1 **Anti-HIV-1 HSPC-based gene therapy with safety kill switch to defend against and attack**

2 **HIV-1 infection**

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22 **Anti-HIV-1 HSPC gene therapy with safety switch**

23

Abstract

24

25   Hematopoietic stem/progenitor cell (HSPC)-based anti-HIV-1 gene therapy holds

26   promise to provide life-long remission following a single treatment. Here we report a multi-

27   pronged anti-HIV-1 HSPC-based gene therapy designed to defend against and attack HIV-1

28   infection. We developed a lentiviral vector capable of co-expressing three anti-HIV-1 genes.

29   Two are designed to prevent infection, including a short-hairpin RNA (CCR5sh1005) to knock

30   down HIV-1 co-receptor CCR5 and a membrane anchored HIV-1 fusion inhibitor (C46). The

31   third gene is a CD4-based chimeric antigen receptor (CAR) designed to attack HIV-1 infected

32   cells. Our vector also includes a non-signaling truncated human epidermal growth factor receptor

33   (huEGFRt) which acts as a negative selection-based safety kill switch against transduced cells.

34   Anti-HIV-1 vector-transduced human CD34+ HSPC efficiently reconstituted multi-lineage

35   human hematopoietic cells in humanized bone marrow/liver/thymus (huBLT) mice. HIV-1 viral

36   load was significantly reduced (1-log fold reduction, p <0.001) in transplanted huBLT mice.

37   Anti-huEGFR monoclonal antibody Cetuximab (CTX) administration significantly reduced

38   huEGFRt+ vector-modified cells (>4-fold reduction, p <0.01) in huBLT mice. These results

39   demonstrate that our strategy is highly effective for HIV-1 inhibition, and that CTX-mediated

40   negative selection can deplete anti-HIV-1 vector-modified cells in the event of unwanted adverse

41   effects in huBLT mice.

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Introduction

Forty years after its discovery, HIV-1 infection remains a significant public health issue with a total of over 39 million cases and more than 1.3 million new cases globally in 2023 alone.[1] Although antiretroviral therapy (ART) has significantly improved life expectancy and health of those living with HIV, it cannot cure HIV-1 infection.[2–6] Life-long treatment is necessary due to the persistence of HIV-1 infection by producing latent HIV-1 viral reservoirs.[4–7] Other shortcomings of ART include patient adherence, administrative availability, drug cost, and adverse side effects. Moreover, ART reduces but does not prevent all of the known complications of HIV-1 infection.[8–12] A novel therapeutic approach for life-long remission without ART or elimination is critical to address these issues.[5,13]

Thus far, HIV-1 cure has only been achieved in a few patients who have undergone hematopoietic stem cell transplantation (HSCT) from HLA type fully or partially matched allogeneic CCR5Δ32/Δ32 homozygous donors.[14–19] In these few patients, allogeneic transplantation with CCR5Δ32/Δ32 HSPC to treat underlying leukemia has also led to long-term HIV-1 remission without the need for ART after successful repopulation of immune cells lacking the HIV-1 co-receptor CCR5. These handful HIV-1 cure cases offer great hope for the development of an HSPC-based anti-HIV-1 gene therapy that provides long-term remission or cure for HIV-1 infection. However, CCR5Δ32/Δ32 homozygous mutation is found in less than 1% of the global population.[20,21] Furthermore, allogeneic stem cell transplantation also requires HLA-matching,[16–18] making it extremely difficult to identify a HLA type matched donor with CCR5Δ32/Δ32 homozygous mutation. Despite this, HSPC-based gene therapy to genetically modify autologous cells with anti-HIV-1 genes holds great promise to provide life-long

69 remission or cure following a single treatment.[22–24] Unlike HSCT, autologous HSPC-based gene

70 therapy uses a patient's own cells and hence does not require HLA-matching.[23,25]

71 　　　　Anti-HIV-1 HSPC-based gene therapy may require a multi-target approach to effectively

72 inhibit HIV-1, similar to the combinatorial drug treatment strategy used in ART.[26,27] We

73 previously identified and proved the potent antiviral activity of a non-toxic short hairpin RNA

74 against CCR5 (CCR5sh1005) to down regulate CCR5 expression by RNA interference to protect

75 cells from HIV-1 entry.[28,29] Although our CCR5sh1005 was efficient for down regulating CCR5

76 in human CD4+ T cells and HIV-1 inhibition through HSPC gene-modification in humanized

77 BLT mice and rhesus macaques, it does not fully ablate CCR5 expression.[30–33] We therefore

78 added a membrane anchored anti-HIV-1 fusion inhibitor C46, which targets gp41 on HIV-1

79 virions to prevent fusion into host cells, a critical viral entry step.[30,31,33] These anti-HIV-1 genes

80 work synergistically to protect cells by inhibiting HIV-1 binding and fusion before viral

81 integration to prevent the establishment of chronic HIV-1 infection. We previously demonstrated

82 that dual anti-HIV-1 combinations (CCR5sh1005 and C46) improved HIV-1 inhibition compared

83 to CCR5sh1005 alone and inhibited both R5-tropic and X4-tropic HIV-1.[33]

84 　　　　In addition to defending HSPC against HIV-1 infection by use of the aforementioned

85 anti-HIV-1 genes, we reasoned that the potential for chronic remission of HIV-1 infection could

86 be increased by simultaneously engineering a host immunological attack on HIV-1 infected cells.

