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1 **Gene transfer of two entry inhibitors protects CD4<sup>+</sup> T cell from HIV-1 infection in**  
2 **humanized mice**

3 - Running title: HIV-1 gene therapy in humanized mice

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6

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12

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17

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21 **Abstract**

22

23 Targeting viral entry is the most likely gene therapy strategy to succeed in protecting the immune  
24 system from pathogenic HIV-1 infection. Here, we evaluated the efficacy of a gene transfer  
25 lentiviral vector expressing a combination of viral entry inhibitors, the C46 peptide (an inhibitor  
26 of viral fusion) and the P2-CCL5 intrakine (a modulator of CCR5 expression), to prevent CD4<sup>+</sup> T  
27 cell depletion *in vivo*. For this, we used two different models of HIV-1-infected mice, one in  
28 which *ex vivo* genetically-modified human T cells were grafted into immunodeficient  
29 NOD.SCID. $\gamma$ c<sup>-/-</sup> mice before infection and one in which genetically-modified T cells were  
30 derived from CD34<sup>+</sup> hematopoietic progenitors grafted few days after birth. Expression of the  
31 transgenes conferred a major selective advantage to genetically-modified CD4<sup>+</sup> T cells, the  
32 frequency of which could increase from 10 to 90% in the blood following HIV-1 infection.  
33 Moreover, these cells resisted HIV-1-induced depletion, contrary to non-modified cells that were  
34 depleted in the same mice. Finally, we report lower normalized viral loads in mice having  
35 received genetically-modified progenitors. Altogether, our study documents that targeting viral  
36 entry *in vivo* is a promising avenue for the future of HIV-1 gene therapy in humans.

## 37 **Introduction**

38

39           Although there is no consensus on a definitive immune correlate of protection, there are  
40 multiple convincing examples linking human genetics and susceptibility to HIV-1 infection. The  
41 best example of a genetic predisposition protecting from HIV-1 remains the  $\Delta 32$  mutation that  
42 prevents CCR5 expression at the cell surface, and thus completely protects 1% of Europeans  
43 from being infected.<sup>1</sup> The so-called 'Berlin patient' was grafted with CCR5-deficient bone  
44 marrow to treat his leukemia and was subsequently cured of both diseases.<sup>3</sup> Simultaneously,  
45 genetic interventions targeting chemokine receptors using DNA -nucleases gave encouraging  
46 results in humanized mice (HuMice)<sup>3-5</sup> but the long term impact of this procedure, as well as the  
47 concerns with off-target cleavages, is still unknown. Clinical trials applying this strategy to  
48 lymphocytes or stem cells have shown that modified cells possessed a selective advantage  
49 compared with non-modified cells,<sup>6</sup> which is one criterion of success for the therapy. Thus, there  
50 is a strong rationale to use gene therapy as an adjunct to current and future treatments.<sup>7</sup>

51           Maraviroc, a CCR5 chemical antagonist, is a powerful medication *in vitro* but resistant  
52 variants rapidly emerge in treated patients for complex reasons, such as mutations in the gp120  
53 coding sequence affecting CCR5 docking.<sup>8</sup> Similarly, the fusion inhibitor Enfuvirtide (a gp41  
54 analog), which is delivered in solution to patients, rapidly becomes ineffective because gp41  
55 mutates to escape Enfuvirtide binding.<sup>9</sup> Thus, the therapeutic arsenal targeting viral entry is  
56 scarce and poorly efficient. However, strategies based on blocking entry are perhaps the most  
57 promising to rapidly restore a pool of functional T cells, the main goal to prevent AIDS.<sup>10</sup> More  
58 recently, it was shown that HIV-1 infection needs not to be productive in CD4<sup>+</sup> T cells to induce

59 cell death by pyroptosis <sup>11</sup>. This mechanism of HIV-1-induced cell death highlights the interest of  
60 strategies aimed at preventing viral entry. We proposed developing a gene transfer vector in  
61 which two viral entry inhibitors in combination would have a better efficacy at preventing viral  
62 entry. In support of this hypothesis, a synergistic effect of Enfuvirtide was demonstrated in cells  
63 with low levels of CCR5 <sup>12</sup>. Importantly, viral variants able to escape gp41 analogs and CCR5  
64 inhibitors at the same time have only been described *in vitro* with a drastic cost on viral fitness, <sup>13</sup>  
65 illustrating the difficulty for the virus to escape both inhibitors at the same time. Using  
66 monocistronic lentiviral vectors, we previously showed a synergistic effect of the P2-CCL5  
67 intrakine with the C46 peptide on HIV-1 infection *in vitro* <sup>14</sup>. The P2-CCL5 intrakine, originally  
68 described as a high affinity CCL5 (RANTES) variant, <sup>15</sup> was later modified to incorporate an ER  
69 retention sequence, sequestering CCR5 away from the cell surface <sup>16</sup>. The C46 peptide is the  
70 optimized membrane-bound form of Enfuvirtide and has been used in several gene therapy  
71 studies since it is effective on both CCR5- or CXCR4-tropic HIV-1, and can be accommodated in  
72 several gene transfer vectors, including lentiviral vectors <sup>17-20</sup>. Here, we aimed to evaluate the *in*  
73 *vivo* efficacy of an optimized lentiviral vector co-expressing those two entry inhibitors. We used  
74 two pre-clinical models of HIV-1 gene therapy, either infusing genetically-modified T cells in  
75 adult immunocompromised NOD.SCID.gc<sup>-/-</sup> (NSG/PBL) mice or grafting genetically-modified  
76 hematopoietic progenitors in NSG neonates (NSG/CD34).

