1 **Systemic HIV-1 Infection Produces a Unique Glial Footprint in Humanized Mouse Brains**

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3 *Running title:* HIV-1 infection in humanized mouse brain

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15 astrocytes, HIV-1

16

17 **Summary Statement**

18 We created mice with both a humanized brain and an immune system. The animals were used to

19 investigate glial responses to HIV-1 infection. At a transcriptional level we defined the

20 interactions between human glia and immune cells in the presence of the systemic HIV-1

21 infection. Noticeably, altered transcriptional changes were human specific. At five weeks after

22 viral infection humanized mouse brain displayed potent interferon-mediated antiviral innate

23 immune responses and alteration of neuronal progenitors differentiation and myelination. This

24 model can be used to tests both diagnostic and therapeutic interventions for cure HIV-associated

25 brain impairment.

26

Abstract

Studies of innate glial cell responses for progressive human immunodeficiency virus type one (HIV-1) infection are hindered by the availability of relevant small-animal models. To overcome this hindrance, a mouse model reconstituted with humanized brain and immune systems was created. Newborn NOD/SCID/IL2Rγc$^{-/-}$ mice of both sexes were transplanted with human neuroglial progenitors (NPC) and hematopoietic stem cells. Intraventricular injection of NPC yielded an anatomical symmetrical glia (human astrocyte and oligodendrocyte) repopulation of the mouse brain. The human glia were observed in periventricular areas, white matter tracts, the olfactory bulb and brain stem. HIV-1 infection of these dual humanized mice led to meningeal and perivascular human leukocyte infiltration into brain. The species-specific viral-neuroimmune interactions in the infected animals were identified by deep RNA sequencing. In the corpus callosum and hippocampus overlapping human-specific transcriptional alterations were seen for interferon type 1 and 2 signaling pathways (*STAT1, 2, IRF9, ISG15, IFI6*) and a range of host antiviral responses (*MX1, OAS1, RSAD2, BST2, SAMHD1)* in infected animals. Glial cytoskeleton reorganization, oligodendrocyte differentiation and myelin ensheathment (*MBP, MOBP, PLP1, MAG* and *ZNF488*) were downregulated. The data sets were confirmed by real-time PCR. The viral defense signaling patterns observed in these mice parallels the neuroimmune communication networks present in the HIV-1 infected human brain. In this manner, the new mouse model can facilitate discovery of therapeutics, viral eradication targets for virus induced nervous system diseases, and simplify HIVCure research approaches.

Introduction

47

48 Despite sustained investigation of the pathophysiological mechanisms of human

49 immunodeficiency virus (HIV)-associated neurocognitive disorders (HAND), virus-induced

50 changes in neuroimmune communications are incompletely understood (Gelman et al., 2012;

51 Saylor et al., 2016). As the virus cannot infect rodent cells co-morbid disease conditions that lead

52 to impaired immune, glial, and neural cell function have not yet been reflected in a small animal.

53 Transgenic mice containing the whole viral genome or individually expressed proteins and

54 animals injected with viral proteins or human infected cells in brain tissue can only mirror

55 disease components (Gorantla et al., 2012). Lentiviral infections of non-human primates, cats,

56 and ungulates can mirror HIV nervous system comorbidities (Narayan and Clements, 1989) but

57 only some of the natural infectious processes (Jaeger and Nath, 2012). Such limitations are also

58 operative in mice possessing a sustainable human immune system. While humanized animals are

59 susceptible to HIV infection and support persistent viral replication the resultant structural and

60 functional changes that occur in the brain are limited and not always reflective of human disease

61 (Boska et al., 2014; Dash et al., 2011; Gorantla et al., 2010). The absence of human glial cells

62 and differences in viral responses in the setting of divergent patterns of murine and human brains

63 limit mechanistic studies (Oberheim et al., 2009).

64 Astrocytes and oligodendrocytes, the most abundant cell type in the human central

65 nervous system, play pivotal roles in brain homeostasis and metabolism. Astrocytes not

66 oligodendrocytes show limited susceptibility to HIV infection (Chauhan, 2015; Farina et al.,

67 2007; Pfefferkorn et al., 2016; Russell et al., 2017). During disease, communication between

68 astrocytes and oligodendrocytes and infected mononuclear phagocytes (MP; monocyte

69 macrophages and microglia) herald changes in the brain's pro-inflammatory microenvironment

70 (Bednar et al., 2015). The restriction to viral infection reflect absent receptors for viral entry

71 (Schweighardt and Atwood, 2001) and coordinate with less studied intracellular innate immunity

72 protective pathways. Each do not permit the viral life cycle to reach completion (Geffin and

73 McCarthy, 2013

74). Further complicating glial responses during progressive viral infection are the interactions

75 between lymphocytes and monocytes that traffic into the nervous system during viral infection

76 (Dou et al., 2006; Epstein and Gendelman, 1993; Gonzalez-Scarano and Martin-Garcia, 2005;

77 Gorantla et al., 2010). Thus, a disease model that accurately replicates nervous system viral

78   infection must contain both human immune and glial cell elements (Gorantla et al., 2012). This

79   can be achieved by reconstitution of the mouse brain with human neural progenitor cells (NPC).

80   Transplantable NPC readily produce neuronal and glial lineage cells in the rodent brain (Nunes

81   et al., 2003; Uchida et al., 2000). The mice contain human hematopoietic stem progenitor cells

82   (HSPC) readily generating lymphocyte and monocyte-macrophages susceptible to HIV-1

83   infection. Human glial (astrocytes and oligodendrocytes) evolve from intraventricular NPC

84   transplantation at birth (Han et al., 2013; Windrem et al., 2014). Dual blood and brain humanized

85   mice when infected with a macrophage-tropic CCR5 HIV-1$_{ADA}$ can be examined for resultant

86   effects on glial homeostasis. Transcriptional evaluations of two brain regions, the hippocampus

87   and corpus callosum, demonstrated glial disease footprints in affecting antiviral human-specific

88   immune responses. The importance of this model is underscored by specific glial transcriptional

89   signatures that closely mirror the human disease (Gelman et al., 2012; Sanfilippo et al., 2017;

90   Sanna et al., 2017).

Results

Human NPC engraftment

Mice were reconstituted with human immunocytes and glial cells. Newborn irradiated NSG mice were transplanted intraventricularly with human neuronal progenitor cells (NPC) and intrahepatically with cluster of differentiation 34 (CD34)$^+$ HSPC from the same donor. NPC were isolated from fetal human brains to generate neurospheres, from which single cell suspension was prepared and transplanted at 10^5 cells/mouse (experiments 1 and 2, n = 19) or 0.5×10^5 (experiments 3 and 4, n=26). The fate and distribution of the human NPCs in mouse brain were then evaluated at 6 months of age. To analyze distribution of human glial cells paraffin embedded and formalin fixed tissue was sectioned along the sagittal plane from midline to the anatomical end of the corpus callosum (CC,~ 2.5 mm according to adult mouse brain atlas) from left and right hemispheres. In two experiments (n = 19) the distribution of glial cells was observed. Human glial cells were found in equal measure across both hemispheres on sagittal sections with distributions in the periventricular area (PV), CC, ventral striatum (STRv), cerebral cortex (CCx), midbrain (MB), cerebellum (CB) and hippocampus (Hip) areas. Human cells were present in olfactory bulb (OB), lateral olphactory tract, anterior commissure, caudoputamen, midbrain, pons and medulla (Fig. 1B, C, D, Table 1). A large proportion of the human cells in mouse brains were GFAP positive with typical astrocyte morphology based on size and process. To distinguish human and mouse astrocytes the staining with non-species-specific (rb Dako/Z0034) and human-specific (ms STEM123) antibodies was done. Human astrocytes commonly replaced their murine counterparts with nearly 70–80% occupancy in PV, CC. (Fig. 1B, indicated by star). There was limited occupancy of human astrocytes in the frontal cortex (FC) and CB in 1/3 of analyzed mice (Fig. 1E). Surprisingly, nearly 40% of human nuclei protein (hN, MAB4383) positive cells in the CC and 20-30% in Hip and STR were Olig2$^+$ (rb 1538-Olig2), an oligodendrocyte/progenitor transcriptional factor (Fig. 1D, F). The similar pattern (PV, CC, Hip, SRTv, MB) with less density of distribution of human cells were observed in two next experiments. To confirm glial lineage potential of the NPC we investigated their fate in short-term cultures. Mixtures of neural lineage cells arising from parental progenitors included GFAP/microtubule-associated protein (MAP) glial progenitors, nestin/GFAP neural progenitors (with radial glia characteristics), glutamic acid decarboxylase 67 (GAD67)-negative and class III β-tubulin (Tuj-1)$^+$ neuronal lineage cells (Fig. S1). These data indicated that cells derived from

122 neurospheres retained multi-lineage competence. Expanded human glial cells were integrated in
123 mouse brain tissue without activation of human microglial cells (Fig. 2). We concluded that
124 generated by neurosphere technique NPC transplanted at birth in brain lateral ventricle able
125 efficiently repopulate NSG mouse brain with some variability, differentiate in astrocytes and
126 retain oligodendrocyte development potential.