87 We incorporated a CD4-based anti-HIV-1 CAR that has demonstrated robust HIV-1 viral load

88 reduction when expressed in HSPC, producing anti-HIV-1 CAR T cells to attack and eliminate

89 HIV-1 infected cells.[34] CD4-based anti-HIV-1 CARs are designed to bind a HIV-1 GP120

90 envelope glycoprotein on cell surface with the extracellular CD4 D1D2 HIV-1-binding domain

91 and transmit signals through the intracellular CD3-ζ signaling domain to kill HIV-1 infected cells

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92   by T cell mediated cytotoxicities.[35,36] D1D2CAR 4-1BB is a truncated version of the previously

93   used CD4CAR and includes a 4-1BB costimulatory domain shown to enhance CAR-T cell

94   function and proliferation compared to other anti-HIV-1 CAR-T cell variants *in vivo*.[37]

95   D1D2CAR 4-1BB also does not mediate HIV-1 infection and when coupled with anti HIV-genes

96   such as CCR5sh1005 provide gene-modified cells with extra protection.[37] CD4-based CARs has

97   been co-expressed in dual combination lentiviral vectors with C46 or CCR5sh1005.[34,37] We

98   hypothesize that triple-expression of CCR5sh1005, C46, and D1D2CAR 4-1BB in HSPC will

99   durably protect infection-susceptible progeny cells and target HIV-1 infected cells, thereby

100   inhibiting 3 different steps in HIV-1 infection.

101   Despite a superior safety profile in our humanized mice and NHP studies,[34,37,38]

102   incorporating a negatively-selectable safety kill switch into our gene therapy could prove

103   important for our approach. Anti-HIV-1 CAR T vector-modified cells may potentially cause

104   unexpected health issues such as cytokine release syndrome and CAR-T cell related

105   encephalopathy syndrome in hosts, as seen in cancer immunotherapy.[39–41] To improve safety of

106   our HSPC-based anti-HIV-1 gene therapy, we incorporated a safety kill-switch by co-expressing

107   the non-functional truncated form of human epidermal growth receptor (huEGFRt), which can be

108   targeted with the clinically available chimeric immunoglobulin G1 anti-EGFR monoclonal

109   antibody (mAb), Cetuximab (CTX) (Erbitux™).[42,43] Administration of CTX can negatively

110   select huEGFRt expressing vector-modified cells *in vivo* through antibody-dependent cellular

111   cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC). To prevent off-target

112   activation of CAR T-cells while retaining a marker function for the tracking of vector-modified

113   cells via mAb staining and flow cytometry analysis, huEGFRt lacks the extracellular ligand

114   binding domains I and II and the entire cytoplasmic tail necessary for signaling in EGFR.[42]

115   In this report, we developed a multi-pronged anti-HIV-1 lentiviral vector with a safety

116   kill switch for efficient HSPC transduction and expression of factors capable of protecting cells

117   against and attacking HIV-1 infection. We investigated the ability of our newly developed anti-

118   HIV-1 gene lentiviral vectors to genetically modify human CD34+ HSPC, and the ability to

119   transplant and engraft these vector-modified cells to inhibit HIV-1 infection *in vivo* in huBLT

120   mice. Furthermore, we investigated a safety kill switch by CTX-mediated negative selection of

121   huEGFRt+ vector-modified cells. Together, these elements work together to provide a robust and

122   safe anti-HIV-1 HSPC-based gene therapy strategy.

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124   **Results**

125   **Development of lentiviral vectors with a safety kill switch to defend and attack HIV-1**

126   **infection**

127   We developed two new lentiviral vectors (M1 vector: MNDU-anti-HIV-1-huEGFRt and

128   U1 vector: UbC-anti-HIV-1-huEGFRt) to effectively co-express three anti-HIV-1 genes to

129   defend against and attack HIV-1 infection and to express the huEGFRt cell surface marker as a

130   safety kill switch (Figure 1A). We expressed CCR5sh1005 from a transcriptionally weaker H1

131   RNA Polymerase III promoter to avoid toxic effects of shRNA overexpression, as previously

132   described.[28,29] We first examined a modified Moloney Murine Leukemia virus long terminal

133   repeat promoter (MNDU) and a Ubiquitin C (Ubc) RNA polymerase II promoter to optimize the

134   co-expression of D1D2CAR 4-1BB and huEGFRt expression in M1 vector and U1 vector,

135   respectively. These two transgenes were linked by a self-cleaving T2A sequence for equimolar

