CCR5 Knockout Prevents Neuronal Injury and Behavioral Impairment Induced in a Transgenic Mouse Model by a CXCR4-Using HIV-1 Glycoprotein 120


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CCR5 Knockout Prevents Neuronal Injury and Behavioral Impairment Induced in a Transgenic Mouse Model by a CXCR4-Using HIV-1 Glycoprotein 120

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The innate immune system has been implicated in several neurodegenerative diseases, including HIV-1–associated dementia. In this study, we show that genetic ablation of CCR5 prevents microglial activation and neuronal damage in a transgenic model of HIV-associated brain injury induced by a CXCR4-using viral envelope gp120. The CCR5 knockout (KO) also rescues spatial learning and memory in gp120-transgenic mice. However, the CCR5KO does not abrogate astrocytosis, indicating it can occur independently from neuronal injury and behavioral impairment. To characterize further the neuroprotective effect of CCR5 deficiency we performed a genome-wide gene expression analysis of brains from HIVgp120tg mice expressing or lacking CCR5 and nontransgenic controls. A comparison with a human brain microarray study reveals that brains of HIVgp120tg mice and HIV patients with neurocognitive impairment share numerous differentially regulated genes. Furthermore, brains of CCR5 wild-type and CCR5KO gp120tg mice express markers of an innate immune response. One of the most significantly upregulated factors is the acute phase protein lipocalin-2 (LCN2). Using cerebrocortical cell cultures, we find that LCN2 is neurotoxic in a CCR5-dependent fashion, whereas inhibition of CCR5 alone is not sufficient to abrogate neurotoxicity of a CXCR4-using gp120. However, the combination of pharmacologic CCR5 blockade and LCN2 protects neurons from toxicity of a CXCR4-using gp120, thus recapitulating the finding in CCR5-deficient gp120tg mouse brain. Our study provides evidence for an indirect pathologic role of CCR5 and a novel protective effect of LCN2 in combination with inhibition of CCR5 in HIV-associated brain injury. The Journal of Immunology, 2014, 193: 1895–1910.

The chemokine receptors CCR5 and CXCR4 are coreceptors besides CD4 for HIV infection (1). CCR5 deficiency owing to a congenital deletion mutation, named CCR5Δ32, leads to absence of this receptor from the cell surface and confers protection against infection with CCR5-prefering HIV-1 (2). Experimental knockdown of CCR5 in human macrophages also can prevent infection of the cells with CCR5-prefering virus (3). CCR5 deficiency is not associated with a pathologic phenotype in human beings (2) or in mice (4, 5). However, CCR5 knockout (KO) and wild-type (WT) animals differ in their responses to infections, including the migration of peripheral monocytes and macrophages into the CNS (4, 5). Moreover, upon stimulation CCR5-deficient macrophages show reduced cytokine production of GM-CSF, IL-1β, and IL-6 compared with their WT counterparts (4). Independently of a function in HIV entry, CCR5 and its endogenous ligands CCL3, CCL4, and CCL5 can delay progression to AIDS (6, 7). However, once HIV infection is established, dual tropic and CXCR4-prefering viruses often evolve and usually herald progression to AIDS and HIV-associated dementia (1, 8–11).

Transgenic (tg) mice expressing a soluble viral envelope gp120 of HIV-1 LAI in the brain have been described previously (12). The CXCR4-using HIV-1 LAI isolate infects lymphocytes and macrophages (13). We recently showed in mixed neuronal-glial cerebro-cortical cell cultures from mice genetically deficient in CCR5 or CXCR4, or both coreceptors, that the specificity of HIV gp120 coreceptor usage in the mouse model resembled that in human cells (14). The soluble transgenic gp120 is expressed in astrocytes under the control of the promoter for glial fibrillary acidic protein (GFAP) and can be detected by immunoblotting in brain extracts (15). HIVgp120g mice manifest several neuropathologic features observed in AIDS brains, such as decreased synaptic and dendritic density, increased numbers of activated microglia, and astrocytosis (12, 16–19). HIVgp120tg mice also develop behavioral impair-
ment, such as reduced escape latency, swimming velocity, and spatial retention before 12 mo of age (20, 21).

In this study, we show that genetic ablation of CCR5 prevents neuronal injury and microglial activation in HIV gp120tg mice even though the transgenic gp120 utilizes CXCR4. CCR5 deficiency also protects gp120tg mice against impairment of spatial learning and memory; however, CCR5-ablation fails to abrogate astrocytosis. Genome-wide gene expression analysis shows that gp120tg brains upregulate, among other factors of the innate immune system, the acute phase protein lipocalin (LCN2) 2, which promotes activation while also priming the demise of microglia (22, 23). We find that LCN2 is itself neurotoxic in a CCR5-dependent fashion. Moreover, we observe that blockade of CCR5 signaling and LCN2 cooperate to diminish microglial cell numbers and to prevent neurotoxicity of a CXCR4-using gp120, thus recapitulating the findings in CCR5-deficient gp120tg mice.

Materials and Methods

Animals

Two founder lines of HIV gp120tg mice were provided by Dr. Lennart Mucke (Gladstone Institute of Neurological Disease, University of California, San Francisco, CA) (12). CCR5 knockout mice (CCR5KO, B6.129P2-Ccr5<sup>−/−</sup>/J) were purchased from The Jackson Laboratory. CCR5KO and HIV gp120tg mice were crossed, and F2 animals heterozygous for CCR5 and HIV gp120 were used to generate colonies of the new mouse lines (referred to as lines 1 and 2) comprising all necessary genotypes. Genotyping was performed according to protocols published in the literature (24) and were approved by the Institutional Animal Care and Use Committees of the Sanford-Burnham Medical Research Institute and The Scripps Research Institute.

Behavioral testing

WT, gp120, CCR5KO, and CCR5KO x gp120 mice were between 8 and 9 months of age at the beginning of testing that occurred over a period of 5 weeks.

Light/dark transfer test. The light/dark transfer procedure has been used to assess anxiety-like behavior in mice (24) and was performed as described previously (25). The apparatus is a rectangular box made of Plexiglas divided by a partition into two environments. One compartment is dark, and the other compartment is highly illuminated by a light source located above it. The compartments are connected by an opening located at floor level in the center of the partition. The time spent in the light compartment is used as a predictor of anxiety-like behavior. Mice were placed in the dark compartment to start the 5-min test.

Locomotor activity test. Activity levels were measured in polycarbonate cages placed into frames mounted with two levels of photocell beams at 2 and 7 cm above the bottom of the cage (San Diego Instruments, San Diego, CA) as recently published (26). These two sets of beams allowed for the recording of both horizontal (locomotion) and vertical (rearing) behavior. A thin layer of bedding material was applied to the bottom of the cage. Mice were tested for 2 h.

Barnes maze test. The Barnes maze test assesses spatial learning and memory (27, 28). In addition, the test reveals the strategies used by the animals to perform the task. The test was performed as previously published with minor modifications (29). The Barnes maze used was an opaque Plexiglas disc that was 75 cm in diameter and elevated 58 cm above the floor by a tripod. Twenty holes, 5 cm in diameter, were located 5 cm from the perimeter, and a black Plexiglas escape box was placed under one of the holes. Distinct spatial cues were located all around the maze and were kept constant throughout the study. Mice were tested once a day for 9 d for the acquisition portion of the study. For the Probe Test, the 10th test, the escape tunnel was removed and the mouse was allowed to explore the maze freely for 3 min. The time spent in each quadrant was determined, and the percent time spent in the target quadrant was compared with the average percent time in the other three quadrants. Each session was videotaped and scored by an experimenter who was blind to the genotype of the mouse. Measures recorded included the latency to escape, the number of errors made per session and the strategy used by the mouse to locate the escape tunnel. Search strategies were determined by examining each mouse’s daily session and classifying it into one of three operationally defined categories: 1) random search strategy, using localized hole searches separated by crossings through the center of the maze; 2) serial search strategy, using systematic hole searches (every hole or every other hole) in a clockwise or counterclockwise direction; or 3) spatial search strategy, reaching the escape tunnel with both error and distance (number of holes between the first hole visited and the escape tunnel) scores of less than or equal to 3.