127

128 **Efficient peripheral hemato-lymphoid humanization of NSG mice**

129 A human peripheral hemato-lymphoid system was established in the NSG mice (Fig. 3). Flow
130 cytometric analyses of peripheral blood of humanized mice at 24 weeks of age showed that
131 among human $CD45^+$ cells, B ($CD19^+$) and T lymphocytes ($CD3^+$) were present (Fig. 3A). The
132 $CD14^+$ monocyte population in peripheral blood were also present (Fig. 3B). Human hemato-
133 lymphoid reconstitution was similar to previously published (Arainga et al., 2016; Dash et al.,
134 2011; Gorantla et al., 2010). In the brain, human peripheral blood leukocytes infiltrated the
135 meningeal and perivascular spaces, parenchyma. The majority of HLA-DR cells were located in
136 the meningeal space, with fewer numbers in the perivascular space and brain parenchyma (Fig.
137 3C and D). As we published previously (Gorantla et al., 2010), among these, the greater part
138 were $CD163^+$ macrophages, and some showed branching appearances. Less than 20% of HLA-
139 DR cells were $CD4^+$, and 9% were $CD8^+$ T lymphocytes (Fig. 3C and E). The data were
140 collected in animals with similar levels of human cells in peripheral blood (n = 7).

141

142 **HIV-1 infection of lymphoid and brain tissues in dual humanized mice**

143 At 24 weeks of age, mice were divided into two groups. One group was infected
144 intraperitoneally with HIV-1$_{ADA}$ at 10^4 infectious particles in a volume of 100 µl. We did not
145 observe a significant decline in human $CD4^+$ cells at the time of infection. At ~5 weeks flow
146 cytometric analyses showed ~ 20% drop in circulating $CD4^+$ T cell numbers among the $CD3^+$
147 cell population (p = 0.023) in selected animals with similar levels of human cells in peripheral
148 blood (n = 6/group). An increase in the $CD8^+$ T lymphocytes in proportion to $CD4^+$ T cells was
149 observed (Fig. 4A and B). After infection, HIV-1 $p24^+$ stained cells were present in spleen (Fig.
150 4C) and mice showed a median of 1.6×10^5 viral RNA copies/ml in blood (Fig. 4D). The brain
151 of infected animals (frontal cortex and striatum) were analyzed for the presence of viral RNA
152 (Fig. 4E). The levels of viral RNA expression in the brain tissue remain low (<50 copies/µg of

153   RNA) compare to systemic infection. HIV-1infected human cells were observed in meninges

154   (Fig. 4 F). HIV-1 infection influenced immunocyte infiltration in the brain and glial

155   reconstitution. Numbers of human immunocytes were analyzed for animals with comparable

156   levels of peripheral blood leukocyte repopulation by counting HLA-DR$^+$ and CD163$^+$

157   cells/sagittal section in HIV-1-infected (n = 5), uninfected (n = 6) dual transplanted. A

158   statistically significant two-fold increase in the number of brain infiltrating immunocytes

159   following HIV-1 infection was observed in meninges compared to uninfected dual reconstituted

160   mice 218.2 ± 8.6 versus 112.0 ± 13.3 cells/section (p = 0.0002). In the brain parenchyma

161   activated human HLA-DR$^+$ cells were 29.8 ± 2.8 and 17.0 ± 3.2 cells/section in infected and

162   uninfected mice, respectively (p = 0.017). The majority of human immunocytes were CD163$^+$

163   macrophages (Fig. 4G). In the spleen HIV-1p24 cells were present (Fig. 4C), and proportion of

164   stained cells for the HIV-1 Nef and p24 proteins in spleen were similar (not shown). To analyze

165   changes in glial cells the animals from two experiments with similar and broad distribution of

166   human glial cells were selected. These sections were stained for human-specific GFAP,

167   counterstained with hematoxylin and then subjected to unbiased digital image analysis

168   (DEFINIENS Tissue Studio®, Cambridge, MA, USA) to assess human glial cell repopulation in

169   5-μm sections. The total number of nucleated cells/sagittal section remained consistent across the

170   animal groups 107,061 ± 5,147 and 110,908 ± 6,483 cells/section (Fig. 4H). In comparison to

171   uninfected controls (n = 18), HIV-1-infected mice (n = 19) had a lower proportion of human

172   astrocytes/sagittal section 20.7 ± 1.5 % versus 15.9 ± 1.4 % of human-specific GFAP$^+$ cells,

173   respectively (p = 0.022). Serial sections also were stained for hu-GFAP, HIV-1p24 and Nef. No

174   astrocytes were identified stained with p24 and Nef viral proteins (data not shown). Systemic

175   HIV-1 infection of dual humanized animals increased influx of human macrophages and affect

176   number of hu-GFAP expressing cells.

177

Transcriptional changes in the humanized mouse brain induced by HIV-1 infection

179   To assess the pathobiological outcomes of viral infection following peripheral immune

180   and brain humanization, we applied deep sequencing to evaluate the transcriptional profiles seen

181   as a consequence of virus-immune cells-glia crosstalk. We selected three uninfected and three

182   infected animals with similar patterns of human immune cell repopulation, similar pattern of

183   astrocyte distribution and density, and similar peripheral viral loads (for infected mice) for study

184 (Fig. 5A). Three equivalent aged unmanipulated NSG mice served as controls. Hip and CC
185 repopulated by human glial cells were dissected for sequencing (Fig. 5B). All sequenced reads
186 (~100 bp length) were aligned to the mouse database University of California Santa Cruz
187 Genome Browser database (GRCm38/mm10). The experiment was designed to search for
188 differentially expressed mouse genes as a result of human cell engraftment and HIV-1-infection
189 (Breschi et al., 2017). Subsequently, the reads unmapped to the mouse genome were re-aligned
190 to the human genome (GRCh38/hg19) to find human-specific transcriptional changes. The
191 overall mapped mm10 reads average ratio was 92% for all 9 Hip samples and 94% for all 9 CC
192 samples. For the HIV-1-infected and uninfected humanized mice, the unmapped to mouse
193 database reads were run against its human genome. The average ratio for mapped reads was 32%
194 and 26% for the Hip and CC, respectively. Analyses using the mouse database identified 365
195 differentially expressed transcripts in the Hip and 93 in the CC as a result of the engraftment of
196 human glial cells. When compared to humanized mouse transcripts, few mouse transcripts were
197 differentially expressed as a result of HIV-1 infection in Hip only (Fig. 5E). When the reads
198 unmapped to the mouse genome were realigned to the human reference genome, 115 and 58
199 human transcripts were found differentially expressed in the Hip and CC, respectively, following
200 infection (Fig. 5F and G). The expression (FPKM) of human lineage-specific genes CD163, CD4
201 (macrophages and lymphocytes) were significantly lower compared to human glial transcription
202 factors OLIG1, OLIG2 (progenitors and oligodendrocyte commitment), GFAP and Glutamate-
203 Ammonia Ligase (glutamine synthase family), genes expressed by astrocytes. HIV-1 infection
204 reduced expression of human GFAP (FPKM $\log_2$ changes -0.55, p = 0.033). Expression of mouse
205 cell lineage-specific markers was not statistically significantly affected (data not shown).
206 Region-specific alterations of mouse transcriptome by engrafted human glial cells we
207 summarized in Supplemental Table 1, Fig. S2 and S3). Glial humanization of mouse brain
208 induced regions-specific transcriptional changes associated with multiple biological processes
209 and is of interest beyond this report (Goldman et al., 2015).