136   expression. C46 was expressed from a shorter version of the eukaryotic translation elongation

137   factor 1α (EF1alpha) promoter to maintain efficient expression as the last transgene inserted near

138   the 3'LTR. Despite the multiple promoters and transgenes, the titers of our newly developed

139   anti-HIV-1 vectors in 293T cells were high ($2.75 \times 10^8 \pm 3.03 \times 10^7$ IU/mL for the M1 vector and

140    $1.00 \times 10^8 \pm 1.95 \times 10^7$ IU/mL for the U1 vector) (Figure 1B), which is consistent with our

141   previously developed lentiviral vectors.[31] Normalized % CCR5 expression was reduced to 76.1%

142   and 69.8% in M1 and U1 vector transduced huEGFRt+ MT4-CCR5 cells, respectively,

143   compared to the normalized %CCR5 in Non-CCR5sh1005 vector transduced huEGFRt+ cells

144   (100%) (Figure 1C). Mean fluorescent intensity (MFI) of CCR5 expression in huEGFRt+ cells

145   was reduced to 1491 and 1238 in M1 and U1 vector transduced huEGFRt+ cells, respectively,

146   compared to 10526 in Non-CCR5sh1005 vector transduced huEGFRt+ cells. These results show

147   CCR5 expression was efficiently down-regulated in M1 and U1 vector-transduced huEGFRt+

148   MT4-CCR5 cells. We noticed that MFI of huEGFRt expression were lower in M1 (1197) and U1

149   (1139) vector transduced cells than that of non-CCR5sh1005 vector transduced cells (5665) (Fig

150   1C), suggesting that huEGFRt expression might be compromised due to 4 multiple transgene

151   expressions from one vector. C46 was efficiently expressed in both M1 (94.6% C46+) and

152   U1(98.9% C46+) vector-transduced MT4-CCR5 cells (Figure 1D). D1D2CAR 4-1BB and

153   huEGFRt were efficiently co-expressed in vector-transduced human primary CD8+ T cells by

154   both the M1 vector (36.4%) and U1 vector (49.1%) (Figure 1E). These results show that our

155   newly developed vectors are highly efficient for co-expressing three anti-HIV-1 genes and

156   huEGFRt in human T-cell line and primary human T cells *in vitro*. Both R5-tropic HIV-1$_{NFNSX-}$

157    $_{SL9}$ and X4-tropic HIV-1$_{NL4-3}$ infection were inhibited in M1 and U1 vector transduced MT4-

158   CCR5 cell line *in vitro* (Figure 1F). To examine cell killing activity of M1 and U1 vector-

159   transduced CD8+ CAR T cells, we performed cytotoxic T lymphocyte (CTL) assays by co-

160   incubating vector-transduced CD8+ T cells with either ACH2 cells stimulated to express high

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161   levels of HIV-1 envelope (Env+) or unstimulated ACH2 cells (Env-). We observed

162   approximately 60% specific killing for both M1 and U1 vector-transduced CAR T cells at an E:T

163   ratio of 5:1 (p <0.01, p <0.05, respectively) (Figure 1G). These results reinforce the potential of

164   D1D2CAR 4-1BB to specifically target and induce cellular cytotoxicity in HIV-1 envelope

165   expressing cells. Altogether, these results demonstrate successful construction of a multi-pronged

166   anti-HIV-1 lentiviral vector that can block both R5- and X4-tropic HIV-1 infection *in vitro* and

167   direct a cellular immune response against infected cells via a chimeric antigen receptor.

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169   **Efficient engraftment of vector-modified HSPC for HIV-1 viral load reduction in huBLT**

170   **mice**

171   We next investigated the efficiency of vector transduction and transplantation of human

172   CD34+ HSPC to assess the engraftment, multi-lineage hematopoietic cell reconstitution,

173   transgene expression and HIV-1 inhibition *in vivo* in the huBLT mouse model (Figure 2A). M1

174   and U1 vectors efficiently transduced human fetal liver derived CD34+ HSPC (FL-CD34+

175   HSPC) at multiplicity of infection (MOI) 3 *ex vivo*. D1D2CAR 4-1BB+/huEGFRt+ co-

176   expressing cell population reached 79.9% and 51.7% in M1 and in U1 vector-transduced FL-

177   CD34+ HSPC at day 4 post vector transduction (Figure 2B). The MFI of D1D2CAR 4-1BB

178   (13080) and huEGFRt (14806) expression in the M1 vector-transduced HSPC was notably

179   higher than that of the U1 vector-transduced HSPC D1D2CAR 4-1BB (889) and huEGFRt

180   (1344), similar to the vector-transduced human primary CD8+ T cells (Figure 1E). Multi-lineage

181   colony formation in *ex vivo* culture showed similar % of multi colony-forming units between

182   untransduced, M1, and U1 vector-transduced FL-CD34+ HSPC *ex vivo* (Figure S1). After

183   transplantation of vector-transduced FL-CD34+ HSPC in huBLT mice, total CD45+, CD3+,

