viral DNA form is absent from the HIV-1 inoculum and its amplification by qPCR provides a quantitative measure of HIV-1 infection de novo in mouse brain. As in the experiment shown in Figure 3A, IFNRKO mice in both backgrounds failed to control HIV-1 RNA expression in the brain as efficiently as did WT mice (Fig. 3C). It is striking, however, that there were no significant differences in viral DNA burden between IFNRKO and WT mice in either background (Fig. 3D). These findings extend the effects of the IFNRKO phenotype on HIV-1 expression from peripheral mononuclear cells to brain tissue, and they suggest that some IFN-responsive genes mitigate HIV-1 replication at the level of transcription in the brain.

In HIV-1–infected patients and in various animal models, HIV-1 neuropathogenesis has been associated with induction of a large number of cytokines, chemokines, and IFN-related genes (15, 29, 57–60). Here, we tested whether efficient EcoHIV infection of mouse brain also causes cellular gene dysregulation in the tissue and whether HIV-1 effects require Type I IFN signaling (Fig. 4). We chose 15 transcripts that have been described as being elevated either in HIV-1–infected patients (*IL-1β*, *IRF-7*, *Stat-1*, *MyD88*, and *IL-6*) (61, 62) or SIV-infected macaques (*CD8* and *CD163*) (63, 64) or in brain cells stimulated with HIV-1 or Tat in tissue culture (*MCP-1*, *C3*, and *IP-10*) (65–67). WT or IFNRKO mice on a 129x1 background were injected intracerebrally (IC) with EcoHIV or PBS and brains were collected 4 weeks later for isolation of RNA. Cellular transcripts as well as GAPDH were amplified by qPCR, the expression of each transcript was normalized by GAPDH expression, and finally, the ratio of expression of each transcript in infected compared to PBS-injected brain tissue was calculated to determine its induction during HIV-1 infection (Fig. 4). Fourteen transcripts were significantly upregulated in the brain of infected WT and IFNRKO mice; induction ranged from roughly 1.2-fold for synaptic protein SNAP23 to more than 200-fold for CD8 in IFNRKO mice (Fig. 4). Seven transcripts were induced to significantly greater levels in IFNRKO than in WT brains, consistent with their enhanced expression of HIV-1 RNA shown in Figure 3. However, 7 other transcripts, including 3 encoded by genes linked to the Type I IFN pathway (*IRF-7*, *STAT-1*, and *MyD88*), were significantly induced by HIV-1 independent of the presence of a functional IFN receptor (Fig. 4). Expression of *CD163*, a marker of perivascular macrophages implicated in SIV encephalitis (64), was unchanged and serves as a specificity control in this panel. Thus, EcoHIV causes extensive dysregulation of cellular gene expression in