210

211 **Alterations in the mouse transcriptomes following viral infection**

212 HIV-1-induced transcriptional changes analyzed with alignment to mouse genome identified 25
213 DEG (Fig. 5E) and according to GeneCards® integrative database identified Gene Ontology (GO)

214   biologic processes gene sets could be associated with metabolic processes, blood vessel

215   development and extracellular matrix re-organization (*ALDH1A2, TCF7L2, COL1A2, COL5A1,*

216   *FMOD, BMP7*) as well as neurotransmitters, sugars, organic acids, anion transmembrane

217   transport (*SLC6A13, SLC6A20, SLC22A6, SLC13A4*), and cellular responses to hormone

218   stimulus (*IGFBP2, RAMP3*). Few downregulated genes were observed (*ST18, XIST*) and

219   expression levels of these genes could be found in supplemental data (Supplemental Table S2).

220   The changes in the neurotransmitters expression in Hip of infected humanized mouse brains

221   could affect cognition (Zhou and Danbolt, 2013) and need further evaluation. For example,

222   downregulated by humanization expression of *IGFBP2* was up-regulated by HIV-1 infection and

223   associated with inflammatory responses in HIV-1 infected patients (Helle et al., 2001; Suh et al.,

224   2015). Another example is downregulation of *XIST* initially upregulated by glial humanization

225   and related to the control of gene expression (Groen and Morris, 2013). These changes do not

226   reflect ongoing viral infection.

227

Species-specific alterations in the human transcriptomes following viral infection

229   The major observation is species-specific transcriptional changes in dual humanized HIV-1

230   infected mice brains. In contrast to mouse transcriptome, alignment to human genome reveals

231   overlapping pattern of upregulated and downregulated genes in Hip and CC (Fig. 5F and G, Fig.

232   S3, Supplemental Table S2). The upregulated genes were strongly associated with type 1 and 2

233   IFN responses and specific signaling pathways. These were linked to host viral defense

234   responses (*MX1, IFIT* families, *PLSCR1, EIF2AK2, IFI16, ADAR, OAS3, MX1, BST2, RSAD2*),

235   RNA catabolism (*CMPK2, PNPT1*), ISG15-protein conjugation, regulation of type 1 IFN

236   production (*HERC6, ISG15, DXT3L, DDX58, PPAR9, PPM1K*) and cytokine-mediated signaling.

237   The spectrum of Hip upregulated genes overlapped with that of the CC. In addition, upregulated

238   genes associated with norepinephrine metabolic processes (*EPAS1, LY6E*) and those related to

239   neural and glial differentiation (*PARP10, GARS, AGRN, SOX10, LINGO1*) were found. In the CC

240   and Hip, the identified downregulated genes were linked to myelination and axon ensheathment

241   (*MBP, MOBP, MAG, PLP1, CLDN11*), neural progenitor differentiation (*STMN2, ZNF488,*

242   *IGFBP5, BIN1, APLP1*), glial structural integrity, lipid metabolism, ion transportation and cell

243   cycle regulation (Fig. S3, Supplemental Table S2). Identified transcriptional changes show

244   ongoing human glial antiviral response to HIV-1 infection.

Human astrocytes produce type 1 IFN during infected macrophage-astrocyte co-cultivation

The upregulated expression of genes related to type 1 IFN responses in the chimeric murine brains following HIV-1 infection need investigation of the possible source of interferon. Though almost all nucleated cells can produce type 1 IFN, plasmacytoid dendritic cells are the most potent IFN producers (Steinman, 1991). However, prior studies have demonstrated that astrocytes abortively infected by neurotropic viruses are also a potent source of IFN beta (IFN-β) in mice (Pfefferkorn et al., 2016). To identify the source of type 1 IFN in response to HIV-1 infection in dual humanized mice, we used transwell systems to co-culture human fetal astrocytes and HIV-1-infected monocyte-derived macrophages (MDM). After 3 days of co-culture with HIV-1-infected MDM and astrocytes, astrocytes were found to produce IFN-β. The levels of IFN-β reached a peak at 5 days. Three independent donors were used; a representative experiment is shown in 6. The concentration of IFN-β produced by HIV-infected MDM alone remained at relatively low levels (approximately 15 pg/ml, detection limit). The concentration of IFN-β in control astrocytes and MDM were below detectable throughout the experimental observation period.

RT-PCR confirmation of canonical interferon stimulated human genes expression in humanized mice brain tissues

To confirm the altered expression and the species-specificity of the IFN signaling pathway related transcripts, we applied real-time PCR assays to reexamine the selected genes found affected in RNA-sequencing using human and mouse specific primers and probes. As critical elements of the upstream of type 1 IFN response pathway, *STAT1, STAT2* and *IRF9* were found 3.72, 1.69, and 3.64 times (p = 0.0003, 0.039, 0.00002) upregulated in the CC of HIV-1-infected dual reconstituted mice, respectively, compared to that of control dual reconstituted mice. Upregulation of these three genes was found in the Hip of HIV-infected humanized mice as well (Fig. 7, p = 0.004, 0.020, 0.010). The down-stream IFN inducible genes such as *MX1, ISG15, IFI6* and *CMPK2* were also found significantly upregulated in both CC (12.09, 11.69, 9.41 and 13.69 times, respectively, p<0.000001) and Hip (19.90, 15.90, 9.17 and 21.25 times, respectively, p<0.000001) of HIV-1 infected humanized mice compared to the control animals. We confirmed species-specific upregulation of IRF9/STAT1/STAT2 interferon stimulated response elements that mediate resistance to viruses (Cheon et al., 2013). Among the myelination and glial integrity

10

276 associated genes, the expression of *MBP* was reduced 2.94 and 2.5 times (p = 0.0058, 0.0013) in

277 the Hip and CC of HIV-1 infected mice, respectively. The expression of ZNF488 was only found

278 significantly downregulated in the CC of HIV-1 infected group (p = 0.0129), but not in the Hip.

279 Mouse MBP RT-PCR failed to demonstrate gene expression difference between the HIV-1

280 infected and control animal groups (Fig. 7).

281

Discussion

A mouse model reflecting HIV-1 infection of the nervous system was created that contains both human immune and glial cells in lymphoid organs and brain, respectively. This model represents the combination of NPC transplantation to generate human glia (Nunes et al., 2003; Uchida et al., 2000) and CD34^{+} HSPC reconstitution to forge a human immune system (Ito et al., 2002). This model builds on previous work demonstrating that transplantation of human neurosphere-derived NPC can occur in a living mouse with functional human-derived neurons and glia (Tamaki et al., 2002; Uchida et al., 2000). Early transplantation proved essential to optimize migratory responses of human glia in the mouse brain to facilitate replacement of their murine counterparts through brain subregions (Han et al., 2013; Windrem et al., 2014). We successfully show that human NPC when transplanted at an early postnatal age demonstrate broad glial distribution through the brain. By 6 months of age, considerable numbers of human astrocytes were seen to have repopulated multiple brain regions along whiter matter tracts. Moreover, we found considerable numbers of human Olig2 positive cells (Fig. 1) and signaling the emergence of significant oligodendrocyte differentiation Olig1 (Fig. 3D). The broad variability in glial cell distribution and density were associated, in part, with the number of transplanted cells, NPC gestation and collection times and their stem cell properties that are effected by the number of cell passages and the accuracy of intraventricular injections.

The distribution of human immunocytes in the brain vary and were comparable to what was observed in humanized mice created by CD34^{+} cell transplantation and subsequent HIV-1 infection (Gorantla et al., 2010; Honeycutt et al., 2016). Notably, the majority of human immunocytes present in the brain were observed in the meninges. These were predominantly CD163-positive human macrophages. High levels of systemic HIV-1 infection with infected meningeal and brain-infiltrating cells at lower numbers than what were seen in peripheral tissues were detected. Thus, the model reflects three principal features of human brain disease that includes a functional immune system with mobile brain penetrant monocyte-macrophages, sustained high levels of systemic viral infection and the presence of glial target cells that determine alterations in the brain microenvironment. Most importantly, each of the cell types were able to interact, one with the other, in a physiological manner. In contrast to a number of operative disease models, the effects of HIV-1 infection on human brain cells and human-specific immune changes that result from persistent systemic infection can now be fully evaluated.

313   We applied RNA-sequencing for dissection of species-specific changes to assess the

314   relevance of the model to human disease. Indeed, our findings in the transcriptome of HIV-1-

315   infected dual reconstituted mice corroborated the relevance of the animal model system. As the

316   most abundant cell type in the brain, astrocytes react to injury or viral infection through cellular

317   activation (Sabri et al., 2003). While HIV-1 infection of $CD4^+$ T lymphocytes, perivascular

318   macrophages and microglia are well recognized (Yadav and Collman, 2009), infection of

319   astrocytes remains unresolved. The question that has remained could low level infection of

320   astrocytes or mere exposure of astrocytes to infected cells or HIV-1 itself cause important

321   physiological changes that alter the brain's functional capacities? Indeed, in contrast to high

322   levels of systemic infections, low levels of HIV-1 RNA were seen in analyzed tissue samples.