Immunofluorescence and deconvolution microscopy

For neuropathologic assessment, mice were deeply anesthetized with Isoflurane and immediately transcardially perfused with 0.9% saline. Brains were removed quickly and fixed with 4% paraformaldehyde in PBS (pH 7.4) for 48 h at 4°C. All experimental animals were coded before the process of analysis. For histopathologic analysis, 30-μm-thick sagittal sections were permeabilized with 1% Triton X-100 (freshly prepared) in PBS for 30 min. Nonspecific binding sites were blocked with 10% heat-inactivated goat serum in PBS containing 0.05% Tween 20 (PBS-T) for 1.5 h. Floating sections were immunostained with an anti–MAP-2 mAb (1:500; Sigma) and a polyclonal rabbit anti-synaptophysin (1:500; Dako) as neuronal markers, or ionized calcium binding adaptor molecule 1 (Iba1; 1:125; Wako) and GFAP (1:500; Dako) as microglial and astrocytic markers, respectively. Rhodamine-conjugated goat anti-mouse (1:200; Jackson ImmunoResearch) and Alexa Fluor 488-labeled goat anti-rabbit (1:200; Molecular Probes/Life Technologies) in 5% goat serum in PBS-T were used as secondary Abs. Nuclear DNA was labeled with H33342 (12 μM in PBS).

Observers blinded to genotype analyzed neuronal damage and gliosis in neocortex and hippocampus using an inverted Axiovert 100M fluorescence microscope (Zeiss) fitted with a computer-controlled three-dimensional stage, the appropriate filter sets, and a charge-coupled device camera. We analyzed per animal and marker at least three sagittal sections spaced 300 μm from each other medial to lateral. For each section, we recorded five fields (cerebral cortex) and three fields (hippocampus), using 40× image stacks at 0.5-μm steps along the z-axis. Deconvolution microscopy and fluorescence and volumetric quantitation was performed with the Slidebook software package (Intelligent Imaging Innovations, Denver, CO) (12, 20, 21). After deconvolution with a constrained iterative algorithm, the percentage of neuropil occupied by MAP-2<sup>+</sup> dendrites and Syn<sup>+</sup> presynaptic terminals was determined by threshold segmentation and compared among the different genotype groups. GFAP fluorescence was quantified in 10× images using masks for areas of interest. For quantification of Iba1<sup>+</sup> microglia, we counted cell bodies on the lateral side of three sagittal sections per animal in the area of interest in cerebral cortex and hippocampus.

For analysis of microglial TNF-α, additional brain sections were incubated with mouse anti–TNF-α mAb (1:100; Santa Cruz Biotechnology) in combination with the rabbit anti-Iba1 Ab. A donkey anti-rabbit IgG labeled with Alexa Fluor 647 (1:400; Invitrogen) and Rhodamine–conjugated goat anti-mouse IgG served as secondary Abs. DNA was labeled with H33342. Staining with irrelevant IgG of the same isotype and omission of primary Ab served as controls. Threshold segmentation for Iba1<sup>+</sup> fluorescence was used to define the areas occupied by microglial cell bodies. Specific fluorescence intensities were estimated for TNF-α within microglial cells using the Slidebook software package.

LCN2 was detected using a rat anti-mouse LCN2 IgG (1:500; R&D Systems) and a secondary chicken anti rat Ab labeled with Alexa 488 (1:400; Invitrogen). Staining for LCN2 was combined with one of the aforementioned Abs for Iba1, GFAP, or MAP-2 and the matching secondary Abs. Nuclear DNA was labeled with H33342 (12 μM in PBS).

Microarray

For analysis of whole-genome gene expression, mice were placed under deep general anesthesia with Isoflurane and immediately transcardially perfused with 0.9% saline. Brains were removed quickly and snap frozen in liquid nitrogen. All RNA was purified in our laboratory using the Qiagen RNeasy Lipid Tissue Midi Kit (Qiagen, Valencia, CA). RNA purification was performed with an RNeasy Lipid Tissue Midi Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Sample amplification, labeling, and hybridization on Illumina mouse WG6 v2 Sentrix BeadChips were performed for all arrays in this study according to the manufacturer’s instructions (Illumina, Inc.), and primary hybridization data were collected using an Illumina BeadStation (Sanford Burnham Institute Microarray Core). For line 1 and line 2, replicate array data were each divided into 20 sets of replicates by genotype and age; numbers indicate months of age, and numbers in brackets show biological replicate samples in the group (n): Line 1: months 1.5/3/6/12/20; WT: (6/6/4/6/6); gp120: (7/6/5/6/5); CCR5KO: (3/5/6/6/5); CCR5KO x gp120: (5/3/4/6/5). Line 2: months 1.5/3/6/12/16; WT: (7/6/5/6/3);...
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C5R5KO: (7/6/4/8/3); CCR5KO x gp120: (6/7/7/8/4). The data were treated independently as line 1 (104 arrays) and line 2 (121 arrays). Initially, the primary Immulina mouse WG-6 array data underwent quality control with the Immulina BeadStudio detection $p < 0.05$ as a cutoff for continued inclusion in the analysis. After filtering, 37,349 and 34,749 probes were retained for the line 1 and line 2 experiments, respectively. The data were then quantile normalized and log2 transformed, and the limma function removeBatchEffects was used to alleviate any chip-dependent fluctuations. GeneSpring GX12 expression analysis software was used to perform two-way ANOVA examining the variation in CNS gene expression as a function of mouse age and HIV gp120 genotype separately in CCR5WT and CCR5KO backgrounds and for both mouse lines 1 and 2. The samples were paired by age for each series. The $p$ value cutoff of 0.05 was used to determine significance for differential expression between the conditions. This was followed by intersecting the significant results from the two lines to obtain those genes that were commonly differentially expressed (core common set of genes). Hypergeometric tests found the overlaps between line 1 and 2 to be highly significant ($p < 1 \times 10^{-30}$). The microarray data generated in this study have been deposited in the GEO database under accession number GSE47029 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=jhgnxkscaigtwac&acc=GSE47029).

Bioinformatic s

For comparison of differentially expressed genes in brains of HIV patients (31) with the core common set of genes for CCR5KO x HIV gp120tg mouse lines 1 and 2, the published human gene probe lists were matched with corresponding mouse probes using the BioMart Gene ID Converter tool (http://www.ensembl.org), and the comparison was performed using GeneSpring GX12. The data from the core common set of genes for CCR5KO x HIV gp120tg mouse lines 1 and 2 were exported and uploaded to the commercially available bioinformatic tools of Ingenuity Pathway Analysis (IPA, Ingenuity Systems; http://www.ingenuity.com). Using these tools, the lists were evaluated for enrichment in biologically related genes and involvement in biological canonical pathways. Canonical pathway analyses were generated with IPA and heat maps using the tool HierarchicalClusteringImage with the default color map of GenePattern 3.5.0 software (BROAD Institute).

Quantitative RT-PCR

RNA was isolated from frozen mouse brain tissue as described above for microarray experiments. Reverse transcription (RT) was performed using SuperScript II reverse transcriptase (Invitrogen, Life Technologies, Carlsbad, CA) following the manufacturer’s instructions with some modifications. RNA (500 ng) purified from mouse brain tissue was used to generate cDNA with specific primers and preparations were aliquoted and conserved at $-20˚C$ until use. Real-time PCR was performed using Power PCR SYBR Green Master Mix (Applied Biosystems, Foster City CA; Life Technologies, Carlsbad, CA). The amplification reactions were performed in a total volume of 20 µl containing 2 µl RT, 10 µl Power PCR SYBR Green Master Mix (including HotStart DNA polymerase, reaction buffer, dNTP mix, and SYBR Green dye), 0.5 µM specific primers (Table I), and 8 µl PCR-grade water. PCR amplifications were performed with the 7300 Real-Time PCR System (Applied Biosystems) using universal thermal conditions: 10 min at 95˚C and 40 cycles of 15 s at 95˚C and 1 min at 60˚C. A denaturation step was added at the end of the amplification reaction for melting temperature analysis. The results obtained were analyzed using SDS version 1.2 software (Stratagene, Agilent Technologies). RNA samples corresponding to three to six biological replicates were analyzed. The signal for GAPDH was used as an internal reference. The relative amount of mRNA of each gene versus the internal controls (GAPDH) was calculated following the $2^{-DDCt}$ method. Murine gene sequences were retrieved from the GenBank database (http://www.ncbi.nlm.nih.org) for primer design. Primers were designed using three different sources: 1) PrimerBank (http://pga.mgh.harvard.edu/primerbank/), 2) Primer3 (http://frodo.wi.mit.edu/), and 3) Primer Express software sequences from the 7300 Real-Time PCR System (Applied Biosystems).