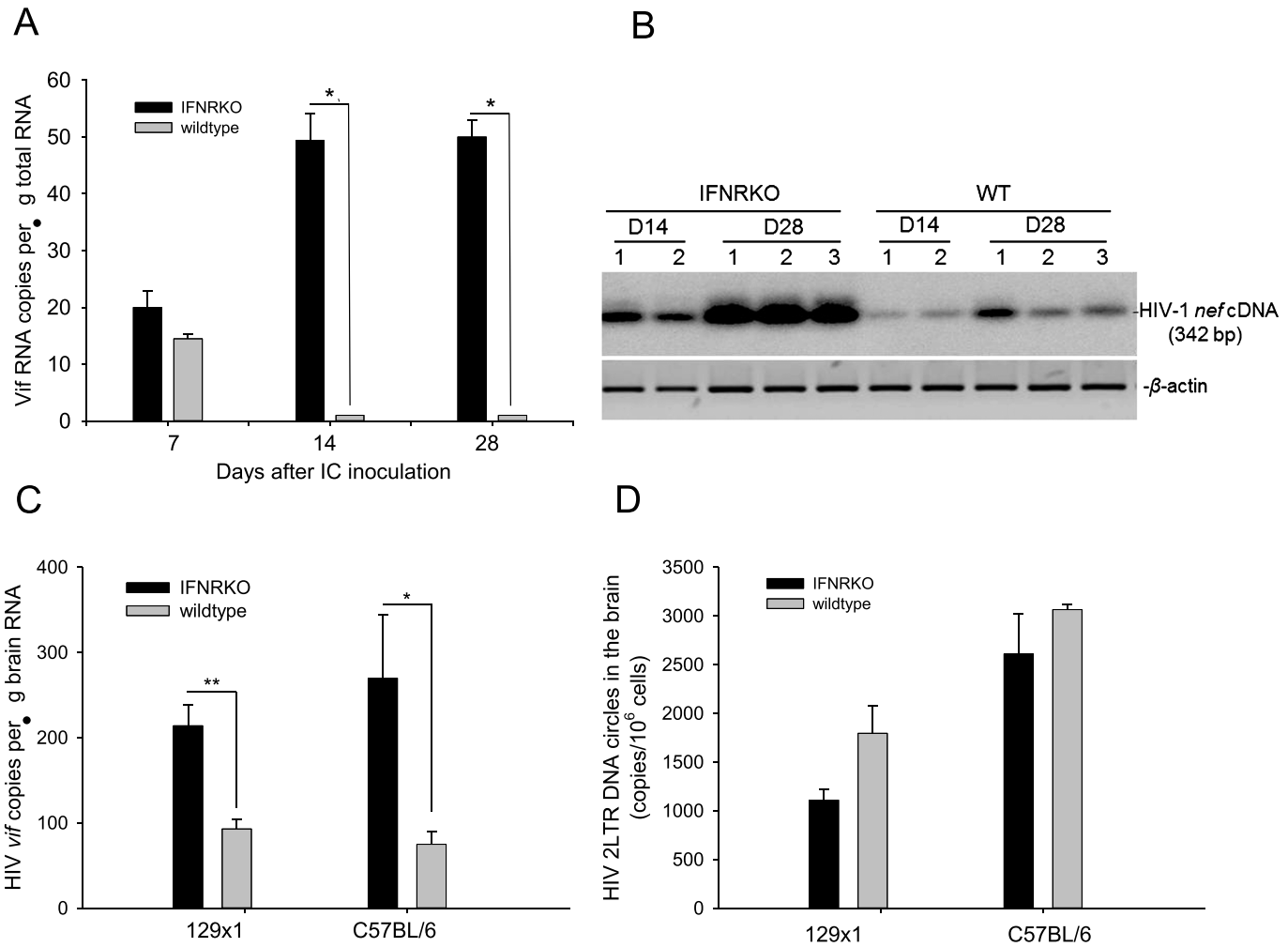

FIGURE 3. Human immunodeficiency virus Type 1 (HIV-1) expression is readily detectable after intracerebral (IC) inoculation of EcoHIV into mouse brain and virus expression is significantly greater in interferon-α/β receptor–negative (IFNRKO) mice versus wild-type (WT) mice. **(A, B)** IFNRKO and WT mice (129x1 strain) were inoculated IC with 10^6 pg of p24 of EcoHIV/NDK, and brain tissues from groups of mice (n = 3) were tested at the indicated time points for HIV-1 expression by QPCR amplification of the *vif* transcript **(A)** or RT-PCR and Northern blot hybridization for *nef* transcript **(B)**. **(C, D)** The experiment was repeated using 2 IFNRKO mouse strains, 129x1/IFNRKO and C57BL/IFNRKO and their matching WT controls. Brain tissues were tested for *vif* expression **(C)** and HIV-1 2LTR circular DNA **(D)** by QPCR 30 days after EcoHIV IC inoculation. *, p = 0.05; **, p = 0.01.

mouse brain, which can be partially mitigated by functional IFN responses.