323   While restriction of astrocyte infection can occur at the level of cell entry (Gorry et al., 2003),

324   this in no way detracts from the notion that significant innate immune responses could still occur

325   by the limited virus that is present and explain what was observed at transcriptional levels.

326   Indeed, more than 30 human genes significantly upregulated in the CC and Hip was found to be

327   closely related to innate and adaptive immunity, HIV host restriction factors, and IFN stimulated

328   factors (Fig. 5, Fig. S3). These findings support the idea that low levels of virus can induce

329   considerable affects in the brain's innate antiviral immune responses. Noticeably, STAT1/STAT2

330   and IRF9 form a transcriptional complex upon activation of type 1 IFN receptors; this complex

331   can subsequently translocate to the nucleus and induce the expression of a broad spectrum of

332   IFN-stimulated genes that serve to contain infection (Schreiber, 2017). Among such IFN

333   stimulated factors, *MX1, OAS3, RSAD2* (viperin), and all the *IFI* and *IFIT* family are involved in

334   combating viruses at nearly every stage of the viral life cycle (Espert et al., 2005; Fricke et al.,

335   2014; Laguette et al., 2011; Lu et al., 2011; Nasr et al., 2012; Neil et al., 2008; Okumura et al.,

336   2006; Rivieccio et al., 2006; Spragg and Emerman, 2013). In the Hip, in addition to the

337   upregulated genes introduced previously, class I HLA genes were found upregulated, which is

338   yet another downstream effect of the type 1 IFN response.

339   The upregulation of the human specific IFN signaling related genes IRF9/STAT1/STAT2

340   were confirmed by real-time PCR assays using human specific probes. We also confirmed by RT-

341   PCR upregulation of rare mRNA transcript identified by RNA sequencing for *CMPK2*, which

342   could belong to infiltrating human macrophages as well as glial cells, and further affirm that the

343   factors serve as a viral restriction factor (Lazear et al., 2013). However, no change in expression

344   of the host restriction factors were reflected in the mouse transcriptional profile as result of HIV-

345   1 infection. This confirms that HIV-1 can neither efficiently enter mouse cells due to the absence

346   of specific receptors, nor trigger a pattern recognition response by murine cellular defense

347   machinery after viral infection. We cannot exclude that extension of the observation time in the

348   fact that few months of infection could be required to trigger mouse cell responses to HIV-1 and

349   as such change the profile of human cell responses and damage.

350   This vigorous type 1 IFN signaling response further leads to the question of what cell

351   source produces type 1 IFN in these dual reconstituted mouse brains. Due to the increased

352   infiltration of macrophages into the brain during systemic HIV-1 infection in these mice, we

353   tested these two-cell types interaction for type 1 IFN production in response to HIV-1 infection.

354   The astrocyte production of IFN-β in response to various neurotropic viruses was documented

355   (Pfefferkorn et al., 2016). Neither macrophages nor astrocytes produce type 1 IFN in significant

356   amounts that are comparable to plasmocytoid dendritic cells seen in response to HIV-1

357   (Fitzgerald-Bocarsly et al., 2008; Wijewardana et al., 2009). We were able to confirm the

358   possible source of IFN-β occurs through their interactions with infected macrophages. The

359   transwell co-culture of HIV-1$_{ADA}$-infected MDM and astrocytes revealed the temporally

360   dependent dynamics of IFN-β production.

361   The cellular machinery that glia use to protect themselves from infection could also

362   secondarily create an inflammatory cascade. This could then lead to restricted astrocyte infection

363   and programmed cell death (Thompson et al., 2001). Through the use of unbiased image analysis

364   we found a limited yet significant decrease in the number of human GFAP^{+} astrocytes in the

365   brains of chimeric mice infected with HIV-1 and reduced expression of GFAP (Fig. 4 and 6). A

366   similar scenario is known to occur in quiescent CD4 T cells, which undergo an abortive infection

367   with accumulation of incomplete reverse viral transcripts (Monroe et al., 2014). In support of this

368   theory, our data showed significant upregulation of genes serving as cytoplasmic sensors for

369   nucleic acids, such as *DDX58, DDX60* and *IFI6* (Li et al., 2016). Therefore, the downstream

370   signaling and effect of these RNA sensors in glial cells are certainly worthy of further

371   investigation. We also cannot exclude compromised neurogenesis by HIV-1-induced interferon-

372   related pathways, which preclude astrocyte's differentiation (Carryl et al., 2015; Geffin and

373   McCarthy, 2013).

374   The identified downregulated genes are mostly critical for oligodendrocyte differentiation

14

375   and myelination. Among them, ZNF488 is a critical transcriptional factor that interacts and

376   cooperates with Olig2 to promote oligodendrocyte differentiation (Wang et al., 2006). *PLP1,*

377   *MAG, MOBP,* and *MBP* are encoding structure proteins of the myelin sheath. *CLDN11* encodes a

378   crucial component of the tight junctions between the myelin sheath (Gow et al., 1999; Morita et

379   al., 1999). Upregulation of LONGO1 expression also could inhibit myelination and

380   oligodendrocyte differentiation (Jepson et al., 2012). Together, all of these transcriptional

381   changes suggest early alterations in the structure and function of white matter during HIV

382   infection. This may potentially lead to a compromise in the integrity of the glial system and

383   consequent neural dysfunction.

384   In summary, we successfully established the dual reconstituted chimeric mouse model

385   that contained both a human immune system and human glia. This model allowed us to access

386   how glial-immunocyte crosstalk may be affected by HIV-1 infection. Using sequencing we found

387   that the transcriptional profiles from the chimeric brains overlap, in large measure, with the

388   disease profile found in human HIV-1 infected patients with cognitive impairments, encephalitic

389   brains and acute SIV infection (Borjabad et al., 2011; Cserhati et al., 2015; Gelman et al., 2012;

390   Sanfilippo et al., 2017; Sanna et al., 2017; Siangphoe and Archer, 2015; Winkler et al., 2012).

391   These findings underscore the protective role astrocytes have in defending the brain from HIV-1

392   infection and damage of myelination processes. This model also provides a platform to further

393   investigate the pathobiology of HIV nervous system infection and viral cell reservoirs needed to

394   combat novel disease combating therapies and eradication measures.

15

Materials and Methods

Animals, cell transplantation, and HIV-1 infection

Newborn NOD/SCID/IL2Rγc$^{-/-}$ (NSG, https://www.jax.org/strain/005557) mice were bred at the University of Nebraska Medical Center (UNMC). Animal procedures strictly followed the Institutional Animal Care and Use Committee approved protocols at UNMC and in adherence to the Animal Welfare Act and the Public Health Service Policy on Humane Care and Use of Laboratory Animals. P0-1 litters were irradiated with 1 Gy (RS 2000 X-ray Irradiator, Rad Source Technologies, Inc., Suwanee, GA, USA). Pups were intrahepatically injected with 5×10^5 CD34$^+$ HSPCs. These mice were simultaneously transplanted with $0.5 – 1 \times 10^5$ NPC into the right lateral ventricle. Two samples of 97 gestation days (experiments 1 and 2) and two samples of 117 days gestation days (experiments 3 and 4) of liver and brain were used to create 4 cohorts of experimental animals. NPC were generated by 4 - 6 passages. Total 45 dual reconstituted animals were created, 23 females and 22 males. Blood samples collected from the facial vein were evaluated by flow cytometry starting 8 weeks post-engraftment to monitor expansion of human leukocytes. Mice with established human hemato-lymphoid reconstitution (~5 months of age) were intraperitoneally infected (n = 22) with the macrophage-tropic ADA HIV-1 strain and euthanized 5 weeks after the infection was established (Table 1).

NPC isolation and culture

Human NPC were isolated from human fetal brains provided by the University of Washington Medical Center Laboratory of Developmental Biology (R24HD000836-51). The UNMC Scientific Research Oversight Committee (SROC) and Institutional Review Boards at the University of Washington Medical Center and UNMC approved the protocol. Cells were isolated from human fetal brains as previously described (Nunes et al., 2003). Briefly, single cell suspensions were cultivated in NS-A medium (STEMCELL, Mountain View, Canada) containing N2 supplement (1:100; Fisher Scientific, Waltham, MA, USA), neural survival factor-1 (NSF1; 1:50; Lonza, Walkersville, MD, USA), LIF (10 ng/ml; Sigma-Aldrich, St. Louis, MO, USA), epidermal growth factor (EGF; 20 ng/ml; Fisher, MA, USA), and basic fibroblast growth factor (bFGF; 20 ng/ml; STEMCELL, Mountain View, Canada) for 14 days before passage. Four hours before injection, the neurospheres were dissociated into single cell suspension with 0.25% Trypsin (Gibco, Grand Island, NY, USA). The single cell suspensions were plated on

16

426   polystyrene-coated coverslips and cultured for 48 hours in vitro. Immunofluorescent cytology

427   was applied to investigate the differential potential of NPC.