Flow cytometry

Brain mononuclear cells were enriched and analyzed following a published protocol (36). Cell suspensions from five mice were pooled and then fractionated on a discontinuous Percoll gradient. For comparison of CD45 high and CD45 low populations, spleens were also collected, minced, and stained with the same Abs as the brain mononuclear cells. After blocking Fc receptors with anti-FcγRI/III, cells were stained for extracellular markers with Abs to CD11b conjugated to PerCP-Cy5.5 (clone M1/70) and CD45 conjugated to APC (clone 30-F11), or with isotype controls for the respective Ab (all from eBioscience, San Diego, CA). After washing, cells were fixed for 20 min in 2% PFA/PBS on ice. Data were acquired with a FACSCanto flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo (Tree Star, Ashland, OR). Using the forward/side scatter (FSC/SSC) plot, gates were set on live cells, and these were further gated on singlets in the forward scatter area/height (FSC-A/SSC-H) plot to eliminate doublets or aggregates. Next, singlets were analyzed with CD11b-PerCP-Cy5.5 and CD45-APC expression. To quantify CD45low and CD45high-expressing cells, splenocytes were analyzed in the same way, and gates were set according to the CD45high-expressing cells. Each plot represents the analysis of 4–10× 106 events.

In vitro cell culture experiments

Cerebrocortical cell cultures containing neurons, astrocytes, and microglia were generated from E14.5 embryos of mouse line 2 as described previously with minor modifications (14, 37). Dissociated cerebrocortical cells were plated at $7 \times 10^5$ cells per well in 96-well, flat-bottom plates for imaging (BD Falcon, Franklin Lake, NJ). Generally, the cells were used for experiments at day 17 in vitro. We exposed cerebrocortical cell cultures to the CXC4r4 using gp120 of HIV-1$_{inm}$ (1 nM) in the presence or absence of recombinant murine LCN2 (4 nM; R&D Systems) or the CCR5 inhibitor Maraviroc (MVC; 5 nM) for 3 d. After fixation and labeling of microglial cells and nuclei with DNA with Tomato lectin, anti–MAP-2 Ab, and Hoechst 33342, respectively, the cerebrocortical cultures were analyzed with fluorescence microscopy and cell counting (five fields each per 9–10 replicates per condition) as described previously (14, 37). Recombinant gp120 of HIV-1$_{inm}$ and MVC were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program.

For analysis of microglial TNF-α and ARG1, exposed, fixed, and permeabilized cerebrocortical cultures on glass coverslips (14, 37) were incubated with mouse anti–TNF-α (1: 100; Santa Cruz Biotechnology) or rabbit anti-ARG1 IgG (1:200; Santa Cruz Biotechnology). Goat anti-mouse and donkey anti-rabbit IgG conjugated to Alexa Fluor 488 (1:400; Invitrogen) served as respective secondary Abs. Fluorescence staining with irrelevant IgG of the same isotype served as controls. The immunostaining was then combined with the Texas Red-conjugated Tomato lectin (1:200; Vector) and H33342 for labeling of microglia and nuclear DNA, respectively. Quantitative microscopic analysis was performed as described above for mouse brain sections using the Slidebook software package. Threshold segmentation for Tomato leucine was used to define the areas occupied by microglial cell bodies. Specific fluorescence intensities were estimated for TNF-α and ARG1 within microglial cells.

ELISA

Protein extracts from brains were prepared using a modification of an earlier described protocol for cell lysis (37). In brief, 250 mg brain tissue was submerged in 2 ml of ice-cold cell lysis buffer and carefully homogenized with a bounce homogenizer. After sonication in ice water, the extract was centrifuged at 13,000 $\times g$ for 10 min at 4˚C. The clear lysate supernatant was aliquoted for storage at $-80˚C$. Lysates were standardized for protein content per brain using a BCA protein assay kit (Pierce, Thermo Fisher, Rockford, IL) before analysis of proteins with specific ELISAs (R&D Systems, Minneapolis, MN) according to the supplier’s instructions.

Statistical analysis

Data sets from quantification of immunoreactive neuropil, ELISA, cell counts, FACS, quantitative RT-PCR (qRT-PCR) and indicated components of the behavioral test battery were subjected to ANOVA followed by Fisher’s protected least significant difference (PLSD) post hoc test using the StatView software package (version 5.0.1; SAS Institute, Cary, NC). Use of spatial strategy was compared using a $\chi^2$ test. Significance level for all analyses was $p = 0.05$.

Results

CCR5KO abrogates neuronal injury associated with expression of CXC4r4 using HIV gp120

To assess in vivo whether CCR5 exerted effects beyond its function as gp120 coreceptor and as such influenced brain injury initiated by a CXCR4-prefering gp120, we cross-bred CCR5-deficient mice with CCR5KO x HIV gp120tg animals of two founder lines (4, 5, 12). The resulting two new mouse lines were viable and fertile and were designated as CCR5KO x HIV gp120tg mice, Line 1, and Line 2.
CCR5+/+ gp120+ or gp120; CCR5+/+ gp120— or WT and CCR5 MAP-2+ neuropil (The gp120 mice of line 2 showed significantly more loss of comparison with all the other three genotypes (Fig. 1B, 1C).

reduction in the percentage of neuropil-positive for MAP-2 or Syp that in both lines 1 and 2, gp120 mice displayed a significant frontal cortex (layer 3) and hippocampus (molecular layer) showed synaptophysin (Syp) in sagittal brain immunolabeled neuronal dendrites for the marker MAP-2 and expression of gp120 in the presence and absence of CCR5. We harvested brain tissue and assessed injury of the CNS due to gp120

interest to our study (CCR5

respectively. Both mouse lines generated the four genotypes of interest to our study (CCR5+/− gp120+ or CCR5KO x gp120; CCR5+/+ gp120+ or gp120; CCR5+/− gp120− or WT and CCR5+/− gp120− or CCR5KO) at expected Mendelian ratios. At 6 months, we harvested brain tissue and assessed injury of the CNS due to expression of gp120 in the presence and absence of CCR5. We immunolabeled neuronal dendrites for the marker MAP-2 and presynaptic terminals for synaptophysin (Syp) in sagittal brain sections (Fig. 1A). Analysis using deconvolution microscopy in frontal cortex (layer 3) and hippocampus (molecular layer) showed that in both lines 1 and 2, gp120 mice displayed a significant reduction in the percentage of neuropil-positive for MAP-2 or Syp in comparison with all the other three genotypes (Fig. 1B, 1C). The gp120 mice of line 2 showed significantly more loss of MAP-2+ neuropil (p < 0.01) and a trend to less Syp (p < 0.066) in the cortex than their line 1 counterparts did. Most notably for both lines, in the absence of CCR5, viral gp120 no longer caused a loss of neuronal dendrites and presynaptic terminals.

CCR5KO prevents microglial activation but not astrocytosis in HIV gp120tg brain

We next labeled sections for astrocytic GFAP or the microglial marker Iba1 and used quantitative immunofluorescence microscopy for analysis. Increased GFAP immunoreactivity indicating astrocytosis occurred in brains of gp120tg animals of both lines regardless of CCR5 deficiency, although the increase of GFAP fluorescence did not reach significance in the hippocampus of CCR5KO x gp120 mice of line 2 (Fig. 1D). Of note, all animals of line 2 expressing gp120 showed significantly less astrocytosis in the cortex than their line 1 counterparts did, independently of the CCR5 genotype (p < 0.01).

### Table I. Primers for qRT-PCR

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<td>NM_011581.3</td>
<td>mThbs2 Fwd3</td>
<td>TGGCCGCGGAGGAGGAGTGA</td>
<td>20</td>
<td>61</td>
</tr>
<tr>
<td>TNFp</td>
<td>NM_013693.2</td>
<td>mTNFp Fwd</td>
<td>TGGCCGCGGAGGAGGAGTGA</td>
<td>20</td>
<td>61</td>
</tr>
<tr>
<td>Tgm</td>
<td>NM_001161715.1</td>
<td>mTgm1 Fwd</td>
<td>TGGCCGCGGAGGAGGAGTGA</td>
<td>20</td>
<td>61</td>
</tr>
</tbody>
</table>

*aSee Ref. 23.