184   CD4+ and CD8+ T, and CD19+ B multilineage human hematopoietic cells were reconstituted

185   and continued to expand in peripheral blood in huBLT mice, as previously reported.[44,45] There

186   were no significant differences between untransduced control, M1, and U1 vector-transduced

187   FL-CD34+ HSPC transplanted huBLT mouse groups (herein referred to as untransduced, M1,

188   U1 huBLT mice, respectively) (Figure 2C). The average vector DNA copies/human cell were

189   higher in M1 vs U1 huBLT mice (~2 copies/cell vs ~1 copy/cell) and stably maintained during

190   the experiment (Figure 2D). The higher vector copy number in M1 huBLT mice compared to U1

191   huBLT mice could be attributed to a higher packaging efficiency of a lentiviral vector with an

192   MNDU promoter, as previously reported.[46] In a subsequent experiment, similarly high vector

193   copy levels (~1.5 copies/cell) were detected in M1 huBLT mice (Figure S2). Absolute numbers

194   of huEGFRt+ human CD3+, CD4+ and CD8+ T cells, but not CD19+ B cells significantly

195   increased in M1 vector-modified huBLT mice compared to U1 vector-modified and

196   untransduced huBLT mice after 4 to 6-weeks post-R5 tropic HIV-1$_{NFNSX-SL9}$ challenge in

197   peripheral blood (Figure 2E), suggesting CCR5sh1005 and C46 may provide protection for M1

198   vector-modified human CD4+ T cells for selective growth advantage, and D1D2CAR 4-1BB

199   may provide a CAR-dependent proliferation advantage. Percentage of huEGFRt+ cells did not

200   show increase in peripheral blood in M1 huBLT mice and in U1 huBLT mice because huEGFRt-

201   cells also increased in the huBLT mice (Figure S3, S4, and S5) as total human hematopoietic

202   cells continue to increase in huBLT mice (Figure 2C).

203   To examine if vector-modified cells from the huBLT mice could respond to HIV-1

204   envelope protein, we performed *ex vivo* cytokine release assays using HIV-1 Env+ ACH2 cells

205   as targets. When mixed with HIV-1 Env+ ACH2 cells, human CD8+ splenocytes from M1

206   huBLT mice exhibited significantly higher IFN-γ expression compared to CD8+ splenocytes

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207   from M1 huBLT mice mixed with HIV-1 Env- ACH2 cells (~3 fold increase, p <0.05) and CD8+

208   splenocytes from control untransduced huBLT mice mixed with HIV-1 Env+ ACH2 cells (~5

209   fold increase, p <0.05) consistent with an HIV-1 envelope-specific cytokine response (Figure 2F,

210   Figure S6). The HIV-1 plasma viral load was significantly reduced (p <0.001, 1-log reduction)

211   for 6 weeks post HIV-1 challenge in M1 huBLT mice compared to untransduced huBLT mice,

212   which served as our negative control. We also observed reduction of HIV-1 viral load for up to 4

213   weeks post HIV-1 challenge in U1 huBLT mice (~4 fold reduction, p <0.05), but viral load

214   reduction was not significant at 6 weeks post infection. Because the M1 vector showed more

215   significant viral load reduction in our donor 1 experiment, we further investigated the M1 vector

216   and validated the effectiveness of HIV-1 viral load reduction in M1 huBLT mice in a repeat

217   experiment with donor 2 (~1 log-fold reduction, p <0.05) (Figure 2G). These results demonstrate

218   that our multi-pronged anti-HIV-1 HSPC-based gene therapy strategy with M1 vector can

219   achieve efficient *ex vivo* CD34+ cell transduction, support multi-lineage human hematopoietic

220   cell reconstitution, stable transgene expression and greater viral load reduction compared to the

221   U1 vector in huBLT mice.

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223   **CTX-mediated negative selection of huEGFRt+ vector-modified cells as a safety kill switch**

224   Although adverse effects have not been reported in anti-HIV-1 HSPC-based gene therapy

225   preclinical studies or in clinical trials, potential adverse side-effects from lentiviral vector

226   transduced HSPC or the induction of anti-HIV-1 CAR T cells must be approached prospectively.

227   We therefore incorporated a safety kill switch into our anti-HIV-1 gene lentiviral vector,

228   huEGFRt, triggered by the cognate CTX antibody. We investigated CTX-mediated negative

229   selection of huEGFRt+ vector-modified cells in huBLT mice (Figure 3A). In our first

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230   experiment, we observed transient reduction of huEGFRt+ vector-modified cells in M1 huBLT

231   mice (Figure S7). Since reconstituted human immune function in humanized mouse models is

232   suboptimal, we hypothesized that our initial modest results were due to the limited number of

233   human NK cells in huBLT mice.[47,48] To enhance the number of functional human NK cells for

234   ADCC, we injected human NK cells and an IL-15 expressing lentiviral vector to promote

235   survival and function of human NK cells. In this augmented humanized mouse model, the