428

429   **Human hematopoietic stem progenitor cells isolations**

430   Matched to the brain sample, the fetal liver tissue was processed with 0.5 mg/ml DNase,

431   collagenase, and hyaluronidase (all from Sigma-Aldrich, St. Louis, MO, USA) using

432   gentleMACS™ Dissociator (Miltenyi Biotec, Auburn, CA, USA). Cell suspensions were passed

433   through a 40-μm cell strainer and then centrifuged through leukocyte separation medium (MP

434   Biomedicals, Santa Ana, CA, USA) at 100 g for 30 min, with acceleration set to 9, and

435   deceleration set to 1. The intermediate layer of cells was collected for positive isolation of

436    $CD34^+$ cells using a magnetic isolation kit (Miltenyi Biotec, Auburn, CA, USA). $CD34^+$ cells

437   were stored in liquid nitrogen using a freezing medium with 50% fetal bovine serum (FBS), 40%

438   Roswell Park Memorial Institute (RPMI)-1640 medium (Hyclone, Logan, UT, USA), and 10%

439   dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA).

440

441   **Flow cytometry**

442   Blood samples were collected from a facial vein in ethylenediaminetetraacetic acid (EDTA)-

443   containing tubes (BD Microtainer, Franklin Lakes, NJ, USA) and centrifuged at 300 g for 5 min.

444   Plasma was stored for HIV viral load measurements. Blood cells were reconstituted in a buffer of

445   2% FBS in phosphate buffered saline and incubated with antibodies against human cell markers,

446   including $CD45^+$ fluorescein isothiocyanate (FITC, BD/555482), $CD3^+$ Alexa700 (BD/561805),

447    $CD19^+$ R-phycoerythrin (PE) cyanine 7 (Cy7, BD/555414), $CD4^+$ allophycocyanin (APC, BD/

448   561841), $CD8^+$ Brilliant Violet^{TM} 450 (BV450, BD/562428), and $CD14^+$ PE (BD/555398) for 30

449   min at 4°C. Samples were analyzed using a BD LSR2 flow cytometer using acquisition software

450   FACS Diva v6 (BD Biosciences, USA), and data was analyzed using FLOWJO analysis

451   software v10.2 (Tree Star, USA). Gates were assigned according to the appropriate control

452   population.

453

454   **Measurements of viral load in plasma and brain tissues**

455   Viral RNA copies in the murine plasma were determined by using a COBAS Amplicor System

456   v1.5 kit (Roche Molecular Diagnostics, Pleasanton, CA, USA). Expression of HIV-1 group-

17

457 specific antigen (gag) RNA in brain tissues were analyzed by reverse transcription (RT)-PCR

458 with previously published primers and probes (Arainga et al., 2016). Briefly, cortex and striatum

459 from three HIV-infected dual reconstituted mice and three control dual reconstituted mice were

460 micro-dissected and transferred to 700 µl QIAzol solution (QIAGEN, Valencia, CA, USA) for

461 RNA isolation using an RNeasy Mini kit (QIAGEN, Valencia, CA, USA). A Verso cDNA

462 synthesis kit (Thermo Fisher Scientific, Grand Island, NY, USA) prepared cDNA according to

463 the manufacturer's instructions. For the first round of conventional PCR, we used the following

464 primers: sense 5' TCAGCCCAGAAGTAA-TACCCATGT 3' and antisense 5'

465 TGCTATGTCAGTTCCCCTTGGTTCTCT 3'. The PCR settings were as follows: 94°C for

466 3 min, 15 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min. The product of

467 the first PCR was subsequently used as a template in the second semi-nested real-time PCR

468 amplification performed on the ABI StepOne Plus real-time PCR machine (Applied Biosystems,

469 MA, USA) using TaqMan detection chemistry. The primers and probe used for the second PCR

470 were: sense 5' TCAGCCCAGAAGTAATACCCATGT 3', antisense 5'

471 CACTGTGTTTAGCATGGTGTTT 3', and TaqMan probe FAM-

472 ATTATCAGAAGGAGCCACCCCACAAGA-TAMRA. The real-time PCR settings were as

473 follows: 50°C for 2 min, 95°C for 10 min, 45 cycles of 95°C for 15 sec, and 60°C for 1 min.

474 ACH2 cells (8×10^5) containing one integrated copy of HIV-1 per cell were used in triplicate

475 as standards, with cell and HIV copy numbers ranging in serial 10-fold dilutions from 10^5 to

476 10^2 deoxyribonucleic acid (DNA) copies/reaction.

477

478 **Immunohistochemistry**

479 Brain tissue was cut sagittally and the left and right hemispheres from experiments 1 and 2, and

480 right hemispheres from experiments 3 and 4, were fixed with 4% paraformaldehyde for 24 hours

481 at 4°C, and then embedded in paraffin. Paraffin-embedded 5-µ thick tissue sections were

482 processed with Declere Solution (Sigma-Aldrich, St. Louis, MO, USA) according to the

483 manufacturer's instructions. Tissue was blocked with 10% normal goat serum in Tris-buffered

484 saline and 0.05% Tween 20 (TBST) for 45 min and then incubated with mouse (ms) anti-human

485 GFAP (1:1000; Y40420/STEM123, TaKaRa Bio, USA), ms anti-human nuclear antigen (1:200;

486 Millipore/MAB4383, Billerica, MA, USA), ms anti-human cytoplasmic marker (1:1000;

487 Takara/STEM121, Mountain View, Canada), ms anti-Nef (1:200, Santa Cruz/sc-65904), ms anti-

18

488   HLA-DR (1:500; Dako/M0746, Carpinteria, CA, USA), ms anti-CD163 (1:50; Thermo/MA5-

489   11458, Denver, CO, USA), rabbit (rb) anti-GFAP (1:1000; Dako/Z0034, Carpinteria, CA, USA),

490   and rb anti-human CD4 (1:500; Abcam/ab133616). Secondary antibodies were goat anti-ms and

491   goat anti-rb immunoglobulin G (IgG) horseradish peroxidase (HRP; Dako, Carpinteria, CA,

492   USA). Bright field images were captured and photographed using $20\times$ and $100\times$ objectives on a

493   Nuance Multispectral Tissue Imaging system (CRi, Wobum, MA). Immunostained sections were

494   scanned using a high-resolution scanner (Ventana Medical Systems, Inc., Oro Valley, AZ, USA).

495   DEFINIENS Tissue Studio® software (Definiens AG, Munich, Germany) was used to analyze

496   the brain sections stained for human glial and immune cells. Spleen and brain tissue sections

497   were analyzed for HIV-1 p24 protein staining with anti-HIV-1 p24 (1:10; Dako/M0857,

498   Carpinteria, CA, USA). None of human-specific antibodies had cross-reactivity and stained

499   mouse brain sections not reconstituted with human cells.