*bSee Ref. 32.

*cSee Ref. 33.

dSee Ref. 34.

eSee Ref. 35.

Following from [http://www.jimmunol.org/](http://www.jimmunol.org/) by guest on July 29, 2017
Counts of Iba1+ cells indicated that gp120 mice displayed significantly more microglial cells than the three other genotypes did, although there was a much smaller, yet significant increase of microglia in the hippocampus of CCR5KO x gp120 mice from line 1 (Fig. 1E). In addition, CCR5KO x gp120 mice of line 2 displayed significantly lower numbers of Iba1+ microglia than their WT controls. However, microglial cell numbers in lines 1 and 2 were not significantly different. Altogether, we concluded that CCR5 is required in our mouse model for HIV gp120 to cause microglial activation and neuronal damage, but not to produce astrocytosis.

**CCR5KO rescues spatial learning and memory in HIV gp120tg mice**

Because gp120 mice of line 2 displayed the most pronounced loss of MAP-2+ neuropil and synaptophysin in the cortex, we next subjected mice of this line to behavioral testing. The optomotor test of vision showed that all mice had intact vision, and the light/dark transfer test found no differences between the genotypes. In the locomotor activity test, we detected a moderately significant effect of genotype on ambulation, but not center activity or rearing behavior. Fisher’s PLSD post hoc test revealed significantly lower levels of ambulation in CCR5KO x gp120 mice relative to WT (Fig. 2A). Thus, the loss of CCR5 (and to a lesser extent gp120 expression) was associated with decreased ambulatory, but not center or rearing activity. In addition, there was no evidence of either amelioration or exacerbation of the effect on ambulatory activity in the CCR5KO x gp120 mice.

The Barnes maze test revealed significant differences between gp120 mice and the other genotypes in three of four measures. Latencies to escape were reduced across the blocks of three trials in all genotypes (Fig. 2B). The numbers of incorrect choices (errors) indicated a significant difference between WT and gp120 mice, with the latter making more errors in the final training trials (Fig. 2C). In the probe test following training, the WT, CCR5KO, and CCR5KO x gp120 groups all spent significantly more time in the target quadrant of the maze relative to the average of time spent in the incorrect quadrants (Fig. 2D). Only gp120 mice failed to show this significant difference, suggesting that they lacked in the use of spatial cues to locate the escape chamber. Chi-square tests comparing each group with the WT group indicated that only the gp120 mice used significantly less spatial strategy on the final day of acquisition compared with WT control mice (Fig. 2E).

None of the behavioral tests showed any sex-dependent differences. Taken together, these data suggested that gp120 mice have impaired spatial learning and memory that is restored by genetic ablation of CCR5.

**HIVgp120 affects gene expression in brains of CCR5WT and CCR5KO mice**

To characterize further the neuroprotective effect of CCR5 ablation in the presence of HIVgp120, we next performed a microarray-based genome-wide gene expression analysis. We collected brain tissue and prepared whole brain RNA of all four genotypes at 1.5, 3, 6, 12, and 20 (line 1) or 16 mo of age (line 2) to identify genes for which differential regulation may be affected by an interaction of...
HIVgp120 expression and age. GeneSpring GX12 expression analysis software was used for two-way ANOVAs examining the variation in CNS gene expression as a function of mouse age and HIVgp120 genotype separately in CCR5WT and CCR5KO backgrounds and for lines 1 and 2 (Fig. 3A). Next, we compared genes differentially expressed in association with the gp120+ genotype or an interaction of age and gp120+ genotype between the two mouse lines separately for the respective CCR5WT and CCR5KO (Fig. 3B). This approach indicated that the gp120+ genotype of lines 1 and 2 shared 1195 differentially expressed genes in the presence of CCR5 and 1766 genes in the absence of the HIV coreceptor. A subsequent comparison of the two lists of shared genes identified a core common set of genes for mouse lines 1 and 2 comprising CCR5WT-specific, common (CCR5WT and KO) and CCR5KO-specific, differentially regulated genes that were represented by 734, 461, and 1305 gene probes, respectively (Supplemental Tables IA, IB, II).

FIGURE 2. CCR5-deficiency protects spatial learning and memory performance in HIVgp120tg mice. (A) Locomotion (ambulation). ANOVA and Fisher PLSD post hoc test: *p < 0.007 (CCR5KO), p < 0.03 (CCR5KO x gp120), p < 0.05 (gp120) compared with WT. (B–E) Barnes Maze test for spatial learning and memory. (B) Latencies. (C) Errors. (D) Probe test. (E) Spatial strategy. ANOVA and Fisher PLSD post hoc test: Latencies, p < 0.26; Errors, *p < 0.03 for gp120 compared with WT; Probe test, **p ≤ 0.003 (WT), p < 0.0002 (CCR5KO), p < 0.0012 (CCR5 KO x gp120); Spatial strategy, χ² = 5.4, *p < 0.02 for gp120 compared with WT. Eight- to nine-month-old gp120 (n = 9), CCR5KO x gp120tg mice (n = 17) and WT (n = 8) and CCR5KO control (n = 21) animals of line 2 were studied using the indicated behavioral tests. Values are mean ± SEM (A–D) or mean (E). n.s., not significant.

FIGURE 3. Genome-wide gene expression analysis for brain identifies a core common set of genes for mouse lines 1 and 2. (A) Two-way ANOVA of gene expression data obtained by microarray with whole brain RNA of gp120, CCR5KO x gp120 mice and WT and CCR5KO control animals of lines 1 and 2, respectively, at five different time points (1.5, 3, 6, 12, and 20 [line 1] or 16 [line 2] mo of age; n = 3–8 per genotype and age group). (B) Identification of a core common set of genes for mouse lines 1 and 2 from overlap in differentially expressed genes owing to HIVgp120tg genotype or an interaction of age and gp120+ genotype between the two mouse lines separately for the respective CCR5WT and CCR5KO (Fig. 3B). This approach indicated that the gp120+ genotype of lines 1 and 2 shared 1195 differentially expressed genes in the presence of CCR5 and 1766 genes in the absence of the HIV coreceptor. A subsequent comparison of the two lists of shared genes identified a core common set of genes for mouse lines 1 and 2 comprising CCR5WT-specific, common (CCR5WT and KO) and CCR5KO-specific, differentially regulated genes that were represented by 734, 461, and 1305 gene probes, respectively (Supplemental Tables IA, IB, II).
To explore possible explanations for the protective effect of CCR5 deficiency against gp120 neurotoxicity, we next investigated whether CCR5 deficiency affected RNA expression in an age-dependent fashion in both mouse lines, but with slightly different kinetics (as primers, see Table I). The differences in gp120 RNA expression between lines 1 and 2 (Fig. 5) suggested that activation of pathways in association with gp120 expression might be attenuated in the absence of the CCR5 receptor.

In contrast, qRT-PCR revealed age-dependent significant increases of CCR5 RNA expression in association with gp120 increases in CCR5 expression in macrophages. In contrast, CCR5KO-specific regulated impairment (Fig. 4A, 4E). One pathway was specifically CCR5 of the five canonical pathways affected by differential gene expression specific to CCR5WT explicitly implicated macrophages in gp120-associated neuronal injury and behavioral impairment (Fig. 4A–J). Notably, two CCR5WT/KO common gene sets all belonged in the categories of immune system and inflammation (Fig. 4A–J). Of note, the five highest scoring canonical pathways each for CCR5WT-specific and CCR5WT/KO canonical pathways (Table III, Fig. 4). The three sets of differentially expressed gene sets all affected components of different canonical pathways. The three sets of differentially regulated human gene probes were recently reported to be differentially regulated in the brains of patients with HIV encephalitis (HIVE) and neurocognitive impairment (NCI) (31). Therefore, we further investigated how far the results from our microarray study recapitulated findings in human patients (32) but with corresponding mouse probes and compared with matched hippocampi from CCR5WT and KO and both mouse lines ( Supplementary Table I). Five of these genes were recently reported to be differentially regulated in the brains of patients with HIV encephalitis (HIVE) and neurocognitive impairment (NCI) (31); therefore, we further investigated how far the results from our microarray study recapitulated findings in human patients (32) but with corresponding mouse probes and compared with matched hippocampi from CCR5WT and KO and both mouse lines ( Supplementary Table I). Five of these genes were recently reported to be differentially regulated in the brains of patients with HIV encephalitis (HIVE) and neurocognitive impairment (NCI) (31).


differentiated expression in brains of HIVgp120tg mice (Table II; 4O) and one inflammation-related pathway (Fig. 4L). To assess biological pathways associated with gp120-induced CNS injury and affected by CCR5 deficiency, we used IPA software to analyze how the differential gene expression patterns affected canonical pathways in the presence and absence of CCR5. Of note, the five highest scoring canonical pathways were revealed by IPA analysis as the most pronounced loss of MAP2-positive neuronal dendrites in cerebral cortex.