236   percentage and absolute cell number of huEGFRt+ M1 vector-modified cells were significantly

237   reduced following CTX treatment. We observed substantial reductions in CD45+, CD3+, CD4+,

238   CD8+, and CD4+/CD8+ multi-lineage hematopoietic cells in peripheral blood of CTX-treated

239   animals compared to CTX-untreated mice after 1 week of CTX injections; this difference

240   persisted for 4 weeks (~13 fold reduction, $p < 0.01$, and ~13 fold reduction, $p < 0.05$,

241   respectively, averaged across all cell lineages at week 4 post-CTX treatment) (Figure 3B and

242   3C). HuBLT mice were euthanized at 4 weeks post CTX injections; huEGFRt+ vector-modified

243   cells were significantly reduced in spleen and BM (~ 9 fold reduction, $p < 0.01$, and ~ 2.5 fold

244   reduction, $p < 0.01$, respectively, averaged across all cell lineages at week 4 post-CTX treatment)

245   in CTX-treated vs CTX-untreated control M1 huBLT mice (Figure 3D, Figure S8). Within

246   HSPC population, huEGFRt+ vector-modified CD34+/CD90+/CD38- HSPC were likewise

247   significantly reduced (~3 fold reduction $p < 0.05$) in the BM of CTX-treated M1 huBLT mice

248   (2.12% ± 1.20%) compared to CTX-untreated M1 huBLT mice (21.67% ± 7.92%) (Figure 3E

249   and 3F). HuBLT mice remained healthy in CTX-treated and untreated groups, suggesting no

250   apparent health adverse effects (Figure S9).

251   Finally, we utilized Phycoerythrin (PE) conjugated CTX (CTX-PE) to stain huEGFRt+

252   cells and to analyze the level of expression by flow cytometry. To test whether CTX-mediated

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253   negative selection could impede detection of huEGFRt+ cells with the same antibody, we

254   compared CTX-PE to another anti-huEGFR PE-conjugated mAb, Matuzumab (MTZ-PE), which

255   binds to a different epitope on huEGFR (Figure S10).[49] MTZ-PE staining confirmed that CTX-

256   treated huEGFRt+ splenocytes from M1 huBLT mice were significantly reduced in multiple cell

257   lineages (CD45+, CD3+, CD4+, CD8+) compared to CTX-untreated splenocytes from M1

258   huBLT mice (~13-fold reduction, $p < 0.01$ averaged across all cell lineages) (Figure S11). The

259   lower %huEGFRt expression estimated by MTZ-PE staining may reflect the lower binding

260   affinity of MTZ-PE than CTX-PE. Despite this difference, both MTZ-PE and CTX-PE stained

261   CTX-treated M1 huBLT mice splenocytes showed significant reductions in huEGFRt

262   expression. These results demonstrate that our CTX-mediated negative selection strategy is

263   highly effective for depleting huEGFRt+ vector-modified human HSPC and progeny cells for

264   diverse cell and gene therapies in huBLT mice.

265

266   **Discussion**

267   In this study, we investigated a multi-pronged anti-HIV-1 HSPC based gene strategy to

268   defend against and attack HIV-1 infection in humanized BLT mice. We developed a novel

269   lentiviral vector that successfully co-expressed three anti-HIV-1 genes. These anti-HIV-1 genes

270   include an shRNA against CCR5 HIV-1 co-receptor and C46 fusion inhibitor to protect cells

271   against HIV-1 infection, and a truncated CD4-based CAR with 4-1BB costimulatory domain

272   (D1D2CAR 4-1BB) to attack HIV-1 infected cells. We also incorporated huEGFRt, to allow for

273   efficient negative selection of vector-modified cells as a safety kill switch in case of potential

274   adverse effects. Our results demonstrate that vector-modified HSPC efficiently reconstituted

275   anti-HIV-1 vector-modified cells and significantly reduced viral load *in vivo* in huBLT mice. We

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276   used huBLT mice since the development of human HSPC derived anti HIV-1 gene modified T

277   cells occurs in the donor matched human thymus tissue. In other humanized mouse models,

278   human T cell development occurs in mouse thymus and it is not efficient nor physiological due

279   to the human leukocyte antigen (HLA) and mouse MHC mismatch.[44,50,51] Administration of

280   CTX, a clinically available anti-huEGFR monoclonal antibody, significantly reduced huEGFRt+

281   gene-modified cells, improving the safety of our anti-HIV-1 gene therapy strategy.