500

501   **Immunofluorescence**

502   For immunofluorescent staining of paraffin-embedded tissue, sections were processed and

503   blocked as described above. Ms anti-human GFAP, ms anti-human nuclei, rb anti-GFAP, and rb

504   anti-Oligo2 (1:500; PhosphoSolution/1538, Aurora, CO, USA) were applied. A Leica DM6

505   system (Leica Microsystems, Wetzlar, Germany) were used for immunofluorescent imaging. The

506   intensity of illumination and the position of the sub-region on the microscope were consistent

507   across all images. For immunofluorescent characterization of cultivated NPC, ms anti-human

508   GFAP (1:200; Abcam, Cambridge, MA, USA), ms anti-MAP2 (1:200; Santa Cruz, Santa Cruz,

509   CA, USA), ms anti-glutamic acid decarboxylase 67 (GAD67; 1:500; Millipore, Billerica, MA,

510   USA), rb anti-nestin (1:200; Millpore, Billerica, MA, USA), and rb anti-Tuj-1 (1:500; Biolegend,

511   San Diego, CA, USA) were applied. Secondary antibodies were goat anti-ms IgG Alexa 488

512   (1:00; Invitrogen, Grand Island, NY, USA), goat anti-ms IgG Alexa 594 (1:00; Invitrogen, Grand

513   Island, NY, USA), goat anti-rb IgG Alexa 488 (1:100; Invitrogen, Grand Island, NY, USA), and

514   goat anti-ms IgG Alexa 594 (1:00; Invitrogen, Grand Island, NY, USA). A Leica DM6 system

515   (Leica Microsystems, Wetzlar, Germany) and a Zeiss LSM510 confocal system (Carl Zeiss

516   Microscopy GmbH, Jena, Germany) were also used for immunofluorescent imaging.

517

518   **Next generation sequencing**

19

519   Left hemispheres from experiments 3 and 4 of the reconstituted mouse brains (0.5×10^5

520   cells/mouse) were dissected by regions and flash-frozen for RNA extraction. The brain of three

521   age-matched NSG mice were also collected for non-reconstituted control. For sequencing

522   hippocampus (Hip) and corpus callosum (CC) RNA from brain tissue from three uninfected

523   dual-reconstituted animals, 3 HIV-1 infected mice and 3 NSG non-reconstituted controls were

524   preserved (Invitrogen, Grand Island, NY, USA). Tissue was transferred to 700 µl of QIAzol

525   solution (QIAGEN, Valencia, CA, USA) for RNA isolation using an RNeasy Mini kit

526   (QIAGEN, Valencia, CA, USA). After nucleic acid integrity was analyzed, samples were deep

527   sequenced using 100 bp/read, up to 40 million reads/sample using an Illumina HiSeq 2500

528   Sequence Analyzer (Illumina, Inc., San Diego, CA, USA). Reads were trimmed using the fqtrim

529   software to remove ambiguous bases from the reads. Read alignment and differential gene

530   expression was performed using the programs from the Tuxedo RNA-seq tool pipeline. The

531   trimmed reads were then aligned to the mouse genome (version mm10/GRCm38) and the hg19

532   human genome (when necessary) using the tophat aligner, version 2.1.1. The ratio of aligned

533   reads per total reads was calculated to assure quality alignments. Unaligned reads coming from

534   the humanized samples were remapped to the hg19 database. FPKM values (Fragments Per

535   Kilobase of transcript per Million mapped reads) were calculated for each gene in each sample

536   using the cufflinks software, version 2.2.1. This was done to calculate each gene's expression

537   level in each sample. An FDR adjusted p-value of 5% was used in all cuffdiff sample

538   comparisons. For both regions (Hip and CC) the control samples (NSG unmanipulated) to the

539   humanized mouse samples (blood and brain reconstituted), and the humanized mouse samples to

540   the humanized mouse samples with HIV were compared. The reads were mapped to the mm10

541   mouse genome and the mouse transcriptome. For both tissues, the unmapped reads, which did

542   not map to the mouse genome, were collected and remapped to the human genome. This was

543   done in order to see what human genes were expressed in these tissues. Cuffdiff was specifically

544   used because it normalizes the expression values and protects against overdispersion so that the

545   values fit into a normalized bell curve (Trapnell et al., 2012).

546

547   **Macrophage and human fetal astrocytes isolation and cultivation**

548   Human monocytes were isolated by leukopheresis from HIV-1/2 and hepatitis seronegative

549   donors. Human monocytes (2×10^5) were seeded in the inserts of 24-well transwell plates with a

550   pore size of 0.4 µm. (Corning Costar, Corning, NY, USA). Dulbecco's Modified Eagle's

551   Medium (DMEM; Invitrogen, Grand Island, NY, USA) with 10% heat-inactivated pooled human

552   serum, 2 mM L-glutamine, 50 µg/ml gentamicin, 10 µg/ml ciprofloxacin, and macrophage

553   colony-stimulating factor (MCSF) enriched conditioned medium maintained at 37°C in a 5%

554   CO_2 incubator was used (Gendelman et al., 1988). On day 7, differentiated monocyte-derived

555   macrophages (MDM) were infected with HIV-1$_{ADA}$ (multiplicity of infection (MOI) = 0.01) for

556   12 hours. Infected macrophages were maintained in the DMEM medium containing 10% heat-

557   inactivated pooled human serum, 2 mM L-glutamine, 50 µg/ml gentamicin, and 10 µg/ml

558   ciprofloxacin for another 6 days before the inserts were transferred to wells with astrocytes at the

559   bottom. Human fetal astrocytes (HFA) were isolated and cultured from fetal donors (Nath et al.,

560   1995). HFA (5×10^5) were seeded at the bottom of 24-well transwell plates and maintained in

561   DMEM/F12 (Invitrogen, Grand Island, NY, USA) containing 10% FBS and antibiotics.

562   Supernatants from HIV-1-infected and control MDM cultures were collected from 2 to 6 days

563   before MDM and astrocyte co-cultivation. Supernatants from the transwell system were collected

564   at 24 hours, 3, 5, and 7 days.

565

566   **IFN-β enzyme-linked immunosorbent assay**

567   IFN-β was measured in the supernatants of astrocytes, a co-culture of astrocytes and control

568   macrophages, a co-culture of astrocytes and HIV-infected macrophages, HIV-infected

569   macrophages, and control macrophages using the Human IFN-β bioluminescent ELISA kit

570   (http://www.invivogen.com/lumikine-hifnb) according to the manufacturer's instructions.

571

572   **RT-PCR for human and mouse transcripts**

573   First-strand cDNA was prepared from CC and Hip using the same method described above. For

574   both brain regions, we compared the control humanized mouse samples to the humanized mouse

575   samples with HIV. The catalog numbers of all TagMan® real-time PCR assays from Life

576   Technologies are as listed below: hu-STAT1 (Hs01013996_m1), hu-STAT2 (Hs01013123_m1),

577   hu-IRF9 (Hs00196051_m1), hu-MX1 (Hs00895609_m1), hu-ISG15 (Hs01921425_s1), hu-

578   CMPK2 (Hs01013364_m1), hu-IFI6 (Hs00242571_m1), hu-MBP (Hs00921945_m1), hu-

579   ZNF488 (Hs00289045_s1), hu-GAPDH (Hs03929097_g1), ms-MX1 (Mm00487796_m1), ms-

580   MBP (Mm01266402_m1), ms-GAPDH (Mm999999_g1). The real-time PCR settings were as

581   follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec, and 60°C for 1 min.

582   All real-time assays were carried out with an Applied Biosystems® StepOnePlus Real-Time

583   PCR Systems. The fold change of each target gene mRNA relative to GAPDH between the HIV^{+}

584   group and the control group was determined. This was done by using threshold cycle (Ct) and

585   the $2^{-\Delta\Delta C_T}$ method, where $\Delta Ct = Ct_{target} - Ct_{GAPDH}$ and $\Delta(\Delta Ct) = \Delta Ct_{HIV} - \Delta Ct_{control}$.

586

587   **Statistical analysis**

588   Values from all quantified astrocytes, oligodendrocytes, macrophages and lymphocytes and cell

589   groups were averaged and presented as mean $\pm$ SEM; multiple t-tests using the Holm-Sidak

590   method for multiple comparisons correction was applied to examine significant differences

591   between groups. Two-way analysis of variance (ANOVA) using Sidak's multiple comparisons

592   correction was used to examine significant differences between groups. For RNA sequencing

593   data, the tophat/cuffdiff pipeline was run on the reads for each sample in order to identify

594   differentially expressed genes (Trapnell et al., 2012).

595

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823

824 **FIGURE LEGENDS**

825 Figure 1. **Glial humanization of the mouse brain.** *A,* Scheme of the transplantation of human

826 blood and brain progenitors at birth and the period of observation. *B,* Representative view under

827 a 1× objective lens of 5-μm thick paraffin-embedded sagittal section stained for human-specific

828 GFAP (brown). *C,* Human astrocytes specific antibodies (hu-GFAP, red) and non-species-specific

829 total glial fibrillary acidic protein (tGFAP; green) staining was performed in different brain

830 regions. Representative images are shown. Original images were collected by a Leica DM6

831 system (Leica Microsystems, Wetzlar, Germany) with 1000× magnification. Nuclei were stained

832 with 4',6-diamidino-2-phenylindole (DAPI; blue). Bar = 20 μm. *D,* Oligodendrocyte specific

833 nuclear staining (Olig2; green) and total human-specific nuclear (hN, red) staining was done in

834 different brain regions and representative images are shown. The original magnification was

835 400×. Bar = 50 μm. Dotted line on (**B**) demarcates the corpus callosum (CC) from the frontal

836 cortex (FC). *E, F,* Quantitative data showing the number of human glial cells from one of four

837 experiments with ten animals. The average percentage of human cells was assessed on 2–4

838 representative sagittal sections from each mouse for a selected field of view of the same brain

839 region under 400× magnification (indicated by star). *E,* Distribution and proportion of hu-GFAP$^+$

840 astrocytes among total number of GFAP$^+$ cells. *F,* Distribution and proportion of hN$^+$ cells

841 positive for Olig2. Each symbol represents an individual mouse and lines indicate mean and

842 standard error of the mean (SEM). Animals were analyzed at 6 months of age and reconstitution

843 characteristics is shown in Table 1.