**Table II. Comparison of Differential CNS Gene Expression in HIV Patients and HIVgp120tg Mice**

<table>
<thead>
<tr>
<th>Human CNS Specimen (National NeuroAIDS Tissue Consortium)</th>
<th>Frontal Cortex</th>
<th>Neostriatum</th>
<th>White Matter</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR5KO x HIVgp120tg Mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR5WT-specific (734 genes)</td>
<td>32 (0.345)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CCR5WT/KO-common (461 genes)</td>
<td>30 (0.007)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CCR5KO-specific (1305 genes)</td>
<td>87 (3.1x10^-6)</td>
<td>0.39</td>
<td>0</td>
</tr>
<tr>
<td>HIV/NCI</td>
<td>593 Genes</td>
<td>HIV/NCI</td>
<td>62 Genes</td>
</tr>
<tr>
<td>CCR5WT-specific (734 genes)</td>
<td>39 (2.79 x 10^-4)</td>
<td>1 (0.85)</td>
<td>3 (0.49)</td>
</tr>
<tr>
<td>CCR5WT/KO-common (461 genes)</td>
<td>83 (&lt;1 x 10^-23)</td>
<td>7 (2.0 x 10^-4)</td>
<td>4 (0.087)</td>
</tr>
<tr>
<td>CCR5KO-specific (1305 genes)</td>
<td>64 (4.18 x 10^-5)</td>
<td>3 (0.66)</td>
<td>6 (0.336)</td>
</tr>
</tbody>
</table>

The specimens are described in detail in Ref. 31. The numbers refer to genes mapped by Ingenuity Pathway Analysis (Ingenuity Systems). Some genes were represented by more than one gene probe. Comparisons were performed using Biomart and Genespring softwares as mentioned in Materials and Methods. All gene numbers are versus HIV-negative controls.
Markers of microglia and macrophage activation can readily be detected in mRNA preparations from whole brain (23, 38, 39). To explore further the activation status of microglia in gp120tg brains, we analyzed the expression of genes that characterize classically activated macrophages (M1) and their alternatively activated counterparts (M2) (23, 38, 40, 32). qRT-PCR showed an age-dependent increase of the M1 markers CD68 and TNF-α that was more pronounced in gp120tg compared with control brains (Fig. 7A, 7C). CD68 was significantly higher in gp120tg compared with control brains despite a pronounced upregulation of LCN2 in brains of gp120 tg animals. Thus, CCR5 deficiency made a significant difference for TNF-α only between WT and the evenly higher expressing CCR5KO and CCR5KO x gp120 brains at 12 mo of age. No significant changes were found in the expression of iNOS between the different genotypes or age groups (Fig. 7B).

In contrast, all three M2 markers showed gp120-associated alterations of expression in at least one age group (Fig. 7D–F). Arginase-1 was significantly upregulated in gp120tg brains compared with controls in both CCR5WT and KO backgrounds at 1.5 mo (Fig. 7D). The same was true for CD163 at 6 mo of age (Fig. 7E). MRC-1 expression was significantly increased in CCR5KO x gp120 compared with CCR5KO and WT brains at 1.5 and 6 mo (Fig. 7E). In 6-mo-old mice, CCR5 deficiency made a difference in that expression of both CD163 and MRC-1 was increased in CCR5KO x gp120 compared with gp120 brains. Thus, despite a pronounced upregulation of LCN2 in brains of gp120 and CCR5KO x gp120 mice, we found only in some age groups a slight increase of M1 markers CD68 and TNFα compared with controls. At the same time, M2 markers were upregulated in association gp120 expression, at least in 1.5- and 6-mo-old brains.

Increased expression of GFAP and LCN2 has been linked to astrocytosis (12, 41–43). Our observations indicated that, in the presence of gp120, CCR5 deficiency protected neurons but left astrocytosis largely unchanged. To explore the effect of CCR5KO on astrocytes, we analyzed in brain samples of line 2 mice at 1.5, 6, and 12 mo of age six additional astrocyte-specific factors that have been implicated in astrocytosis (43). GLAST and GLT1 are astrocytic glutamate transporters that have an important role in keeping the extracellular concentration of the major excitatory

Table III. Canonical pathways affected by differential gene expression

<table>
<thead>
<tr>
<th>Genesa</th>
<th>p Value</th>
<th>Molecules in Canonical Pathway</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR5WT-specific genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis</td>
<td>1.87E-03</td>
<td>20/290</td>
<td>0.07</td>
</tr>
<tr>
<td>B. Pathogenesis of multiple sclerosis</td>
<td>2.16E-03</td>
<td>3/9</td>
<td>0.33</td>
</tr>
<tr>
<td>C. CTL-mediated apoptosis of target cells</td>
<td>3.82E-03</td>
<td>6/654</td>
<td>0.11</td>
</tr>
<tr>
<td>D. NF-κB activation by viruses</td>
<td>4.15E-03</td>
<td>8/73</td>
<td>0.11</td>
</tr>
<tr>
<td>E. CCR5 signaling in macrophages</td>
<td>5.57E-03</td>
<td>7/67</td>
<td>0.10</td>
</tr>
<tr>
<td>CCR5WT and KO common genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F. Communication between innate and adaptive immune cells</td>
<td>2.90E-07</td>
<td>11/82</td>
<td>0.13</td>
</tr>
<tr>
<td>G. Hepatic fibrosis / Hepatic stellate cell activation</td>
<td>2.66E-06</td>
<td>14/132</td>
<td>0.11</td>
</tr>
<tr>
<td>H. IFN signaling</td>
<td>4.60E-06</td>
<td>7/31</td>
<td>0.23</td>
</tr>
<tr>
<td>I. Type 1 diabetes mellitus signaling</td>
<td>4.75E-06</td>
<td>12/104</td>
<td>0.12</td>
</tr>
<tr>
<td>J. TREM1 signaling</td>
<td>5.33E-06</td>
<td>9/58</td>
<td>0.16</td>
</tr>
<tr>
<td>CCR5KO-specific genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K. GABA receptor signaling</td>
<td>4.37E-04</td>
<td>10/47</td>
<td>0.21</td>
</tr>
<tr>
<td>L. LPS/IL-1β-mediated inhibition of RXR function</td>
<td>5.83E-03</td>
<td>22/196</td>
<td>0.11</td>
</tr>
<tr>
<td>M. Mineralocorticoid biosynthesis</td>
<td>8.56E-03</td>
<td>3/7</td>
<td>0.43</td>
</tr>
<tr>
<td>N. Huntington disease signaling</td>
<td>9.28E-03</td>
<td>23/216</td>
<td>0.11</td>
</tr>
<tr>
<td>O. Clathrin-mediated endocytosis signaling</td>
<td>1.29E-02</td>
<td>19/172</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Note that A–O in this table correspond to panels A–O in Fig. 4.

expression in both mouse lines (Fig. 6A, Supplemental Fig. 1A). Considering that the CCR5KO completely abrogated neuronal injury and behavioral impairment in our model, an increase of the chemokine receptor’s expression presumably promoted the occurrence of neuropathology.

Effect of CCR5KO on other relevant host factors

Next, we tested whether expression of the second HIV coreceptor, CXCR4, was affected by gp120 and CCR5-deficiency as suggested by the microarray (Supplemental Table IA). qRT-PCR showed that CXCR4 RNA was upregulated in gp120tg brains of mouse lines 1 and 2, and CCR5-deficiency ameliorated that effect (Fig. 6B, Supplemental Fig. 1B).