77 **Results**

78

79 *A lentiviral vector expressing two inhibitors of HIV-1 entry*

80

81           With the general aim to validate the combination of the C46 peptide and the P2-CCL5  
82 intrakine for HIV-1 gene therapy *in vivo*, we used an optimized version of our previously  
83 described lentiviral vector, which efficiently inhibited HIV-1 infection *in vitro*.<sup>14</sup> To facilitate  
84 detection of genetically-modified cells, we added the GFP reporter gene after the therapeutic  
85 cassette to generate the LvGFP-C46-P2 vector (Fig. 1a). A vector using the same strong promoter  
86 EF1 $\alpha$  but in which the therapeutic cassette was omitted was used as a control (Fig. 1a). We  
87 transduced anti-CD3/CD28 activated PBMCs to monitor transgene expression and function *in*  
88 *vitro* and *in vivo*. Expression of the GFP reporter molecule was well correlated with the  
89 expression of the C46 peptide (detected with the 2F5 monoclonal antibody) (Fig. 1b) and was  
90 also associated with a lower median fluorescence intensity (MFI) of CCR5 *in vitro* (Fig. 1c).  
91 Passive diffusion of the intrakine was ruled out by the observation that GFP<sup>+</sup> cells exhibited  
92 similar CCR5 MFI than non-transduced cells (Fig. 1c), suggesting that this reduction was due to  
93 ER retention of CCR5 through interaction with the P2-CCL5 intrakine.. The MFI of CCR5 was  
94 also reduced two-fold in genetically-modified PBMCs injected *in vivo* in NSG mice (Fig. 1d),  
95 reflecting the expected down-modulation of CCR5 surface expression. Thus, GFP expression  
96 was a faithful reporter of transgenes expression and function, and was thus used to follow  
97 genetically-modified cells *in vivo*.

98

100 *mice*

101

102 As a model for HIV-1 infection of human CD4<sup>+</sup> T cells *in vivo*, we first used adoptive cell  
103 transfer (ACT) in immunocompromised NSG mice (NSG/PBL). A major problem with ACT of  
104 human T cells in NSG mice is the xenogeneic graft-versus-host disease (GVHD) that develops  
105 thereafter and that invariably leads to death.<sup>21</sup> We tested various ACT protocols in NSG mice and  
106 found that injection of 6.10<sup>6</sup> activated T cells in 1 Gy-irradiated mice represented an optimal  
107 trade-off between survival and engraftment efficiency (protocol P3 in Figure S1). To normalize  
108 the number of genetically-modified cells across experiments and vectors, we diluted transduced  
109 cells into non-transduced cells *ex vivo* prior ACT, establishing a number of GFP<sup>+</sup> cells at 10% of  
110 the injected cells. Mice were infected i.v with a CCR5-tropic HIV-1 strain 12 days after ACT.  
111 The frequencies of CD4<sup>+</sup>GFP<sup>+</sup> cells steadily increased in the blood of LvGFP-C46-P2-treated  
112 animals during the course of the infection to reach a plateau where up to 95% of all CD4<sup>+</sup> T cells  
113 expressed the transgene (Fig. 2a). In contrast, the frequencies of GFP<sup>+</sup> cells in control HIV-1-  
114 infected LvGFP-treated mice remained close to the 10% input throughout the experiment (Fig.  
115 2a). The increase in GFP<sup>+</sup> cells with the LvGFP-C46-P2 vector was dependent on HIV-1  
116 infection because it was not observed in non-infected NSG/PBL mice (Fig. S2), showing that the  
117 therapeutic vector did not increase the proliferation of modified cells *per se*. The frequencies of  
118 GFP<sup>+</sup> cells were also superior in the spleen and in the bone marrow of LvGFP-C46-P2-treated  
119 mice compared with LvGFP-treated control mice (Fig. 2b). These increased frequencies  
120 translated into increased numbers of CD4<sup>+</sup>GFP<sup>+</sup> cell in the spleen and the bone marrow of

121 LvGFP-C46-P2-treated mice compared with LvGFP mice (Fig. S3). Altogether, the results  
122 demonstrate that LvGFP-C46-P2-transduced CD4<sup>+</sup> T cells possess a selective advantage relative  
123 to LvGFP-modified T cells.

124 To test the hypothesis that genetically-modified cells resisted HIV-1-induced depletion,  
125 we analyzed longitudinally the frequencies of CD4<sup>+</sup> cells in CD3<sup>+</sup>GFP<sup>+</sup> and CD3<sup>+</sup>GFP<sup>-</sup> T cells in  
126 the blood of LvGFP-C46-P2- and LvGFP-treated mice (Fig. 2c). The frequencies of CD4<sup>+</sup> T cells  
127 in the GFP<sup>-</sup> subset rapidly dropped after HIV-1 infection, showing that non-protected CD4<sup>+</sup> T  
128 cells underwent HIV-1-induced depletion as expected (Fig. 2d). In striking contrast, the  
129 frequency of CD4<sup>+</sup> T cells in the GFP<sup>+</sup> fraction remained constant throughout the experiment,  
130 showing that these cells were protected from HIV-1-induced depletion. Resistance to depletion  
131 was also observed in the spleen and in the bone marrow of LvGFP-C46-P2-treated animals, with  
132 statistically significant differences in the frequencies of CD4<sup>+</sup> T cells in GFP<sup>+</sup> vs GFP<sup>-</sup> T cells  
133 (Fig. 2e). In contrast, the frequencies of GFP<sup>+</sup> cells, like the GFP<sup>-</sup> subset, steadily decreased in  
134 the blood of control LvGFP-treated mice (Fig. S4a), showing that GFP expression *per se* did not  
135 protect from HIV-1-induced deletion. A similar depletion of GFP<sup>+</sup> cells were found in the spleen  
136 and in the bone marrow of control LvGFP-treated mice (Fig. S4b). Thus, CD4<sup>+</sup> T cells expressing  
137 the combination of viral entry inhibitors were protected from HIV-1-induced depletion in  
138 NSG/PBL mice in the blood and in lymphoid tissues.