844

845 Figure 2. **Interaction of human and mouse glial cells**. Sagittal section of paraffin-embedded

846 brain tissues was stained for human astrocytes (hu-GFAP, red) and mouse microglial cells (Iba-1,

847 green) identification. Original images in periventricular region and corpus callosum were

848 collected by a Leica DM6 system (Leica Microsystems, Wetzlar, Germany) with 100×

849 magnification. Selected fields of view (square) were captured with 400× magnification. Bar =

850 100 μm. Mouse microglial cells are in contact with human astrocytes. There were no

851 morphological signs of activation, formation of microglial nodules or active phagocytosis of

852 human cells.

853

854 Figure 3. **Human immune cell repopulation of mouse blood and brain.** *A,* Fluorescence-
855 activated cell sorting (FACS) analysis of peripheral blood. Representative plots of human cluster
856 of differentiation 45 (CD45)$^+$ cells and human CD3, CD19, and CD14 cells gated from the CD45
857 lymphocyte population. *B,* Percentage of human cells in peripheral blood of all dual
858 reconstituted mice used in the studies. Each symbol represents an individual mouse. *C,*
859 Representative images of the distribution of human immune cells in brain are shown. Paraffin
860 embedded 5-μm thick sections were stained for human-specific markers: human leukocyte
861 antigen-antigen D related (HLA-DR), CD163, CD4, and CD8. Representative images from
862 different brain regions are shown. Images were captured by a Nuance multiplex system (CRi,
863 Wobum, MA) with an original magnification of 200×. Insets represent cells with a magnification
864 of 1000×. Bars = 100 μm and 20 μm (inset). *D* and *E*, Quantification of human cells positive for
865 HLA-DR, CD163, CD8, and CD4 in selected regions. Numbers represent frequencies of positive
866 cells per section; 7 mice with similar levels of lymphoid reconstitution were analyzed.

867

868 Figure 4. **HIV-1 infection affects human cell distributions in dual blood and brain**
869 **humanized mice.** *A*, Examples of representative plots showing CD4$^+$ cell decline in peripheral
870 blood of HIV-1-infected mice, with a relative increase in the CD8$^+$ cell compartment. *B,*
871 Quantification of human CD4 and CD8 T cells in the peripheral blood of infected (n = 6) and
872 control (n = 6) mice. Multiple t-tests using the Holm-Sidak method for multiple comparisons
873 correction was applied to examine significant differences between groups. Bars = means ±
874 standard error of the mean (SEM); *Adjusted P = 0.045. *C*, Immunohistology of spleen sections
875 shows HLA-DR positive cells and the presence of HIV-1-infected cells stained for HIV-1 p24.
876 Images were captured by a Nuance multiplex system (CRi, Wobum, MA) with an original
877 magnification of 200×. Bar = 100 μm. *D,* Peripheral viral load determined by a COBAS
878 Amplicor System v1.5 (Roche Molecular Diagnostics, Pleasanton, CA, USA). Each symbol
879 represents an individual infected mouse from all experiments. *E,* Viral RNA levels were
880 determined by semi-nested RT-PCR in the HIV$^+$ group (n = 3, red symbol) and the control group
881 (n = 3, blue symbol) in two brain regions, frontal cortex (FC) and striatum (STR). *F,*
882 Immunohistology of brain sections showing the presence of activated HLA-DR$^+$ cells and HIV-
883 1p24$^+$ infected cells on adjacent serial section. Representative images from the meningeal space
884 containing vascular vessels are shown. Images were captured by a Nuance multiplex system with

32

885 an original magnification of 200×. Insets represent cells with a magnification of 1000×. Bars =

886 100 μm and 20 μm (inset). *G,* Quantification of infiltrating HLA-DR$^+$ and CD163$^+$ cells in the

887 brain regions of HIV-1-infected (n = 5) and uninfected dual reconstituted mice (n = 6). Two-way

888 analysis of variance (ANOVA) using Sidak's multiple comparisons correction was used to

889 examine significant differences between groups. Bars = means ± SEM; *P = 0.017, ****P <

890 0.0001. *H,* Quantification of human glial fibrillary acidic protein (GFAP)$^+$ and all nucleated cells

891 on representative sagittal brain sections of HIV-1-infected (n = 18) and control (n = 19) mice.

892 Unpaired t-tests were used to examine significant differences between groups. Each point is

893 individual animals averaged counts from two sections presented, means ± SEM are shown; *P =

894 0.022, ns = no significance.

895

896 Figure 5. **HIV-1 infection induces transcriptional alterations in brains of dual humanized**

897 **mice.** *A,* Characteristics of peripheral reconstitution and the levels of HIV-1 infection of mice

898 selected for RNA-sequence analysis (n = 3 per group). Percentage of human CD45, CD3, CD4,

899 and CD8 cells, as well as HIV-1 viral load (VL) in the peripheral blood of HIV-1-infected dual

900 reconstituted mice (HIV$^+$, red symbols) and control dual reconstituted mice (control, blue

901 symbols) are shown. Three unmanipulated NSG of the same age were used as a control and were

902 negative for all these parameters. *B,* Representative image of human glial cell reconstitution and

903 scheme of CC and Hip microdissection. *C,* Identification of differentially expressed genes.

904 Sequenced reads were mapped to the mouse database (mm10) and comparisons were conducted:

905 1) between non-manipulated NSG mice and dual reconstituted NSG mice to find differences

906 induced by humanization (humanized mouse mm10, blue color); 2) between uninfected and HIV-

907 1 infected dual reconstituted mice to observe differences of mouse transcriptome induced by

908 infection (humanized mouse mm10, green) and in CC such differences were not identified.

909 Reads unmapped to the mm10 database was realigned to the human database (hg19) and

910 comparisons were conducted between uninfected and HIV-1-infected dual reconstituted mice

911 (human HIV$^+$ hg19, red color). *D,* Confirmation of the expression of human cell lineage-specific

912 genes for Hip (hg19) and effects of infection on human GFAP downregulation were statistically

913 significant in t-test comparison, P = 0.033, but did not reach significance with FDR adjustment.

914 None of mouse cell lineage-specific genes expression was affected by HIV-1 infection. *E,* Up-

915 and downregulated mouse genes in the Hip of HIV-1-infected dual reconstituted mice compared

916   to uninfected mice (mm10). *F*, *G,* Up- and downregulated human genes in the CC and Hip of

917   HIV-1-infected dual reconstituted mice compared to uninfected mice (hg19). *E, F, G*, Genes with

918   1.5 fold changes with FDR adjusted $p < 0.05$ were graphed.

919

920   Figure 6. **Astrocyte production of IFN-β.** Levels of human IFN-β were measured by enzyme-

921   linked immunosorbent assay (ELISA) in the supernatants collected from astrocytes (green), a co-

922   culture of astrocytes and control monocyte-derived macrophages (astrocytes and MDM, red), co-

923   culture of astrocytes and HIV-infected macrophages (astrocytes and HIV-1 infected MDM,

924   blue), HIV-1infected and control MDM purple and orange, respectively). X-axis indicates the

925   time points before and after co-culture (24 hours, 3, 5, and 7 days). Data are expressed as the

926   mean of triplicate technical replicates from one from three independent experiments (each done

927   with distinct donors). Student t-tests were performed to compare Astrocytes with and without

928   HIV-1 infected MDM (red and blue) for each time point (2 and 4 days). Bars = means ± standard

929   error of the mean (SEM); **$p = 0.0029$, ***$P = 0.0004$, and ****$p < 0.0001$.