CXCL12 is the natural ligand of CXCR4 and therefore could potentially compete with viral gp120 for the interaction with its receptor (1, 8). qRT-PCR indicated minor, age-dependent changes in CXCL12 RNA and no effect of CCR5 deficiency (Fig. 6C, Supplemental Fig. 1C).

qRT-PCR also revealed patterns of age-dependent and gp120-associated increases for RNA of CCL5, CCL2, CXCL10, C4b, and LCN2 and less pronounced for CCL3 and CCL4 (Fig. 6D–J). The absence of CCR5 showed effects mostly for RNA of CCL5, CCL2, CXCL10, and C4b. The changes in RNA were similar in both mouse lines for four genes: CXCR4, CXCL12/SDF-1, CCL5, and CCL4 (in CCR5WT). Only for two genes, CCL4 (in CCR5WT) and LCN2, were increases in mRNA higher in line 1 than line 2; however, overall, the patterns of differential RNA expression were similar for the 10 genes in mouse lines 1 and 2.

LCN2, one of the most upregulated proteins in gp120tg brains, and CCR5 have both been reported to affect the activation of macrophages and microglia. LCN2 was found to promote proinflammatory activation and apoptosis in microglia (22, 23). CCR5-deficient macrophages reportedly displayed upon stimulation impaired cytokine production compared with their WT counterparts (4). Markers of microglia and macrophage activation can readily

be detected in mRNA preparations from whole brain (23, 38, 39). To explore further the activation status of microglia in gp120tg brains, we analyzed the expression of genes that characterize classically activated macrophages (M1) and their alternatively activated counterparts (M2) (23, 38, 40, 32). qRT-PCR showed an age-dependent increase of the M1 markers CD68 and TNF-α that was more pronounced in gp120tg compared with control brains (Fig. 7A, 7C). CD68 was significantly higher in gp120tg samples than in controls at 12 mo, whereas the same pattern occurred with TNF-α in the 1.5-mo group. However, CCR5-deficiency made a significant difference for TNF-α only between WT and the evenly higher expressing CCR5KO and CCR5KO x gp120 brains at 12 mo of age. No significant changes were found in the expression of iNOS between the different genotypes or age groups (Fig. 7B).

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Increased expression of GFAP and LCN2 has been linked to astrocytosis (12, 41–43). Our observations indicated that, in the presence of gp120, CCR5 deficiency protected neurons but left astrocytosis largely unchanged. To explore the effect of CCR5KO on astrocytes, we analyzed in brain samples of line 2 mice at 1.5, 6, and 12 mo of age six additional astrocyte-specific factors that have been implicated in astrocytosis (43). GLAST and GLT1 are astrocytic glutamate transporters that have an important role in keeping the extracellular concentration of the major excitatory

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neurotransmitter at physiologic, nontoxic levels (44). qRT-PCR indicated an overall age-dependent upregulation of GLAST, but not GLTI in gp120tg brains in the presence and absence of CCR5. However, in 6-mo-old animals, expression of both transporters was significantly higher in CCR5KO x gp120 than in gp120 mice (Fig. 8A, 8B). Ptx3 and TGM1 are in the brain specifically expressed in astrocytes (43). qRT-PCR showed an overall age-dependent upregulation of both genes in gp120tg brains inde-
dependent of the CCR5 genotype, although induction was up to ∼50-fold higher for TGM1 than Ptx3 (Fig. 8C, 8D). Astrocytes also produce soluble thrombospondins (Thbs), which support synaptogenesis (45). qRT-PCR revealed for Thbs1 an age-dependent increase within the gp120 mouse group; however, the changes were overall not significant compared with WT mice. However, Thbs1 was significantly upregulated at 1.5 mo in CCR5KO x gp120 compared with the other three genotypes (Fig. 8E). In contrast, Thbs2 was significantly upregulated in association with gp120 in both CCR5WT and KOs in an age-dependent manner (Fig. 8F). Thus, overall CCR5 deficiency appeared to have limited effects on the increased expression of genes associated with astrocytosis in gp120tg brains.

Analysis of protein concentrations using an ELISA confirmed microarray and qRT-PCR results suggesting that LCN2 was highly upregulated in gp120tg brain independently of CCR5 genotype (Fig. 9A, Supplemental Fig. 1K). Deconvolution microscopy confirmed that LCN2 was located to astrocytes (Fig. 9B). In contrast to LCN2, CXCL12 protein concentrations assessed by ELISA in extracts from brain tissues of 3, 6, and 12-mo-old mice differed from the mRNA expression pattern by showing significant reductions in association with gp120 expression in both mouse lines although at different ages (Fig. 9C, Supplemental Fig. 1L). CCR5 deficiency did not per se affect CXCL12 protein concentration, but it prevented a significant drop in HIVgp120tg brains, except for the 12-mo samples from line 2. ELISAs also confirmed significantly elevated protein levels for both CCL5 and CCL2.

**FIGURE 5.** RNA expression of HIVgp120 in the CNS in the presence and absence of CCR5. RNA preparation and qRT-PCR analysis was performed as described in Materials and Methods. Values are mean ± SEM. n = 3–6 animals per group/genotype. *p < 0.05, **p < 0.01, ***p < 0.001, ANOVA and Fisher’s PLSD post hoc test.

**FIGURE 6.** Effect of CCR5 deficiency on RNA expression of host factors in the CNS. Analysis of mRNA expression in brain of line 2 mice using qRT-PCR: (A) CCR5; (B) CXCR4; (C) CXCL12; (D) CCL5; (E) CCL3; (F) CCL4; (G) CCL2; (H) CXCL10; (I) C4b; and (J) Lcn2. RNA preparation and qRT-PCR were performed as described in Materials and Methods. Note the different y-axis scale for the various samples. Values are mean ± SEM. See Supplemental Fig. 1 for the corresponding data obtained for Line 1 mice. *n = 3–6 animals per group/genotype. p < 0.05, **p < 0.01, ***p < 0.001, ANOVA and Fisher PLSD post hoc test.
indicated that microglial TNF-α expression was in line with an increased expression of a macrophage M1 marker in the presence and absence of CCR5, whereas the same effect occurred for CCL2 only in line 2.

LCN2 has been reported to stimulate an M1 polarization of microglia (23), and our qRT-PCR had detected increased mRNA for TNF-α in gp120tg brains (Fig. 7). Therefore, we stained brain sections of all four genotypes of 6-mo-old line 2 mice with a combination of Abs against the microglial marker Iba1 and TNF-α. In analogy to the quantitative fluorescence-based analysis of cell markers described above, Iba1 was used to generate masks defining microglial cell bodies in cerebral cortex and hippocampus, and the fluorescence intensity for TNF-α was estimated (Fig. 9F, 9G). Microglia in gp120tg brains showed a significantly higher fluorescence signal for TNF-α in the presence and absence of CCR5 compared with WT controls. This observation was in line with an increased expression of a macrophage M1 marker in the presence of gp120 and LCN2, and it indicated that microglial TNF-α expression can occur in the absence of neuronal injury.

Altogether, the results indicate that the viral envelope protein gp120 can trigger components of an antiviral innate immune response and astrocytosis independently of CCR5 genotype and neuronal injury.

CCKRSKO abrogates accumulation of residential microglia in HIVgp120tg brain

Although immunofluorescence staining with Iba1 only indicated more microglia in gp120tg brain in the presence of CCR5, the strongly elevated CCL2 protein concentrations raised the question if any recruitment of peripheral mononuclear cells occurred. Therefore, we prepared live cell suspensions from whole brains of mice of all genotypes from line 2 and analyzed cell surface expression of CD45 and CD11b by flow cytometry. Cells isolated from spleen served as controls (Fig. 9H). In brain, CD11b+CD45low cells represent resident microglia and macrophages, whereas CD11b+CD45high cells indicate recently infiltrated peripheral monocytes and macrophages (46). Quantification of CD11b+CD45low CNS cells indicated again that CCR5 deficiency was associated with a significant reduction of resident microglia and macrophages in gp120tg brain (Fig. 9H, upper panel, and Fig. 9I). The numbers of CD11b+CD45high cells in brain were comparably low with (in percent ± SEM) 0.39 ± 0.28 (WT), 0.26 ± 0.11 (gp120), 0.49 ± 0.42 (CCR5KO), and 0.12 ± 0.04 (CCR5KO x gp120) and no significant differences between the genotypes. In contrast, CD11b+CD45high cells were readily detected in splenocyte populations (Fig. 9H, lower panel). Thus, increased levels of CCL2 and CCL5 were not associated with infiltration of peripheral CD11b+CD45low CNS cells, whereas CCR5 deficiency appeared to restrict the number of CD11b+CD45low cells in gp120tg brains.