282   HSPC-based gene therapy has been investigated to achieve life-long remission or cure

283   due to the potential of anti-HIV-1 gene modified HSPC to continuously provide HIV-1 protected

284   immune cells.[22–24] Unfortunately, the efficiency of gene modification in HSPC and the level of

285   engraftment are not sufficient to achieve life-long remission with current technologies.[22–25] If the

286   engraftment and reconstitution is incomplete, remaining unprotected cells are subject to

287   infection. CAR T-cells have emerged as a powerful immunotherapy for different forms of

288   cancer.[52,53] Anti-HIV-1 CAR gene can re-engineer host immune cells to target HIV-1 specific

289   antigens such as gp120 on the surface of HIV-1 infected cells and elicit virus-specific

290   cytotoxicity.[37,54,55] This strategy subverts the necessity for complete engraftment of anti-HIV-1

291   vector modified HSPC, as anti-HIV-1 CAR T cells can attack HIV-1 infected cells. In addition to

292   CCR5sh1005 and C46, we successfully developed a lentiviral vector capable of co-expressing a

293   CD4-based D1D2CAR 4-1BB for efficient HSPC gene modification to achieve efficient viral

294   load reduction.

295   We successfully incorporated a safety kill switch by co-expressing huEGFRt in our anti-

296   HIV-1 HSPC based gene strategy to better prepare for potential adverse effects such as clonal

297   outgrowth or malignant transformation of lentiviral vector transduced HSPC by random vector

298   insertional mutagenesis, CAR T cell mediated cytokine release syndrome, or encephalopathy

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299   syndrome.[34,38,40,56–58] We chose huEGFRt cell surface marker gene due to its relatively short

300   cDNA sequence, allowing for its inclusion and efficient co-expression in a complex multi-

301   pronged lentiviral vector. HuEGRt can be targeted with the clinically available chimeric

302   immunoglobulin G1 anti-EGFR monoclonal antibody (mAb), Cetuximab (CTX) (Erbitux™) for

303   efficient negative selection.[42,43] Furthermore, huEGFRt expression can be monitored by

304   fluorescence conjugated anti-huEGFR mAb and flow cytometry, giving it a secondary purpose

305   as a trackable gene marker of vector modified cells.[42]

306   Other negative selection strategies of vector-modified HSPC have been developed using

307   CD20 paired with rituximab, herpes simplex virus-thymidine kinase (HSV-TK) paired with

308   ganciclovir, and inducible caspase 9 (iCas9) paired with AP1903 (Rimiducid) to induce

309   dimerization.[59–61] CTX-mediated negative selection of huEGFRt+ cells stands out as a promising

310   safety switch for several compelling reasons. Unlike rituximab used to deplete CD20+ cells,

311   CTX has not been shown to cause late onset neutropenia in clinical trials.[42,43,62,63] CTX-mediated

312   elimination of huEGFRt+ cells also holds several advantages to the HSV-TK system. The HSV-

313   TK system paired with ganciclovir is only functional on proliferating cells, and a loss of

314   sensitivity of ganciclovir could further stifle this method's effectiveness.[64,65] Studies have also

315   indicated the potential for immunogenicity against HSV-TK, and its interference with host DNA

316   repair, greatly enhancing the potential for unwanted cytotoxicity.[66,67] The iCas9 system has

317   shown promise, with exposure to AP1903 leading to elimination of 85-95% of circulating iCas9-

318   transduced cells *in vitro* and *in vivo*.[68–70] However, there are limitations to the iCas9 system's

319   practical application in clinical trials, as safety switches derived from non-human sequences will

320   likely increase the risk of immunogenicity.[71–73] The significant reduction of huEGFRt+ vector-

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321 modified cells by CTX in huBLT mice in this study combined with prior use in clinical studies

322 suggests its efficacy as a safe and successful kill-switch system for HSPC-based gene therapies.

323 In summary, we provided a proof of concept that our newly developed multi-pronged

324 anti-HIV-1 gene lentiviral vector with a safety kill switch could mediate efficient HSPC CD34+

325 transduction, engraftment, viral load reduction, and negative selection of vector modified cells *in*

326 *vivo* in huBLT mice. Our studies performed in humanized BLT mice provide valuable insight on

327 the development, protection, and efficacy of engineered T cells from anti-HIV-1 gene modified

328 human HSPC developed through the human thymus. For clinical application, we recognize that

329 there are still many obstacles to overcome. Further investigation of our strategies in more

330 clinically relevant animal models, such as non-human primates, could provide us more clinically

331 relevant results. We believe continuous improvements in the level of HIV-1 inhibition by

332 enhancing the engraftment of anti-HIV-1 gene modified HSPC and ensuring safety will

333 ultimately succeed us to translate our HSPC based anti-HIV-1 gene therapy into clinic.

334

335 **Materials and Methods**

336 This study was carried out in strict accordance with the recommendations in the Guide

337 for the Care and Use of Laboratory Animals of the National Institutes of Health ("The Guide"),

338 and was approved by the Institutional Animal Care and Use Committees of the University of

339 California, Los Angeles, protocol #ARC-2007-092. For humanized mice, all surgeries were

340 performed under ketamine/xylazine and isoflurane anesthesia, and all efforts were made to

341 minimize animal pain and discomfort.