Because productive HIV-1 infection in human brains generally correlates with overt pathological changes (68, 69), we next inquired about neuropathological changes in brain tissues from IC-infected mice, also as a function of the IFNR phenotype. C57BL/6 IFNRKO and WT mice received intracerebral injections of virus or PBS and evaluated 2 weeks later (Fig. 5). We counted perivascular lesions in hematoxylin and eosin–stained sections and used computerized image analysis to quantify Iba-1 staining as a measure of macrophage/microglial activation and GFAP staining as a measure of astrogliosis (Fig. 5). Representative fields from the right basal ganglia region in the vicinity of the injection site are shown in each category, and the frequencies of discrete pathological parameters and of their extent in this region are graphed. PBS injection was innocuous, indicating that IC inoculation alone did not lead to constitutive morphological changes in the brain, as previously reported (52). However, at 2 weeks after EcoHIV infection, brains from both WT and IFNRKO mice showed frequent perivascular infiltrates of mononuclear cells, as well as regions of excessive staining of lineage markers of both macrophage/microglia and astrocytes. The distinction between the maintenance and absence of IFN responses in the brains was quantitative, with each measure of neuropathogenesis significantly lower in WT mice, in which IFN responses were intact; when these responses were absent, there were significantly more perivascular lesions and greater Iba-1 and GFAP expression. As an internal control, we also evaluated pathological changes in the respective left (i.e. the uninjected) basal ganglia regions of affected brains and found only occasional low-level macrophage/microglial activation, astrogliosis, and no lesions (not shown). These observations are consistent with our previous results showing the highest expression of IC-injected EcoHIV near the inoculation site, with