139

140 *Resistance of genetically-modified human CD4<sup>+</sup> T cells to HIV-1-induced depletion in*

141 *NSG/CD34 HuMice*

142



143 We next wanted to confirm the potency of the vector to prevent HIV-1-induced CD4<sup>+</sup> T  
144 cell depletion in a more physiological setting. For this, we grafted LvGFP-C46-P2-transduced  
145 CD34-purified cells from cord blood into neonatal NSG mice and monitored human cell  
146 reconstitution and transgene expression overtime. At 17 weeks post-injection,  $11.9 \pm 11.0$  % of  
147 total cells from the blood (excluding erythrocytes) were human CD45<sup>+</sup>CD3<sup>+</sup> T cells in the  
148 animals used for the experiment. The frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> T cells among CD3<sup>+</sup> cells  
149 were less variable representing  $40.0 \pm 5.8$  % and  $47.0 \pm 5.3$  % , respectively (Fig. S5). Among 14  
150 NSG/CD34 HuMice generated with LvGFP-C46-P2-modified CD34<sup>+</sup> cells, only 8 had detectable  
151 GFP<sup>+</sup> cells in CD4<sup>+</sup> T cells 17 weeks after. Four of those mice were infected with a CCR5-tropic  
152 HIV-1 strain, whereas 4 were left uninfected. Because the frequency of GFP<sup>+</sup> cells was highly  
153 variable among NSG/CD34 HuMice, it was not possible to reliably measure a selective  
154 advantage in that setting. To directly assess resistance of genetically-modified CD4<sup>+</sup> T cells to  
155 HIV-1-induced depletion, frequencies of CD4<sup>+</sup> T cells were measured in GFP<sup>+</sup> and GFP<sup>-</sup> cells  
156 (Fig. 3). In non-infected mice, the frequencies of CD4<sup>+</sup> T cells in the blood remained similar in  
157 GFP<sup>+</sup> vs GFP<sup>-</sup> T cells throughout the course of the experiment (Fig. 3a). As expected, frequencies  
158 of GFP<sup>-</sup> cells steadily decreased in HIV-1-infected animals whereas frequencies of GFP<sup>+</sup>  
159 remained stable, showing that CD4<sup>+</sup>GFP<sup>+</sup> T cells resisted HIV-1-induced depletion in the blood  
160 of NSG/CD34 HuMice (Fig. 3b). As expected in non-infected mice, the frequencies of CD4<sup>+</sup> T  
161 cells in lymphoid organs were similar in GFP<sup>+</sup> or GFP<sup>-</sup> subsets (Fig. 3c). In contrast, frequencies  
162 of CD4<sup>+</sup> T cells among GFP<sup>+</sup> and GFP<sup>-</sup> cells significantly differed in the LN, spleen and bone  
163 marrow (Fig. 3d). Of note is the one mouse in which resistance to deletion was not evident in the  
164 blood did not show any sign of resistance in the lymphoid organs. Thus, gene transfer of two

165 entry inhibitors in CD34<sup>+</sup> cells conferred resistance to CD4<sup>+</sup> T cells in 3 mice out of 4 analyzed.

166

167

168 *Gene transfer of entry inhibitors impact viral replication in NSG/CD34 HuMice.*

169

170 To assess the impact that the therapy might have on viral loads, we measured viremia in  
171 LvGFP-C46-P2-treated mice in which GFP<sup>+</sup> cells were observed (n=4) or not (n=6) prior HIV-1  
172 infection. To accommodate the various levels of human cells engraftment among the different  
173 mice (Fig. S5), viremia was corrected by the frequency of CD45<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup> T cells among total  
174 cells of the blood at the time of the analysis. Initially, normalized viremia was similar in both  
175 groups, showing that the therapy was not associated with an immediate effect on viral replication.  
176 However, we observed a tendency for lower normalized viral loads in mice bearing GFP<sup>+</sup> cells  
177 compared to mice in which no GFP<sup>+</sup> cells could be detected (Fig. 4a). To confirm that animals  
178 with GFP<sup>+</sup> cells carried less virus, we analyzed p24 expression in CD4<sup>+</sup> T cells at the end of the  
179 experiment. We found that the frequencies of CD4<sup>+</sup> cells expressing p24 in mice with GFP<sup>+</sup> cells  
180 were lower than in mice without GFP<sup>+</sup> cells and close to background staining obtained in non-  
181 infected HuMice (Fig. 4b). Altogether, we conclude that NSG/CD34 HuMice reconstituted with  
182 gene-modified CD34<sup>+</sup> progenitors were protected from HIV-1-induced CD4<sup>+</sup> T cell deletion and  
183 had a lower number of infected cells, corroborating with lower viral loads.

184 **Discussion**

185

186 Here, we show that a lentiviral vector encoding two viral entry inhibitors confers a  
187 selective advantage to genetically-modified cells *in vivo*, due to their resistance to HIV-1-  
188 mediated depletion. We observed a strong and long-lasting selective advantage in the NSG/PBL  
189 model. A lower selective advantage was reported in a very similar model of NSG/PBL HuMice  
190 using a vector expressing only the C46 peptide.<sup>18</sup> This observation suggest that two entry  
191 inhibitors might be better than one at protecting cells from HIV-1. However, a pre-clinical study  
192 in macaques reconstituted with progenitors expressing the C46 peptide alone showed lower viral  
193 loads correlated to a clear selective advantage<sup>22</sup>. Moreover, recent studies showed that inhibition  
194 of CCR5 expression by shRNA was sufficient to protect CD4<sup>+</sup> T cells from infection and to  
195 confer a selective advantage in chimeric Bone marrow-Liver-Thymus (BLT) HuMice<sup>23,24</sup>. Thus,  
196 targeting gp41 and CCR5 have independently the potential to curb HIV-1 infection, highlighting  
197 the interest of using two inhibitors of this crucial step of HIV-1 infection in the same vector.

198 A strong selective advantage is not always associated with lower viral loads. In CD34-  
199 reconstituted HuMice, Walker et al. reported that expression of a triple combination of anti-HIV-  
200 1 genes did not impact viral replication, although a significant selective advantage was observed.  
201<sup>25</sup> A modest but significant effect on viral loads was reported following CCR5-specific ZFN-  
202 mediated modification in NSG/PBL HuMice.<sup>5</sup> However, only one time point was analyzed in  
203 that study. A kinetics study showed that the reduction in viral loads using the same technology  
204 was much more discrete in NSG/CD34 HuMice despite a considerable selective advantage.<sup>3</sup> Our  
205 PCR and p24 data concur to the hypothesis that selective advantage conferred by our vector had

206 an impact on viral replication. Recently, a complete protection from HIV-1 was observed in BLT  
207 mice reconstituted with human cells modified with a vector very similar to our, encoding the C46  
208 peptide and a shRNA targeting CCR5<sup>19</sup>. This is the first report showing that viral replication can  
209 be totally controlled in humanized mice by gene therapy without prior sorting of genetically-  
210 modified cells, as recently shown for a CCR5 shRNA<sup>26</sup>. This surprising and unique result  
211 suggest that maximal efficacy of HIV-1 gene therapy might necessitate a functional immune  
212 response, that is present in monkeys and BLT HuMice but lacking or severely hampered in other  
213 HuMice models. One must keep in mind though that some HIV-1-specific PCR might amplify  
214 the vector as well<sup>27</sup>. The use of HIV-1-specific PCR discriminating HIV-1 from the vector such  
215 as the one employed in our study should become the gold standard.