930

931   Figure 7. **Real-time PCR confirmation of affected human genes in humanized mice brain**

932   **tissues.** Selected human gene expression profiles in the CC and Hip were confirmed by

933   TaqMan® real-time PCR. The HIV-1 infected (n = 6) and control group (n = 5) are illustrated.

934   The fold change in gene expression of human *STAT1, STAT2, IRF9, MX1, ISG15, IFI6, CMPK2,*

935   *MBP,* and *ZNF488* between the HIV-1 infected and the control groups were determined by using

936   the $2^{-\Delta\Delta C_T}$ method (A-I), where $\Delta Ct = Ct_{target} - Ct_{GAPDH}$ and $\Delta(\Delta Ct) = \Delta Ct_{HIV} - \Delta Ct_{control}$.

937   Multiple t-tests using Holm-Sidak method for multiple comparisons correction was applied to

938   examine significant group differences. Bars = means ± standard error of the mean (SEM); * -

939   FDR adjusted $p < 0.00001$.

34

940 **Table 1. Human glial cell distribution pattern on sagittal plane.**

Donor #, cells	Animal ID	Sex	Brain regions/structures	Viral copies/mL in PB[a], IHC P24^{+} in spleen[b]
#1, CD34^{+} and NPC, 10^5 cells/mouse	842	F	OB, CCx, cc, CP, act, STRv, Hip, PV, TH, HTH, MB, Pons, Cb, Md, meninges	1.89×10^4
	844	F		2.05×10^6
	845	F		P24+
	848	M		
	843	F		
	846	F		
	847	F		
	849	M		
	850	M		
#2, CD34^{+} and NPC, 10^5 cells/mouse	853	F	OB, CCx, cc, CP, act, STRv, Hip, PV, TH, HTH, MB, Pons, Cb, Md, meninges	1.50×10^5
	854	F		6.14×10^5
	855	F		P24+
	857	M		P24+
	858	M		3.30×10^5
	851	F		
	852	F		
	856	M		
	859	M		
	860	M		

#3, CD34$^+$ 10^5 cells/mouse, NPC 0.5×10^5 cells/mouse	861	F	cc, Hip, act, PV, MB, Pons	P24+
	862	F	OB, STRv, cc, act, PV, Hip, MB, Cb	6.30 x 10^4
	864	F	cc, Cb, Pons	P24+
	867	M	CP, cc, PV, MB, Md, BS, meninges	P24+
	868	M	CCx, cc, act, CP, STRv, Hip, Pons, Cb, PV	3.05 x 10^4
	869	M	OB, cc, act, CP, STRv, Hip, MB, Pons	1.15 x 10^5
	865	F	OB, cc, CCx, CP, PV, Hip, MB, BS, meninges	
	866	F	OB, cc, act, CP, STRv, PV, MB, Md, Pons	
	870	M	cc, act, PV, Hip	
	871	M	cc, act, PV, MB	
#4, CD34$^+$ 10^5 cells/mouse, NPC 0.5×10^5 cells/mouse	881	F	died	P24+
	883	F	CCx, cc, act, PV, Cb, Md, cpd,	P24+
	884	F	died	P24+
	885	F	PV, cc, MB, cpd, BS	3.90 x 10^6
	886	F	ot, act, cc, CCx, PV, STRv, MB, CP, BS	9.19 x 10^5
	887	F	CCx, PV, act, STRv, MB, TH, Pons, Cb, BS	P24+
	888	M	PV, TH, HTH, cpd, MB, Cb, Md, Pons,	P24+
	893	M	CCx, cc, PV, STRv, lot, MB, meninges	P24+
	895	M	CCx, Hip, cc, MB, cpd, PV, MB, Md, Pons	P24+
	896	M	CCx, cc, cbp, MB, Md, Pons, Cb	P24+
	882	F	CP, TH, HTH, Md	
	889	M	CP, MB, HTH, Md	

890	M	MB
891	M	cc, CCx, CP, PV, HTH, MB, Pons, Md, BS, Cb
892	M	died
894	M	CCx, cc, PV, MB, Pons

941 [a.] PB, peripheral blood

942 [b.] Animals infected with HIV-1$_{ADA}$ and infection confirmed by the measurement of peripheral blood viral load (VL) and/or with

943 positive staining of human cells for HIV-1 p24 protein in spleen sections (IHC P24).

944 Abbreviations: OB, Olfactory bulb; act, Anterior commissure; cc, Corpus callosum; STRv, ventral striatum; CP, caudoputamen; Hip,

945 hippocampus; MB, midbrain, CCx, Cerebral cortex; Cb, Cerebellum; TH, thalamus; HTH, Hypothalamus; Md, Medulla; PV,

946 periventricular; cpd, Cerebral peduncle, BS, brain stem; ot, optic tract; lot, Lateral olfactory tract.

Figure 1

A

NPC - LV
HSPC -ih

Newborn NSG

B

24 week-old

C

	Hu-GFAP	GFAP	DAPI	Merged
CC				
STR				
CB				

D

	hN	Olig2	DAPI	Merged
CC				
Hip				
STR				

E

hu-GFAP+/
total GFAP+ cells, %

FC   CC   STR   CB

F

Olig2+/hN+ cells, %
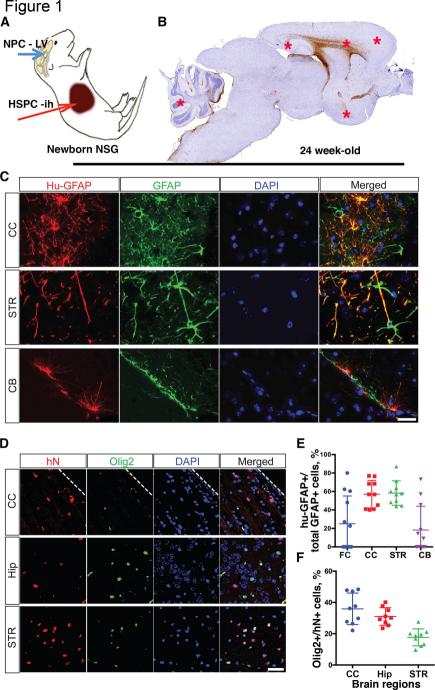
CC   Hip   STR
Brain regions

Figure 2

Periventricular   Corpus Callosum

huGFAP
Iba-1
DAPI

Figure 3

Figure 4

Figure 5

A

CD45 | CD3 | CD4 | CD8 | VL (human cells in PB, %) ... (HIV − / +)

B — CC, Hip

C Differentially expressed genes (DEG)

- human HIV+ (hg19) — red
- humanized mouse (mm10) — cyan
- humanized mouse HIV+ (mm10) — green

Corpus Callosum: 58, 93, 0
Hipocampus: 115, 365, 25

D Human lineage-specific genes

OLIG2, OLIG1, GLUL, GFAP (p=0.033), CD4, CD163
Expression, FPKM
- uninfected (blue)
- HIV+ (red)

E Hip DEG (mm10)

Pnpla3, Tcf7l2, Ramp3, Slc22a6, Aldh1a2, Fmod, Slc6a20a, Slc6a20b, Ptgds, Slc13a4, Lgals3bp, Igf2, Bmp7, Slc6a13, Igfbp2, Col1a2, Car12, Tshz2, Unc13c, Col5a1, Nrep, St18, Cmss1, Xist

F CC DEG (hg19)

MX1, XAF1, CMPK2, IFI44L, IFIT1, IFITM3, IFIT3, RSAD2, IFI6, ISG15, HERC6, USP18, BST2, PLSCR1, HERC5, OAS3, DDX58, IFI44, DTX3L, PARP14, PARP9, STAT1, EIF2AK2, IFIT5, IFIT2, C19orf66, LGALS3BP, IRF9, SHISA5, B2M, PPM1K, LAP3, PNPT1, STAT2, ZNF488, TPPP3, ACBD7, C7orf41, CDK18, STMN2, CLDN11, PLP1, MAG, Sept4, MOBP, MBP

G Hip DEG (hg19)

MX1, ISG15, DONSON, IFIT3, IFIT1, IFITM3, IFI44L, IFI6, HERC6, BST2, PLSCR1, IFI35, OAS3, DDX58, IFI44, HERC5, PARP9, LY6E, DTX3L, IFIT2, STAT1, IFIT5, IRF9, HLA-B, PARP14, HLA-C, RNASE1, LGALS3BP, B2M, EIF2AK2, SHISA5, ADAR, HLA-A, ZNFX1, TRIM14, SAMHD1, PYCR1, PSME1, GABRA3, IFI16, SLC7A5, TRIM62, SPATS2L, UBE2L6, LINGO1, ERAL1, MOV10, NCAPH2, GARS, C1QL1, EPAS1, AGRN, PLLP, ADORA1, SARS, MAG, Sept4, MOBP, MBP

Log2 fold changes, FDR adjusted p<0.05

Figure 6

Figure 7