**Figure 7.** Effect of CCR5 KO on expression of M1- and M2-type markers of microglia and macrophages in brains of line 2 mice: (A) CD68; (B) iNOS; (C) TNF-α; (D) Arginase-1 (Arg-1); (E) CD163; (F) MRC-1. Preparation of RNA and qRT-PCR were performed as described in Materials and Methods. Note the different y-axis scales for the various samples. Values are mean ± SEM. *n = 3 animals per group/genotype. *p < 0.05, **p < 0.01, ***p < 0.001, ANOVA and Fisher PLSD post hoc test.

**Figure 8.** Changes in expression of astrocyte markers in gp120tg brains in the presence and absence of CCR5. (A) GLAST; (B) GLT1; (C) TGM1; (D) TGB1; (E) Thbs1; (F) Thbs2. Preparation of RNA and qRT-PCR were performed as described in Materials and Methods. Note the different y-axis scales for the various samples. Values are mean ± SEM. *n = 3 animals per group/genotype. *p < 0.05, **p < 0.01, ***p < 0.001, ANOVA and Fisher PLSD post hoc test.

**Interruption of CCR5 signaling and LCN2 cooperate to diminish microglial cell numbers and to protect neurons upon exposure to CXCR4-using HIVgp120**

LCN2 has been reported to induce astrocytosis (42), proinflammatory activation as well as apoptosis in microglia (22, 23) and to exert itself neurotoxicity (48). However, CCR5KO x gp120 mice express increased levels of LCN2 without showing neuronal injury, suggesting that CCR5KO might also curb toxicity of LCN2. Therefore, we next assessed a potential functional link between LCN2 and CCR5 in the presence of a CXCR4-using viral gp120. We exposed cerebrocortical...
cell cultures derived from embryos of line 2 WT mice to gp120 of the CXCR4-using HIV-1IIB. In the presence and absence of recombinant murine LCN2 or the CCR5 inhibitor MVC, or combinations thereof, using published effective concentrations (14, 37, 47, 48). Subsequent analysis of microglial and neuronal cell numbers by fluorescence microscopy showed that LCN2 and MVC in combination led to a significant increase of microglia. However, in the presence of the CXCR4-using gp120, LCN2 and MVC caused a significant reduction of microglial cells (Fig. 7B, 7D). The latter condition recapitulated the scenario in CCR5KO x gp120 mice. Moreover, gp120 and to a lesser extent LCN2 caused a significant loss of neurons, although the combination did not significantly increase the toxicity of gp120. MVC alone abrogated the toxic effect of LCN2, but not of the CXCR4-using gp120. However, the combination of LCN2 and MVC completely prevented the loss of neurons in the presence of gp120, again recapitulating the observations in CCR5KO x gp120 mice, (Fig. 7C, 7E).

Analogous to the quantitative fluorescence-based analysis of TNF-α in microglia in brain sections described above, Tomato lectin was used in separate experiments to define microglial cell bodies in cerebrocortical cell cultures. Afterwards, the fluorescence intensities for TNF-α and ARG1 were estimated in microglia (Fig. 10E). Microglia in cerebrocortical cells exposed to LCN2 plus MVC, with and without gp120, contained significantly more TNF-α compared with vehicle controls. However, microglia in cerebrocortical cultures exposed to gp120 alone or the combination of MVC plus LCN2 plus gp120 showed significantly increased ARG1. Therefore, microglia simultaneously exposed to MVC plus LCN2 plus gp120 were reduced in numbers, but expressed in comparison with vehicle controls increased levels of both TNF-α and ARG1.

**Discussion**

Beyond its function as viral coreceptor, little is known about how CCR5 influences HIV-associated neurocognitive disorders. In this study, we show with histologic analysis and behavioral testing that genetic ablation of CCR5 prevents neuronal injury, microglial activation, and impairment of spatial learning and memory in a transgenic mouse model expressing a CXCR4-using HIVgp120 in the brain. These findings implied a crucial indirect role for the β-chemokine receptor in the in vivo brain damage mediated by the second viral coreceptor CXCR4.

Genetic CCR5 ablation also revealed that astrocytosis, another prominent pathologic feature of AIDS brains (31), can occur independently from neuronal injury and behavioral impairment. Interestingly, gp120 mice of line 2 displayed significantly less MAP-2+ neuropil and a trend toward fewer Syp+ presynaptic terminals in the cortex, but also significantly less astrocytosis than in their line 1 counterparts independently of the CCR5 genotype. Together with reports that in human HIV patients NCI can occur without HIV, our findings raised the possibility that astrocytosis reflects an insufficiently protective response of the CNS (31).

Genome-wide gene expression analysis of brains from all four genotypes of line 1 and 2 of CCR5KO x HIVgp120g mice produced three major salient findings. First, a comparison of differentially regulated genes in brains of HIV patients with and without signs of HIV and NCI (31) to those in gp120g mouse brains revealed a significant overlap for both CCR5WT and KO. Thus, HIV gp120g mice share not only significant neuropathologic features, but also a significant fraction of differentially expressed genes with brains of HIV and HIVE patients with NCI. Surprisingly, the human brain array did not report GFAP or LCN2 among the consistently upregulated factors in HIVE patients although astrocytosis with increased GFAP immunoactivity is one of the
FIGURE 10. LCN2 and interruption of CCR5 signaling reduce in combination microglial cell numbers and protect neurons in the presence of a CXCR4-using HIVgp120 from toxicity. Seventeen-day-old mixed neuronal-glial cerebrocortical cell cultures from line 2 WT mouse embryos were exposed to the CXCR4-using gp120 of HIV-IIB (1 nM) in the presence or absence of LCN2 (4 nM) or the CCR5 inhibitor MVC (5nM) for 3 d. Controls received vehicle only. After fixation and labeling of microglia, neurons and nuclear DNA with Tomato lectin, anti–MAP-2 Ab, and Hoechst33342, respectively, the cell cultures were analyzed with fluorescence microscopy (five fields each per 9–10 replicates per condition). (A) Microglial cell numbers. (B) Neuronal cell numbers. Representative images of microglia (C) and of neurons (D) (scale bars, 40 μm). Microglial TNF-α is increased in cerebrocortical cells exposed to LCN2 plus MVC with and without gp120 (E), whereas ARG1 is elevated by gp120 alone and in combination with LCN2 plus MVC (F). Cerebrocortical cell cultures grown on glass coverslips were incubated with LCN2, MVC, and gp120 as described above and stained with Ab against TNF-α or ARG1 and Tomato lectin to label microglia. Immunostaining and microscopy was performed as described in Materials and Methods. Threshold segmentation for Tomato lectin fluorescence was used to define areas occupied by microglial cell bodies. Specific fluorescence intensities for TNF-α and ARG1 were estimated in microglia and normalized for cell number (A). An average of 4845 cells was counted (A, B), and an average of 226 cells were analyzed (E, F) per condition in two independent experiments each for (A)–(D) and (E) and (F). Values are mean ± SEM. ***p < 0.05, **p < 0.01, ***p < 0.001, ANOVA and Fisher PLSD post hoc test.
and inflammation (8, 11, 58). Five of the genes—GLAST, Ptx3, TGM1, Thbs1, and Thbs-2—showed significant differential regulation in association with gp120 expression in at least one age group, but CCR5 deficiency had a comparably small effect. GLT1 was reported to be unchanged in astrocytosis following MCAO or peripheral LPS injection (43), but our results suggest that it can change with astroglia in gp120tg brain in an age-dependent fashion. In addition, GLAST was increased at 6 mo in CCR5KO x gp120 compared with gp120 brains, whereas CCR5-deficiency seemed to prevent a drop in GLT1 expression in the gp120tg brain at the same age. Thus, CCR5 deficiency might, in an age-dependent manner, favor an expression pattern of GLAST and GLT1 that prevents excitotoxic glutamate concentrations.