216         Considering the recent developments of nucleases that target CCR5 in CD34<sup>+</sup> progenitor  
217 cells , we believe that residual expression of the molecule such as the one observed with our  
218 intrakine, might allow for normal hematopoeisis and circulation of modified-cells while total  
219 ablation by genetic means may impact on these processes. Recent advances in lentiviral delivery  
220 of ZN finger nucleases might improve specific targeting of the nuclease to mature CD4<sup>+</sup> T cells, a  
221 protocol that would limit bystander effects<sup>28</sup>.

222         The selective advantage of genetically-modified cells would only be obtained in the  
223 context of high levels of viral replication. Although ART interruptions have been performed in  
224 the past to provoke selective growth of modified cells in small-scale clinical trials for gene  
225 therapy,<sup>29,30</sup> an interruption in therapy is not foreseeable in patients in the long term. Gene  
226 therapy might thus be particularly suitable for patients experimenting treatment failure with high  
227 viral loads.

228 **Materials and Methods**

229

230 *Lentiviral vector design and production*

231 Second-generation self-inactivating (SIN) lentiviral vectors were used in this study.<sup>31</sup>

232 The LvGFP-C46-P2 vector was constructed by adding an eGFP gene and 2A sequence upstream

233 of the therapeutic cassette (construction encoding the C46 peptide and P2-CCL5 analog

234 described in Petit et al., 2014)<sup>14</sup> in the backbone of a lentiviral vector carrying the EF1a

235 promoter. As a control, the LvGFP vector expressing GFP only was used. Details on the cloning

236 procedures are available on request. Lentiviral vectors were produced in mycoplasma-free HEK-

237 293T cells, as described previously.<sup>32</sup> Briefly, 23.3 µg of the Δ8.9 packaging plasmid, 30 µg of

238 the vector plasmid, and 10 µg of the vesicular stomatitis virus (VSV)-G envelope were

239 transfected into 15.10<sup>6</sup> cells in T-175 flasks by calcium phosphate precipitation. Vector

240 supernatants were collected 48 hours post-transfection and concentrated by ultrafiltration

241 (Centricon Plus-70; Millipore, Molsheim, France) at 3500 g at 4°C. Viral stocks were kept frozen

242 at -80° C. Viral titers were determined on HEK-293T cells with various concentrations of vector

243 supernatants in the presence of Polybrene (8 µg/mL; Sigma-Aldrich, Saint-Quentin-Fallavier,

244 France). Seventy-two hours after transduction, the percentage of cells expressing the transgenes

245 was determined by flow cytometry and used to calculate a viral titer as the number of infectious

246 particles per milliliter.

247

248 *Mice and humanization*

249 NOD Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup> (NSG) mice (strain #05557; Jackson Laboratory, USA) were  
250 bred in animal facilities of Centre d'Expérimentation Fonctionnelle (CEF) according to the  
251 Jackson Laboratory handling practice specific to that strain. The regional ethical committee on  
252 animal experimentation Darwin approved all mouse protocols. Primary human cells were  
253 obtained from leukapheresis samples collected from healthy donors at the Etablissement Francais  
254 du Sang after informed consent. Cells were grown at a concentration of  $1.10^6$  cells/mL and  
255 activated in RPMI, 10% FCS, penicillin/streptomycin, interleukin-2 (Proleukin, 600 IU/mL;  
256 Novartis, Basel, Switzerland), and CD3/CD28 beads (Invitrogen, Carlsbad, CA) at 3 beads per  
257 cell. Two days after activation, cells were transduced by spinoculation for 2 hrs at 1000 g at  
258 30°C, with the indicated lentiviral vectors at a multiplicity of infection (MOI) of 6 to 8 in the  
259 presence of protamine sulfate (2 µg/mL, Sigma). Three days after transduction, 1 Gy-irradiated  
260 female 8 to 12-weeks old NSG mice were injected with  $6.10^6$  cells. Twelve days post-adoptive  
261 cell transfer (ACT), mice were infected with 25 ng of p24 of NLAD8 HIV-1 strain in a final  
262 volume of 100 µL of 1X PBS. All mice used in this study were randomly assigned to  
263 experimental group and cages. Investigator was not blinded to the group allocation during the  
264 experiments.

265 Human hematopoietic progenitor cells were obtained from cord blood samples collected  
266 from healthy donors after informed consent. Mononuclear cells from human cord blood were  
267 isolated by Ficoll density gradient and centrifuged at 200 g during 13 min to remove platelets.  
268 Then, CD34<sup>+</sup> progenitors were sorted with the human CD34 MicroBeads kit, according to the  
269 manufacturer's instructions (Miltenyi). CD34<sup>+</sup> cells were incubated at a concentration of  $1.10^6$   
270 cells/mL over night into StemSpan SFEMII medium (StemCell technologies) complemented with

271 human recombinant cytokines (IL-6 and TPO at 20 ng/mL; SCF and FLT3-L at 100 ng/mL;  
272 Peprotech) and antibiotics. Cells were transduced with the LvGFP-C46-P2 lentiviral vector in  
273 StemSpan medium in the presence of cytokines, the proteasome inhibitor MG-132 (1  $\mu$ M;  
274 Sigma), antibiotics and protamine sulfate (8  $\mu$ g/mL; Sigma). CD34<sup>+</sup> cells underwent two rounds  
275 of transduction separated by 3 hours incubation at 37°C and 5% CO<sub>2</sub>. For each transduction  
276 cycle, cells were centrifuged at 1000 g at 30°C for 2 hours with the lentiviral vector at a MOI of  
277 15. Twenty-four to 48-hour-old NSG mice were irradiated at 0.9 Gy and grafted with 0.5.10<sup>5</sup> to  
278 2.5.10<sup>5</sup> transduced CD34<sup>+</sup> cells by the intra-hepatic route. Ten ng of the p24 NL-AD8 HIV-1  
279 strain were injected into the retro-orbital sinus of 17 weeks-old mice in a final volume of 100  $\mu$ L  
280 of 1X PBS.