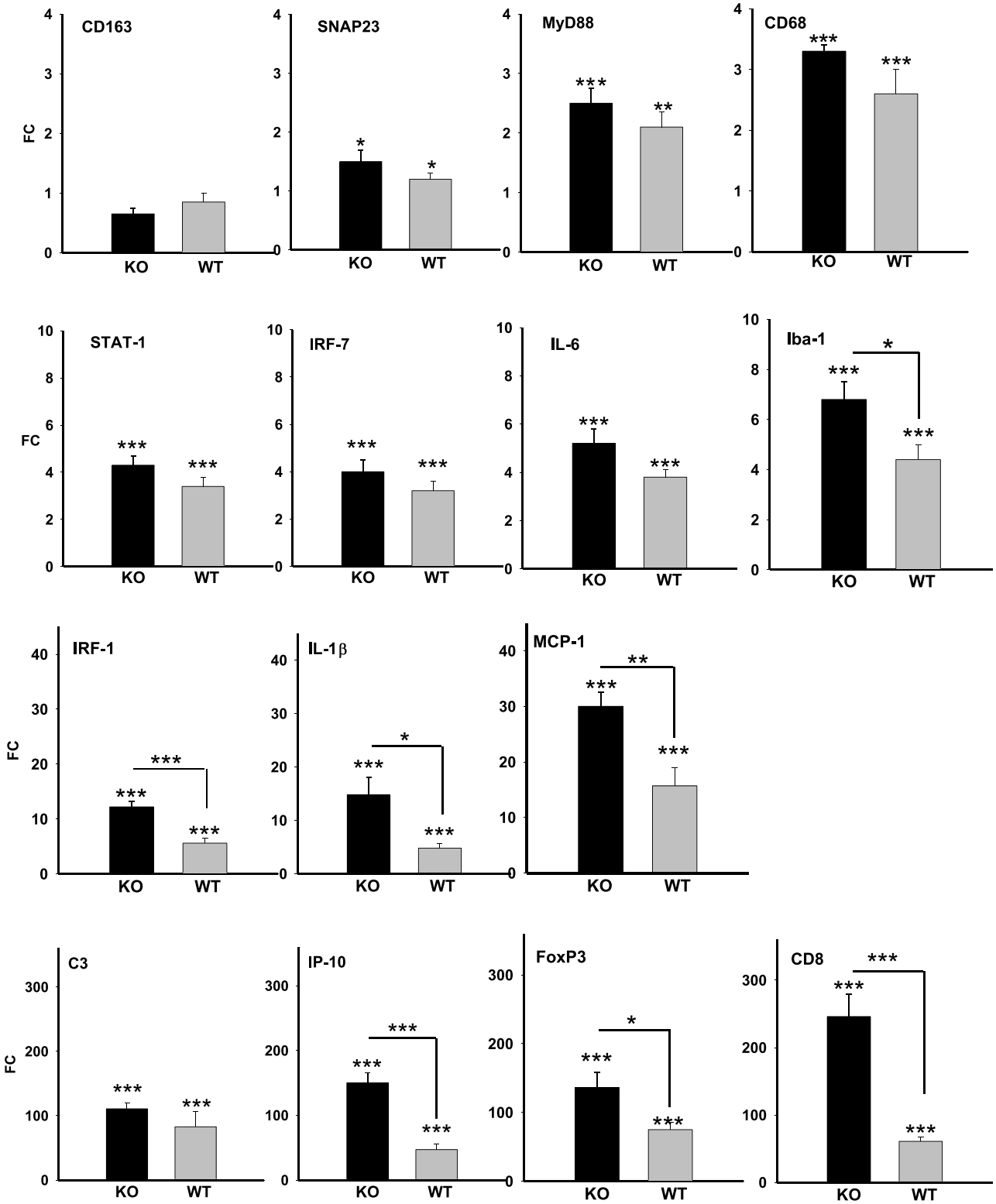

FIGURE 4. EcoHIV-infected interferon-α/β receptor–negative (IFNRKO) mice show greater dysregulation of some inflammatory and immune response genes in the brain versus wild-type (WT) mice. 129/IFNRKO (KO, black bars) and WT mice (gray bars) were infected by intracerebral injection with EcoHIV/NDK or with phosphate-buffered saline (PBS); 4 weeks later, the mice were killed and total cellular RNAs from brain tissues were compared by qPCR for expression of genes known to be involved in human immunodeficiency virus Type 1 (HIV-1) neuropathogenesis in patients. IFNRKO and WT mice inoculated with PBS served as respective controls for measurement of gene expression changes. FC, fold change.