342

343 **Vector Construction**

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344   Our vector construct backbone is derived from the "EQ" plasmid (generously provided

345   by Satiro N. De Oliveira, UCLA, Los Angeles, California).[43] We inserted our previously

346   constructed CCR5sh1005, [29] D1D2CAR 4-1BB previously published by Zhen et al,[37] and also

347   the membrane anchored HIV-1 fusion inhibitor C46 previously published by Burke et al.[30] Final

348   optimized constructs also included the CD8 stalk element after the D1D2CAR 4-1BB

349   extracellular domains and before the CD8 transmembrane domain in the D1D2CAR 4-1BB.

350   Ubiquitin C or modified MLV long terminal repeat (MNDU3) promoters were used in each

351   vector respectively to express D1D2CAR 4-1BB and huEGFRt. C46 was expressed using a

352   truncated elongation factor-1a (EF1a) promoter. The final construct plasmids (M1 and U1) were

353   purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Stellar competent cells

354   from Takara Bio (Kusatsu, Shiga, Japan) were transformed with our constructed plasmids, and

355   plasmid stocks were then produced using Macherey-Nagel Nucleobond Xtra Midi Kit

356   (Macherey-Nagel, Düren, Germany).

357

358   **Cell culture**

359   MT4-CCR5 cells are a human T-lymphotropic virus type 1-transformed human CD4$^+$ T

360   cell line that stably expresses CCR5, and were kindly provided by Dr. Koki Morizono (UCLA,

361   Los Angeles). MT4-CCR5 cells were generated by transducing MT4 cells with a lentiviral vector

362   expressing human CCR5 under the control of the internal SFFV promoter. These cells were

363   cultured in RPMI-1640 (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine

364   serum (FBS), 2mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (GPS).

365   Human primary peripheral blood mononuclear cells (PBMC) were isolated from whole blood

366   from healthy donors obtained by the UCLA CFAR Virology Core Laboratory using Ficoll Paque

367   Plus (GE Healthcare, Uppsala, Sweden). PBMC were cultured in RPMI-1640 supplemented with

368   20% FBS and GPS (20F RPMI). Primary human fetal liver derived (FL) CD34+ HSPC were

369   isolated from human fetal liver obtained from the UCLA CFAR Gene and Cellular Therapy Core

370   Laboratory (Los Angeles, California) or Advanced BioScience Resources, Inc (Alameda, CA)

371   using a CD34$^+$ microbead isolation kit (Miltenyi Biotec, Auburn, CA).

372

373   **Lentivirus Production**

374   Lentiviral vectors were packaged with a VSV-G pseudotype using calcium phosphate

375   transfection, then collected from transfected HEK293T-cells treated with chloroquine and

376   concentrated in 1xHBSS via ultracentrifugation, as previously described.[74–76] Titers for lentiviral

377   vectors were determined using CTX-PE mAb staining and flow cytometry in vector-transduced

378   HEK293T-cells and were based on huEGFRt expression at Day 3 post-transduction.

379

380   *In-Vitro* **HIV-1 inhibition assays**

381   We established our vectors' proof of concept to inhibit HIV-1 and target HIV-1 infection

382   *in-vitro* in cell lines and primary cells prior to *in-vivo* experiments. For our HIV Inhibition assay,

383   MT4-CCR5 cells were transduced with triple-anti-HIV vectors at MOI 1 and infected with R5-

384   tropic HIV-1$_{NFNSX}$ virus (MOI 1) or X4-tropic HIV-1$_{NL4-3}$ virus (MOI 0.005) at 4 days post-

385   transduction. Supernatants collected 7 days post-challenge were analyzed for p24 using Abcam

386   HIV p24 ELISA kit (Abcam, Waltham, MA). To functionally qualify D1D2CAR 4-1BB, CD8+

387   T cells were isolated from healthy-PBMCs provided by UCLA CFAR virology core and

388   expanded in RPMI 1640 (Gibco) with 10% fetal bovine serum (HyClone) containing IL7

17

389   (10ng/mL) and IL15 (5ng/mL). Cells were transduced with lentiviral vectors at MOI 3 and co-

390   cultured with either unstimulated ACH2 cells or ACH2 cells stimulated overnight with

391   PMA/Ionomycin (Invitrogen, Darmstadt, Germany) to increase HIV-1 gp120 surface protein.[77,78]

392   ACH2 cells are a cell line with a single integrated copy of HIV-1 strain LAI. Unstimulated and

393   stimulated ACH2 cells were labeled with CellTrace™ Far Red (Invitrogen) before 16-hr co-

394   culture followed by stain with Zombie (Aqua or Green) Fixable Viability (Biolegend, San Diego,

395   CA) and KC57 antibody (Beckman Coulter, Indianapolis, IN) to detect Gag+ ACH2 cells.

396   Specific killing was calculated as follows: % specific killing =(%live Gag+ ACH2 cells co-

397   cultured with untransduced cell—%live Gag+ ACH2 cells co-cultured with vector-transduced

398   cells)/ %live Gag+ ACH2 co-cultured with untransduced cell × % Gag+ in ACH2 cells alone.