281

#### 282 *HIV-1 production and quantification*

283 HIV-1 molecular clone NL-AD8 was obtained through the AIDS Research and Reference  
284 Reagent Program. HIV-1 stocks were prepared with 30  $\mu$ g of plasmid transfected into 15.10<sup>6</sup>  
285 mycoplasma-free HEK 293T cells in T-175 flasks by calcium phosphate precipitation. The  
286 supernatant was frozen at -80°C and viral titers were quantified by p24 ELISA according to the  
287 manufacturer's instructions (Zeptometrix, Buffalo, NY). Mice were bled on ACD (acid-Citrate-  
288 Dextrose) anticoagulant and plasma HIV-1 RNA viral loads were measured using the Abbott  
289 RealTime HIV-1 RT-PCR assay that do not amplify genomic regions present in lentiviral vectors  
290 contrary to the Roche Cobas PCR (our unpublished observations and De Ravin et al <sup>27</sup>). Due to  
291 the small volumes of plasma from the mice, a dilution was necessary to reach the volume needed  
292 for the assay. Thus, this detection limit varied between 200 and 2000 copies/mL depending on the

293 initial volume of mouse plasma.

294

295 *Flow cytometry*

296 Red blood cells from whole blood were lysed with 4.5 ml of water for 15 s before adding  
297 0.5 ml of 10X PBS. Red blood cells from spleen or bone marrow were lysed with ACK buffer  
298 ( $\text{NH}_4\text{Cl}$  0.15 M,  $\text{KHCO}_3$  10 mM, EDTA 0.1 mM). Cell suspensions were stained with an optimal  
299 quantity of antibodies at a concentration of  $10^7$  cells/mL in a final volume of 100  $\mu\text{L}$  of PBS/FCS  
300 3%. Incubation was performed in the dark at  $6^\circ\text{C}$  for 20 min. The following anti-human mAbs  
301 were used for cell surface staining: CD45 PE-CF594 (clone HI30; catalog number (cat  $\neq$ )  
302 562279, BD Biosciences) anti-CCR5 Alexa Fluor 647 (HEK/1/85a; cat  $\neq$  313712, Biolegend),  
303 anti-CD4 PerCP (RPA-T4, cat  $\neq$  300528, Biolegend), anti-CD8 Alexa Fluor 700 (HIT8a, cat  $\neq$   
304 300920, Biolegend), CD3 PE-Cy7 (UCHT1, cat  $\neq$  300420, Biolegend). The human IgG1 mAb  
305 2F5 specific for a gp41 epitope (cat  $\neq$  AB001, Polymun, Austria) was used to detect the C46  
306 peptide. The KC57-RD1 (cat  $\neq$  6604667, Beckman Coulter) antibody was used to detect  
307 intracellular p24 after cells were treated with permeabilization buffer (eBioscience, fixation and  
308 permeabilization kit). All cell preparations were acquired on an LSR II cytometer (BD) and  
309 analyzed with FlowJo software (Tree Star, Portland, OR). The frequencies of positive cells were  
310 determined according to the fluorescence minus one (FMO) staining negative control.

311

312 *Statistical analysis*

313 No statistical method was used to assess sample size needed to detect an effect. Except for



314 the NSG/CD34 model, which is a single experiment, all the results shown in this study are  
315 compiled from 2 independent experiments. Two-tailed p values indicated on the graphs were  
316 calculated with Prism version 6.0 software (GraphPad Software, San Diego, CA), using the  
317 unpaired Mann-Whitney test with a confidence interval of 95%. The median value are indicated  
318 by horizontal bars on the graphs. Linear and non linear regression analysis were performed using  
319 Prism 6.0 to determine whether slopes significantly differed. Plateau with one phase decay  
320 association or dissociation equations were used to model the data.

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322

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331 Levacher constructed the vectors; Corinne Amiel and Véronique Schneider performed HIV-1  
332 qPCR; Karim Dorgham, Francois Lemoine, Guy Gorochov conceived experiments, contributed  
333 to essential reagents and corrected the manuscript; Nicolas Petit and Gilles Marodon conceived  
334 experiments, analyzed the data and wrote the manuscript.

335

336 **Conflict of Interest**

337 The authors declare no conflict of interest

338

339 Supplementary information is available at Gene Therapy's website

340

341 Figure S1. Survival and graft efficiency after ACT of activated and transduced T lymphocytes in  
342 NSG/PBL HuMice.

343 Figure S2. Selective advantage for LvGFP-C46-P2-modified CD4<sup>+</sup> cells is dependent on HIV-1  
344 infection.

345 Figure S3. Resistance to HIV-1-induced deletion in LvGFP-C46-P2-injected NSG/PBL mice.

346 Figure S4. CD4<sup>+</sup> T cell deletion in LvGFP-control NSG/PBL mice.

347 Figure S5. Human cell reconstitution in 17 weeks-old in NSG/CD34 humanized mice.

348

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352

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443

444 **Figure Legends**

445 **Figure 1. Lentiviral vector design and co-expression of anti-HIV-1 genes and eGFP into a**  
446 **lentiviral vector. (a)** A schematic representation of the structure of the lentiviral vectors used in  
447 the present study is shown. (LTR: long terminal repeat; cypT: central polypurine tract of HIV-1;  
448 EF1 $\alpha$ : Elongation factor 1 promoter; C46: membrane-bound form of T20 (C46 peptide); 2A: 2A  
449 sequence of the foot-and-mouth disease virus; P2i: P2-CCL5 intrakine; WPRE: Woodchuck  
450 Hepatitis virus regulatory element;  $\Delta$ LTR: U3 deleted LTR). Indicated is the reference of the  
451 vector used throughout the study. Not to scale. **(b)** Co-expression of the C46 peptide (detected  
452 with the 2F5 mAb) and of eGFP and **(c)** co-expression of CCR5 and eGFP in human CD4<sup>+</sup>  
453 PBMC activated by CD3/CD28 beads and IL-2 21 to 29 days post transduction with the LvGFP-  
454 C46-P2 vector (NT: Non-transduced; FMO: fluorescence minus one; MFI: median fluorescence  
455 intensity) **(d)** *In vivo* CCR5 expression on CD45<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup> T cells in GFP<sup>+</sup> and GFP<sup>-</sup> cells from  
456 non-irradiated NSG mice grafted with 2.10<sup>6</sup> LvGFP-C46-P2 transduced T lymphocytes and  
457 analyzed in the blood and the spleen 34 to 53 days post-graft.