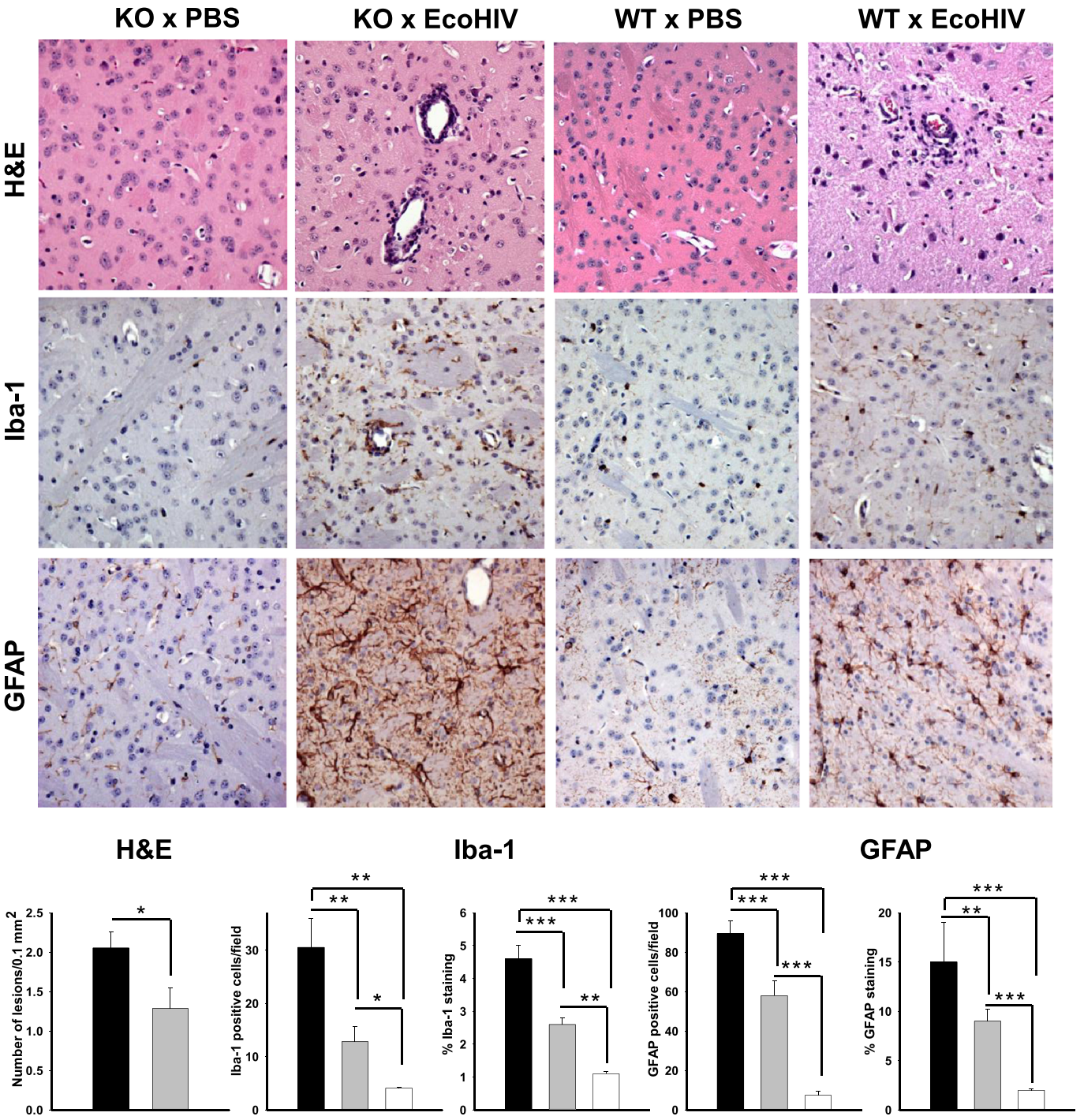

FIGURE 5. EcoHIV-infected interferon-α/β receptor–negative (KO) mice show more extensive brain abnormalities than infected wild-type (WT) mice. KO and WT mice received intracerebral infection with EcoHIV or phosphate-buffered saline (PBS). Two weeks later, the animals were killed, and brains were fixed, embedded in paraffin, and sectioned. Thin sections were stained with hematoxylin and eosin (H&E) or immunostained with an antibody to the macrophage/microglia marker Iba-1 or to the astrocyte marker glial fibrillary acidic protein (GFAP). The photomicrographs show representative fields at 10× from the right basal ganglia region in the vicinity of the injection site in each experimental system. There are perivascular mononuclear cells, extensive macrophage/microglial (MP/MG) activation, and astrocytosis in infected but not in control mice. Minimal pathology was found in the left (uninjected) hemispheres of infected mice (not shown). Graphs show means and SEM of quantitative analysis of perivascular lesion counts, MP/MG activation, and astrogliosis using 15 to 20 representative sections from 2 mice in each condition. Black bars, infected KO mice; gray bars, infected WT mice; open bars, uninfected WT mice. Asterisks indicate Student *t*-test p values for significance of differences among the conditions: *, p = 0.05; **, p = 0.01; ***, p = 0.001.

virus only slowly spreading to other brain regions (52). The differences in the extent of neuropathological changes between infected WT and IFNRKO mice were reproduced in 5 experiments, and they were independent of the EcoHIV backbone (NL4-3 or NDK) and mouse strain used (129x1 or C57BL). These findings support and extend those in Figure 4 showing that IFN responses reduce but do not eliminate cellular gene dysregulation during mouse brain infection.

Finally, we compared brain tissues from EcoHIV-infected WT and IFNRKO mice at 2 weeks after IC inoculation for the presence of HIV-1 p24, a virion structural protein indicative of productive viral infection (Fig. 6A, B). In some experiments, we infected mice with EcoHIV/NL4-3–GFP, an EcoHIV clone modified to express GFP as a convenient surrogate marker of HIV-1–encoded proteins for cellular colocalization (Fig. 6C–K). Using immunohistochemistry and monoclonal anti-p24 antibody for p24 staining in paraffin-embedded brain sections, we found that IFNRKO mice expressed p24 more efficiently and had higher number of p24-positive cells than did WT mice (Fig. 6A, B), a result consistent with their more extensive expression of viral RNA (Fig. 4). The morphology of p24-positive cells in IFNRKO brains resembled that of macrophages/microglia (Fig. 6A), while p24 in WT brains resided mostly in round mononuclear cells (Fig. 6B). To identify the lineage of cells productively infected by HIV-1 in mouse brain, we used frozen brain sections and used fluorescence microscopy staining for Iba-1 to detect macrophages/microglia and GFP to detect HIV-1 (Fig. 6C–K). Brains of IFNRKO mice showed abundant GFP expression that frequently localized to Iba-1–positive cells with multiple cellular processes (Fig. 6C–H). Using Z-stacking and high-magnification confocal microscopy to visualize GFP-expressing HIV-1–infected cells in more detail, it is clear in the IFNRKO mice that they are Iba-1–positive (Fig. 6F–H), with highly ramified processes characteristic of activated microglia. Similarly, brains of infected WT mice contained HIV-1–expressing Iba-1–positive cells but at a lower frequency than IFNRKO mice, with many GFP-positive cells appearing as round cells without processes (Fig. 6I–K). Merged images in Figure 6H and K also show that some GFP-positive cells did not co-stain with a macrophage marker. A detailed study of EcoHIV tropism to mouse CNS cells is underway and will be reported separately. The present study shows that EcoHIV can establish productive infection in brain macrophages/microglia in mice in vivo, and this infection is more prominent in IFNRKO mice.