Our observations support the notion that the overall neuroprotective deficiency of the chemokine receptor has limited effects on astrocytosis and only transiently affects glutamate transporters that are important to maintain nontoxic extracellular levels of the excitatory neurotransmitter.

Our recent in vitro studies showed that CCR5-deficient neurons are not protected against HIVgp120-induced toxicity of microglia and macrophages (14). In this study, CCR5 deficiency was not associated with a reduction of gp120 expression, which could therefore not account for neuroprotection. However, because the transgenic mice express a CXCR4-using gp120, a reduced expression of CXCR4 could potentially contribute to the absence of neuronal damage in CCR5KO x gp120 mice, at least in line 1. Furthermore, we could not exclude that a less diminished CXCL12 concentration contributed at certain times to the neuroprotective effect of CCR5 ablation by reducing the availability of free CXCR4 to viral gp120.

CCR5KO x gp120 brains lacked neuronal injury; therefore, we could not strictly discern whether limitation of microglial cell numbers prevented neuronal damage or whether the absence of injured neurons abrogated the accumulation of microglia. In any case, ample published evidence supports a crucial role for macrophages and microglia in HIV-1 and gp120-induced neurotoxicity (51, 58–63). The implication of microglia in gp120-induced neuronal injury in vivo is also in line with our finding of increased numbers of this cell type in cerebral cortex and hippocampus of gp120 mice. The possibility that CCR5 deficiency modulates in vivo the function of macrophages and microglia resulting in amelioration of gp120 neurotoxicity fits reports that CCR5-deficient macrophages show upon stimulation a diminished cytokine response compared with their WT counterparts (4). Moreover, our observation that CCR5KO x gp120 mice had a significantly reduced number of microglia compared with their CCR5WT gp120 counterparts despite the high expression of CCL2 and CCL5 is in line with a recent report that the CCL5-CCR5 axis is essential for survival of macrophages during viral infections (64).

In addition, the pattern of gene regulation specific to gp120tg brains expressing CCR5 explicitly implicated macrophages in two and CCR5 in one of the top five canonical pathways. Hence, the canonical pathways clearly linked CCR5 and macrophages and microglia to neuronal injury and behavioral impairment. Moreover, the top canonical pathway affected by the CCR5WT/KO common gene set represented communication between innate and adaptive immune cells and the fifth comprised TREM1 signaling, both of which suggested again the involvement of microglia and macrophages. In addition, both CCR5WT-specific and CCR5WT/KO common gene sets pointed to up-regulation of immune activation and inflammation, but lacked a clear indication of a polarized activation of microglia and macrophages along the M1 and M2 spectrum (23, 38, 32). However, the CCR5WT/KO common gene set included significantly elevated CD86 and CXCL10, two markers of M1 polarization (38, 39). Our analysis of three additional markers each for M1 and M2 polarization in three age groups indicated that gp120tg brains provided an environment in which markers of both M1 and M2 type activation were upregulated. Interestingly, CCR5 deficiency did not change that situation. Thus, microglia and macrophages in gp120tg brains can exhibit an intermediate phenotype on the M1-M2 spectrum (65) or comprise a mixed population of M1 and M2 polarized cells. A recent review of pathologic mechanisms in HIV-1 and SIV infections suggested that M1 activation of macrophages and microglia predominates early, whereas M2 type activation can prevail later in HIV-1 infection of the CNS (66). Microscopy-based quantification of microglial TNF-α in mouse brain sections overall confirmed the expression pattern indicated by qRT-PCR, although not all microglial cells appeared to have detectable levels of the cytokine. Future experiments will aim at a more detailed characterization of the microglial activation pattern in gp120tg brains.

CCR5KO x gp120 mice lacked any sign of behavioral impairment and neuronal injury. Canonical pathways specifically affected in CCR5KO-deficient gp120tg brains showed downregulation of many pathway components, except for mineralocorticoid biosynthesis. The combined downregulation of components of GABAergic neurotransmission, of endocytosis, of LPS/IL-1-mediated inhibition of a nuclear receptor, and of a Huntington disease–associated signaling pathway suggested that significant adaptation of the CNS are required to achieve neuroprotection in the presence of gp120.

LCN2 reportedly promotes proinflammatory activation and apoptosis in microglia (22), and it has been linked to astrocytosis (42, 43, 47) and neurotoxicity (47). Thus, elevated LCN2 levels could presumably contribute to neuronal injury in gp120tg mice. However, we found that CCR5 deficiency allowed in vivo for high expression of LCN2 without neuronal injury and that in vitro inhibition of CCR5 blocked LCN2 neurotoxicity. Both findings suggested that the chemokine receptor was crucial for the neurotoxic effect LCN2.

Our experiments in cerebrocortical cell cultures revealed that LCN2 and interruption of CCR5 signaling paradoxically cooperated in the presence of a CXCR4-using HIVgp120 to reduce microglial cells in number and to protect neurons. Given the critical role of macrophages and microglia in HIV neurotoxicity, this observation provided an explanation and mechanism for in vivo protection against CXCR4-mediated gp120 neurotoxicity by CCR5-deficiency in our mouse model.

Quantification of TNF-α and ARG1 in cerebrocortical microglia in vitro was overall in line with the RNA expression pattern seen in 1.5-mo-old mice, although the increase of the M1 marker TNF-α following exposure to a combination of LCN2 and gp120 did not reach significance. Similarly, the increase of ARG1 between exposure to MHC and the combination of MHC, LCN2, and gp120 was not significant. However, the in vitro experiments showed that in a mixed neuronal-glia cell population LCN2 alone failed to increase microglial TNF-α, although the acute phase protein caused neurotoxicity. However, the combination of CCR5 inhibition with MHC in combination with LCN2 showed a trend to increase the cytokine in microglia (p = 0.078) while also preventing neuronal loss. Thus, neurons, astrocytes, and CCR5 blockade appeared to modulate the effect of LCN2 on microglia.

The characterization of the exact cellular and molecular mechanism of this regulation will be the subject of future studies. Interestingly, gp120 triggered an increase of the M2 marker ARG1 in microglia, an effect that was ameliorated in the presence of either LCN2 (p = 0.10) or MVC alone, but not their combination.
Moreover, increased microglial ARG1 was associated with neurotoxicity. In contrast, microglial cells that remained after exposure to the neuroprotective combination of MVC plus LCN2 and gp120 showed simultaneously elevated levels of TNF-α and ARG1, thus recapitulating the mRNA expression pattern observed in brains of CCR5KO x gp120 mice.

In summary, we found in vivo evidence for a pathologic role of CCR5 that appears to be independent of its coreceptor function because the gp120 in the transgenic mouse model uses CXCR4 (13). We also observed that neurotoxicity of LCN2 is CCR5 dependent, and pharmacologically abrogating CCR5 signaling revealed a novel protective effect of LCN2 in HIV/gp120-associated brain injury and behavioral impairment. Considering that transgenic mice are a model with limitations and gp120 is only one of several HIV components that can cause neuronal injury, our findings nevertheless raise the possibility that inhibition of CCR5 might be beneficial against HIV-associated CNS injury, even in the presence of a CXCR4-using virus.

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Disclosures

The authors have no financial interests of interest.

References


NEUROPROTECTION FROM HIV gp120 TOXICITY BY CCR5KO


Supplemental Figure S1 Effect of CCR5-deficiency on host factors in the CNS. Analysis of mRNA expression in brain of Line 1 mice using quantitative RT-PCR (A – J) and ELISA (K – L): (A), CCR5; (B), CXCR4; (C), CXCL12; (D), CCL5; (E), CCL3; (F), CCL4; (G), CCL2; (H), CXCL10; (I), C4b; and (J), Lcn2. RNA preparation and qRT-PCR was performed as described in Materials and Methods. Protein extracts of whole brains were prepared as described in Materials and Methods and analyzed by ELISA, LCN2 (K); CXCL12 (L). Note different scales of y-axis for different samples (A – L). Values are mean ± s.e.m. (*** P < 0.001, ** P < 0.01, * P < 0.05; ANOVA and Fisher’s PLSD post hoc test; n = 3 - 6 animals per group/genotype).