458

459 **Figure 2. Protection of genetically-modified human CD4<sup>+</sup> T cells from HIV-1 infection in**  
460 **NSG/PBL mice. (a-b)** Frequencies of GFP<sup>+</sup> cells in human CD45<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup> T cells in the  
461 blood at various days after HIV-1 infection **(a)** and in the spleen or bone marrow (BM) **(b)** 35 to  
462 45 days after injection of LvGFP- or LvGFP-C46-P2-modified T cells in NSG mice. **(c)**  
463 Representative histograms and dot plots showing the gating strategy to determine the frequencies  
464 of CD4<sup>+</sup> T cells in GFP<sup>+</sup> and GFP<sup>-</sup> human CD3<sup>+</sup> T cells. **(d-e)** Frequencies of CD4<sup>+</sup> cells in the  
465 CD3<sup>+</sup>GFP<sup>+</sup> and CD3<sup>+</sup>GFP<sup>-</sup> populations were determined in LvGFP-C46-P2-injected mice in the  
466 blood at various days after infection **(d)** and in the spleen or bone marrow (BM) **(e)** at the end of  
467 the experiment. The results are compiled from 2 independent experiments using the P3 ACT  
468 protocol (Fig. S1). Non linear regression analysis curve fit are shown. The p value indicate the  
469 significant difference between the two slopes.

470

471 **Figure 3. Resistance of genetically-modified CD4<sup>+</sup> T cells to HIV-1-induced depletion *in vivo***  
472 **in NSG/CD34 HuMice. (a)** Blood frequencies of CD4<sup>+</sup> cells in CD3<sup>+</sup>GFP<sup>+</sup> or CD3<sup>+</sup>GFP<sup>-</sup>

473 populations were determined in non-infected (HIV<sup>-</sup>) or **(b)** infected (HIV<sup>+</sup>) NSG HuMice at  
474 various time points after infection. Linear regression curve fit and p values are depicted on the  
475 graphs. n.s = not significant (p>0.05) **(c)** Frequencies of CD4<sup>+</sup> cells into CD3<sup>+</sup>GFP<sup>+</sup> or  
476 CD3<sup>+</sup>GFP<sup>-</sup> populations in HIV<sup>-</sup> or **(d)** HIV<sup>+</sup> mice in the spleen, the lymph nodes (LN) and the  
477 bone marrow (BM) 11 weeks post-infection.

478

479 **Figure 4. Gene transfer of entry inhibitors impacts viral replication in NSG/CD34 HuMice**

480 **(a)** Viral load was measured by qPCR after HIV-1 infection in LvGFP-C46-P2-treated mice with  
481 undetectable (-GFP) or detectable GFP<sup>+</sup> cells (+GFP) in CD4<sup>+</sup> T cells prior to infection. Shown is  
482 the viral load value normalized by the frequency of human CD45<sup>+</sup>CD3<sup>+</sup>CD4<sup>+</sup> T cells present in  
483 total cells of the blood sample for each time point. **(b)** Frequencies of p24<sup>+</sup> cells in CD4<sup>+</sup> T cells  
484 from the lymph node of NSG HuMice with (+GFP) or without GFP<sup>+</sup> cells (-GFP) 77 days after  
485 infection with NL-AD8 HIV-1 (HIV+) or non infected (HIV-). A representative CD4 vs p24  
486 staining is shown above each group. One mouse from the (+GFP) group was excluded from the  
487 graph since it did not show any protection against HIV-1-induced depletion in the periphery.