DISCUSSION

Our results strongly suggest that innate immune responses mediated by Type I IFN can mitigate, albeit not arrest, systemic HIV-1 infection and neuropathogenesis in vivo. The closest parallel to our findings comes from studies on induction of viral encephalitis during infection of macaques with SIV (36). In this system, SIV entry into the brain early after virus exposure coincides with induction of IFN-β and imposition of SIV transcriptional latency that lasts through the asymptomatic period of disease, suggesting the role of IFN-β in delaying SIV neuropathogenesis (37, 38). Type 1 IFN has been described to control HIV-1 infection in tissue culture in a variety of cell types and at different stages in virus replication (23, 47, 61, 70). While studies in culture also indicate that HIV-1 has evolved mechanisms to counter many innate antiviral responses (44–48), the extent of HIV-1 escape from these constraints in vivo has not been determined. Our results suggest that functional IFN responses in vivo afford measurable advantage to the host in fighting HIV-1 infection, including that in the brain, at least in the animal model tested. In HIV-1–infected human beings, both monotherapy and adjunct therapy with recombinant IFN-α were found to reduce plasma virus burden (71–73), suggesting that augmentation of native IFN responses to HIV-1 can be clinically beneficial. A similar approach could be explored for the amelioration of HIV-1 brain disease.

The conclusions of the present study were made possible by the resource of mice lacking Type I IFN responses (22) and the ability of EcoHIV to infect a range of mouse strains (49, 51, 53, 74). Although the initial expression of IFN proteins themselves is not affected in IFNRKO mice (22), IFN responses are not transduced, and the mice succumb to cytopathic viruses (e.g. vesicular stomatitis virus and Semliki Forest virus) at doses 10^6-fold less than do WT mice (21, 22, 75). Consistent with these studies, we show that mice defective in IFNR signaling maintained significantly higher systemic and CNS HIV-1 burdens than did WT mice (Fig. 2), albeit lesser in extent than that seen with some other viruses (22, 75). Natural killer cells (76) or innate virus resistance factors (45) may also contribute to early HIV control in the mouse host. Because HIV-1 DNA levels were similar in infected WT and IFNRKO mouse brains (Fig. 3), the effect of Type I IFN signaling on EcoHIV infection in mouse brain seems to be mediated at the viral transcription level. This result is consistent with our finding that HIV-1 can infect macrophages/microglia in mouse brains (Fig. 6) and the demonstration by others that infection of cells of this lineage by HIV-1 in culture is blocked by Type I IFN at transcription (34, 35). Transcriptional silencing of HIV DNA in these cells could mitigate neuropathogenesis by reducing virus production and expression of viral neuropathogenic proteins, including Tat and Nef (77, 78). Further studies are needed to clarify the mechanism of HIV-1 inhibition by IFN in mice and, in particular, why this block seems to be incomplete.

Intracerebral inoculation of EcoHIV into both WT and IFNRKO mice caused neuropathological and molecular changes with characteristics similar to those in human HAND (28, 29, 68, 69, 79, 80). However, compared to infected WT mice, higher HIV-1 RNA burdens in brain tissues of infected IFNRKO mice correlated with increased brain pathology in these animals, including significantly greater numbers of perivascular inflammatory lesions, increased microglial and astrocyte activation, and increased induction of inflammatory and other cellular genes previously linked to neuropathogenesis in HIV-1–infected humans (Figs. 4–6). Conversely, because infected IFNRKO and WT mice differ singularly in their ability to signal through IFN receptors (22), our results strongly suggest that functional IFN responses mitigate HIV-1 neuropathogenesis through suppression of HIV-1 expression in the brain. This was most evident in the clear difference between infected IFNKO and WT mice in the presence of HIV-1–positive macrophages/microglia, the main cellular agents of HIV-1–mediated neuropathogenesis (Fig. 6) (15, 16). In fact, the detection of