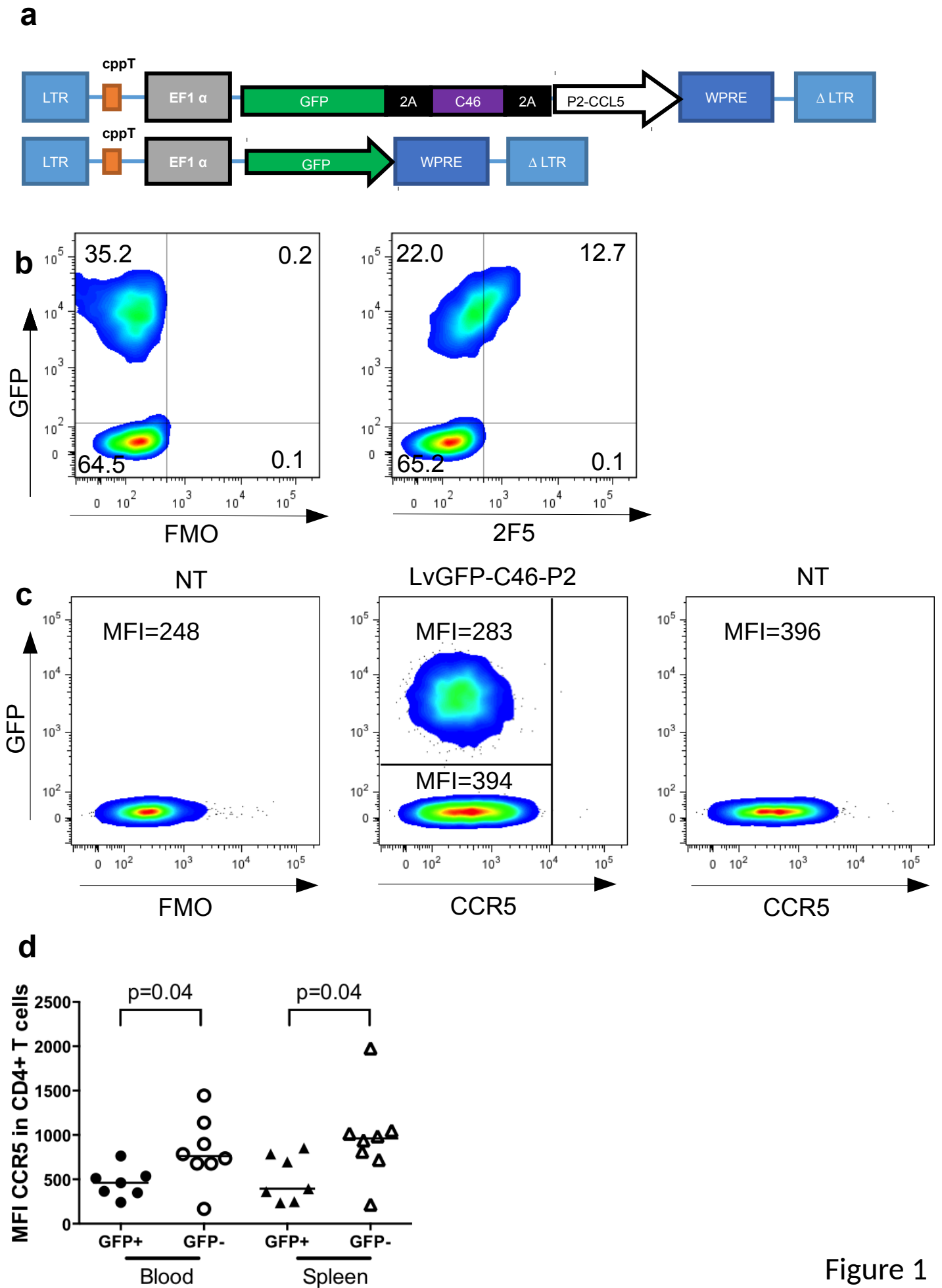


Figure 1

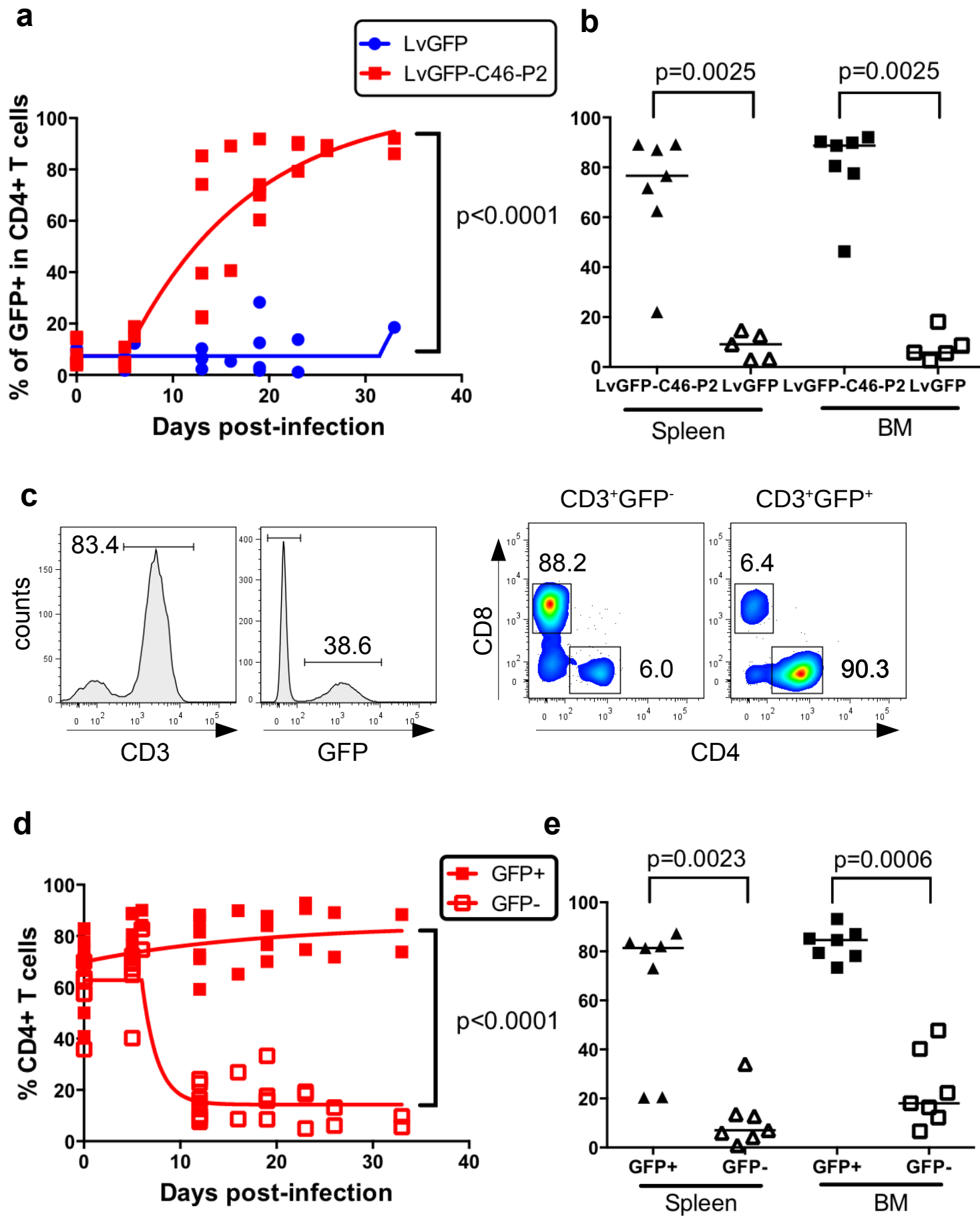


Figure 2

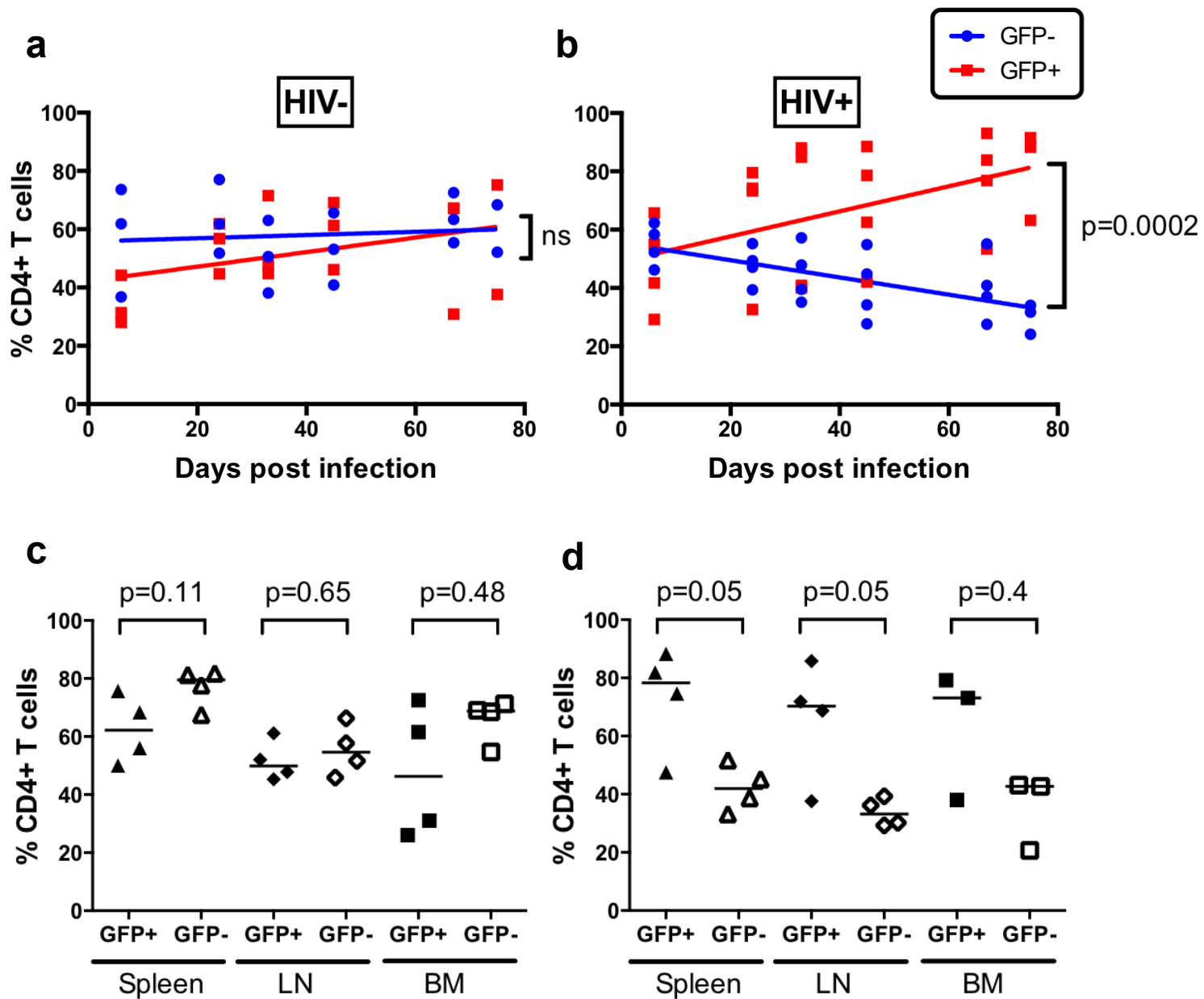


Figure 3

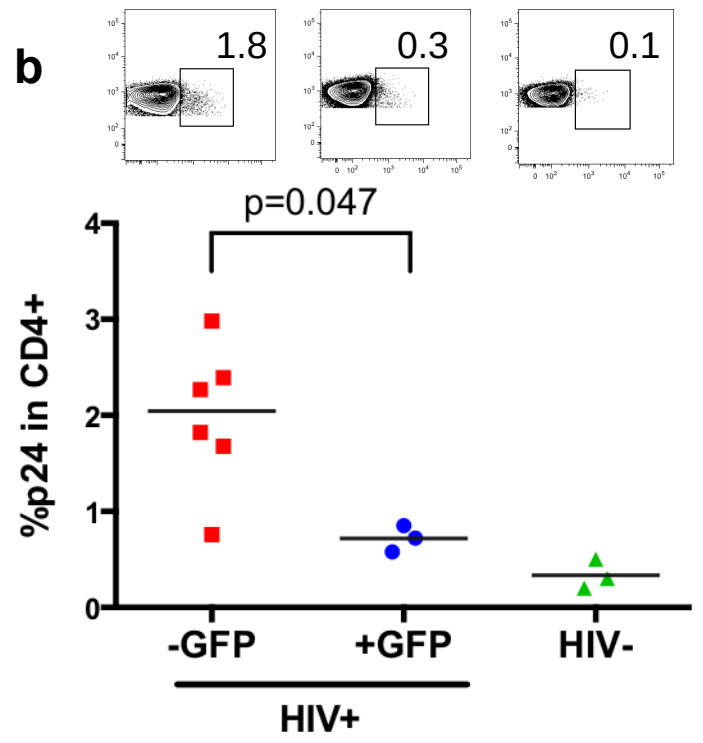
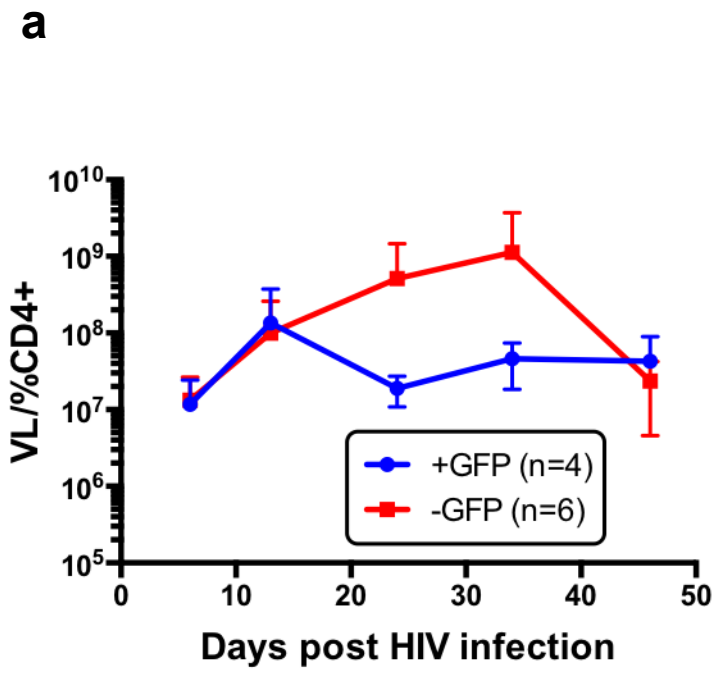


Figure 4