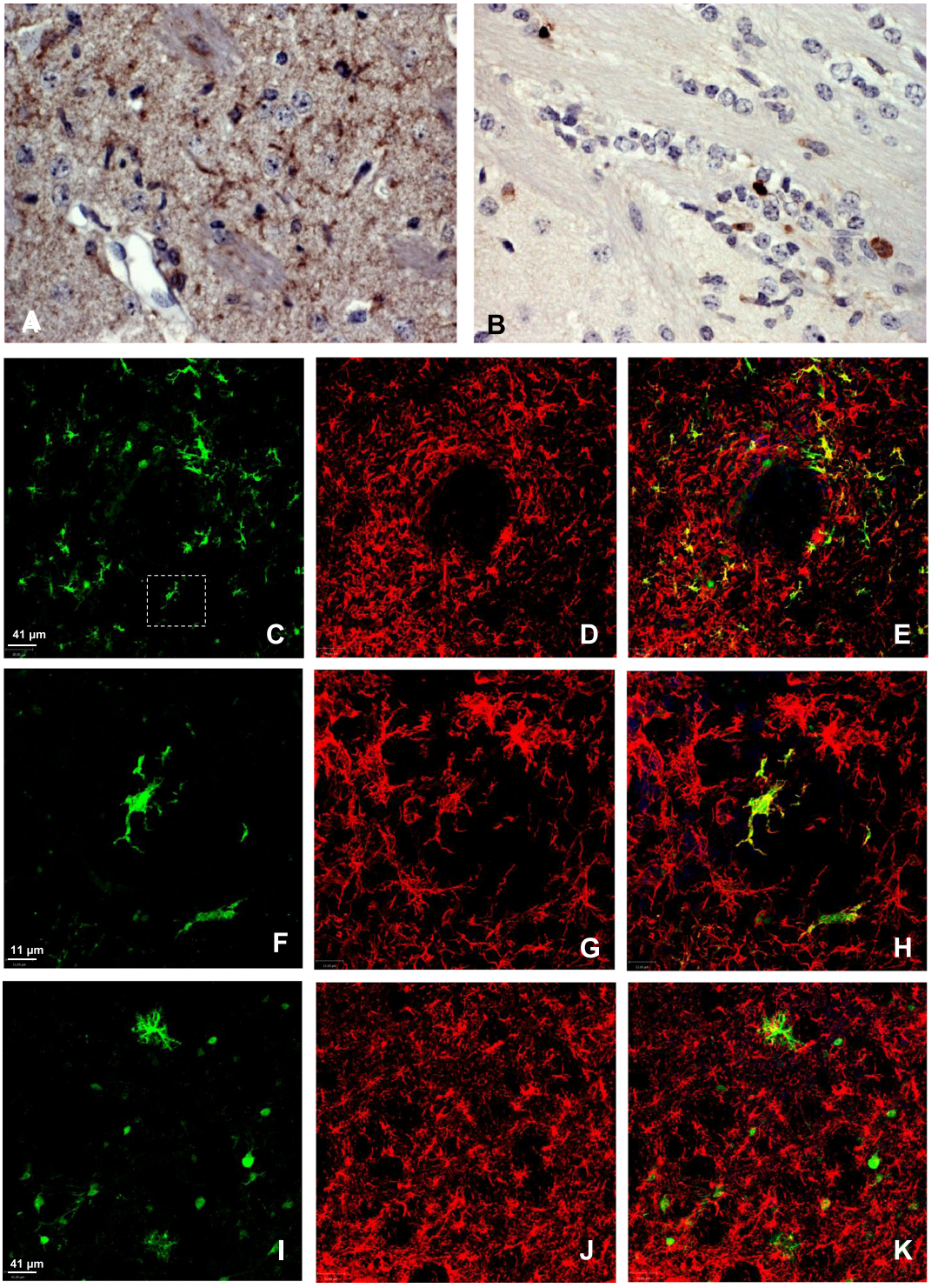

FIGURE 6. HIV-1 p24 and HIV-1–encoded marker green fluorescent protein (GFP) localize to microglia/macrophages in the brains of infected mice and are more efficiently expressed in interferon-α/β receptor–negative (IFNRKO) than wild-type (WT) animals. **(A–K)** At 2 weeks after intracerebral infection of mice with EcoHIV/NDK **(A, B)** or EcoHIV/NL4-GFP **(C–K)**, the brains were removed, fixed, sectioned, and either stained for p24 by immunohistochemistry and evaluated by light microscopy **(A, B)** or co-stained for the macrophage marker Iba-1 and virally encoded GFP and evaluated by fluorescence microscopy **(C–K)**. The figure compares virally coded protein expression in brain tissues of IFNRKO **(A, C–H)** to that of WT mice **(B, I–K)**. Panels **F** to **H** show higher magnification of dashed area in Panel **C** enhanced by Z-stacking for better resolution of HIV-1–positive macrophages/microglia. Original magnification: **(A, B)** 25×. In **B**, p24-positive cells are mostly mononuclear, with Iba-1–negative, HIV-1–positive mononuclear cells also present in **I** to **K** (also WT). **C, F, J**: GFP (green); **D, G, J**: Iba-1 (red); **E, H, K**: merge.

p24-positive (and marker protein GFP-positive) macrophages/microglia in infected IFNRKO mice bears similarity to HIV encephalitis in humans (68, 69) and to experimental HIV encephalitis in other animal models (55, 81–83). These results also support a causal relationship between the extent of HIV-1 expression in the brain and the severity of brain pathology (28, 29, 84). However, IFNRKO mice infected with EcoHIV by intracerebral inoculation do not display the high virus burdens, multinucleated giant cells, and gross morphological changes that are typical of HIV or SIV encephalitis (68, 69, 81).

Recent studies indicate that antiviral inflammatory responses are intact in IFNRKO mice despite the absence of IFN signaling (85), indicating that this strain offers the opportunity to distinguish between these potential pathogenic pathways. With differences in HIV-1 expression in the brain linked to IFN responses, we were able to investigate viral neuropathogenesis in this model as a function of cellular products that are induced by virus expression (Fig. 4). HIV-1 and its proteins have been shown to be directly neuropathogenic (77, 86–88) and induce large number of brain cell gene products implicated in neuroinflammation and brain disease (62, 65, 89, 90). Here, 14 of the 15 cellular transcripts shown were induced by EcoHIV infection in brains of WT and IFNRKO mice, and of these, 7 were more significantly induced in the IFNRKO strain. The latter observation indicates that extracellular Type I IFN is not required for their induction in this system and supports the contention that HIV-1 products and the induction of intermediate cellular products are responsible for the enhanced gene expression. Indeed, if induction of the 7 inflammatory and immune response–linked genes overexpressed in IFNRKO brain is considered pathogenic, it can be concluded that, in this case, Type I IFN protects against HIV-1–associated neuropathogenesis. It should also be noted that EcoHIV expresses all HIV-1 genes except gp120 (53), and therefore, the HIV-1–associated neuropathogenesis observed in this work occurs independently of gp120. Because EcoHIV transcribes well in mouse brain (Fig. 3) and because Tat is expressed systemically in EcoHIV–infected mice (53), the virus likely expresses functional Tat in the brain, which can contribute to the observed pathological findings alone (91) or together with other viral proteins such as Nef or Vpr (88, 92).

We interpret extensive overexpression of lineage-restricted markers like CD8 and FoxP3 as evidence for the migration of CD8-positive and regulatory T cells, respectively, into the brain, as previously observed during HIV-1 or SIV infection (63, 93). IRF-1 is induced very early in HIV-1 infection, and IP-10 can be induced by HIV-1 Nef, providing a potential link between greater induction of these cytokines in IFNRKO mice and increased viral expression (92, 94). However, there are multiple routes to cellular gene induction during HIV-1 infection. For example, interleukin (IL)-1β produced by microglia in HIV-1–infected brain (62), and as shown here, may itself drive the expression of MCP-1 in astrocytes (95). The degree of induction of cellular transcripts during EcoHIV infection is consistent with the neuropathology observed in WT mice and the more extensive neuropathology observed in IFNRKO mice (Fig. 5).

In summary, our studies support a protective role of Type I IFN in HIV-1 expression in the periphery and in the brain. Our work demonstrates that this central, innate immune pathway reduces but does not eliminate HIV-1 neuropathogenesis through its induction of posttranscriptional latency in the brain. Therefore, Type I IFN may supplement existing antiretroviral therapies to minimize HIV-1 pathogenesis.

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