399

400   **Lentiviral Vector Transduction of HSPC for *in vivo* experiments**

401   Fetal liver-derived CD34+ HSPCs were resuspended in Yssel's medium (Gemini Bio

402   Products) with 2% BSA (Sigma-Aldrich) and seeded into 20 µg/mL RetroNectin (Clontech

403   Laboratories)-coated plates. After 1 hour of incubation at 37□, cells were transduced with

404   lentiviral vectors at MOI 3 and cultured overnight at 37□. The following day, vector-transduced

405   CD34+ HSPCs were transplanted into NSG mice. An aliquot of the transduced CD34+ HSPCs

406   were cultured in 10F RPMI, supplemented with cytokine stimulations (SCF, Flt-3, TPO;

407   PeproTech) at a concentration of 50 ng/mL for 3 days. The efficiencies of vector transduction

408   were evaluated by flow cytometry (Fortessa flow cytometers, BD Biosciences) and/or by vector

409   copy number (VCN) using digital PCR as described below (ThermoFisher QuantStudio 3D

410   Digital System/Quantstudio Absolute Q Digital PCR system).

411

Humanized BLT Mouse Construction

NSG (NOD/SCID/IL2rγ -/-) mice were used to generate humanized BLT mice and housed according to UCLA Humanized Mouse Core Laboratory procedures as previously described. [31] Human fetal thymus and fetal liver were obtained from Advanced Bioscience Resources (ABR). Fetal tissues were obtained without patient identifying information. Written informed consent was obtained from patients for the use of tissues for research purposes. Briefly, one day before transplant, CD34+ cells were isolated from fetal livers using anti-CD34+ magnetic bead-conjugated monoclonal antibodies (Miltenyi Biotec) and transduced with vectors described above. NSG mice were conditioned with sub-lethal body irradiation (270 cGy Cesium-137). On the day of transplant, an equal mixture of non-transduced or vector-transduced FL-CD34+ cells (~ 0.5×10^6 per mouse) and CD34- cells (~4.5×10^6 per mouse) were mixed with 5μL of Matrigel (BD Biosciences) and implanted with a piece of thymus under the kidney capsule. Mice were then injected with non-transduced or vector-transduced CD34+ HSPCs (~ 0.5×10^6 per mouse) using a 27-gauge needle through the retro-orbital vein plexus. At 8–10 weeks post-transplantation, blood was obtained from each mouse by retro-orbital sampling and peripheral blood mononuclear cells were analyzed by flow cytometry to quantify human immune cell engraftment.

Colony-forming unit (CFU) assays

Colony-forming units were assayed by culturing transduced and non-transduced FL-CD34+ cells 3 days after transduction in triplicate in a 6 well plate (ThermoFisher) using complete methylcellulose (MethoCult H4435 Enriched, Stem Cell Technologies). 14 days later, colony-forming units (CFU) in each well were then counted by light microscopy, and the colony

435   type was scored based on morphology. Proportions of differentiated hematopoietic colonies =

436   100% × (each colony type CFU counted/total CFU counted) and calculated from each well from

437   triplicates.[79] Total CFU counts ranged from 30-75 in each well.

438

439   **HIV-1 Infection and viral load analysis**

440   NSG huBLT mice were injected with R5 tropic HIV-1$_{NFNSX-SL9}$ (MOI 5) (200 ng p24)

441   retro-orbitally 11 weeks post-vector-modified HSPC transplant [74]. Mice were bled retro-orbitally

442   every 2 weeks after infection, and blood samples were analyzed for HIV-1 viral load via RT-

443   PCR. HPSC engraftment was assessed by vector copy number assay via digital PCR, and cell

444   lineage differentiation and transgene expression were measured via flow cytometry.

445

446   **Depletion of huEGFRt+ transduced cells via Cetuximab**

447   At week 13-14 post-transplantation of vector-modified HSPC, mice were separated into

448   two groups with one group to receive CTX treatment (CTX+) alongside human NK cells and

449   huIL-15 treatment (n=5) and the other group to be left untreated (n=4). Vector-modified

450   huEGFRt+ HSPC transplanted huBLT mice were treated with cetuximab (Erbirtux^{TM}) at a

451   concentration of 1mg per mouse intraperitoneally for 11 consecutive days. Because of the lack of

452   efficient development of human NK cells in NSG mice, which was hypothesized to be the result

453   of a lack of IL15,[80] and to facilitate antibody dependent cellular toxicity, we injected a dose of

454   5×10^6 human NK cells isolated from healthy PBMCs one day before the first CTX treatment and

455   a second dose of 5×10^6 human NK cells from the same donor on day 7 of CTX treatment.

456   Lentiviral vectors expressing IL-15 (2.5×10^5 IU/mouse) were injected retro-orbitally on day 7 of

457   CTX treatment. huEGFRt expression and absolute cell count were monitored in multi-human

20

458   cell lineages by staining peripheral blood cells with CD45-, CD3-, CD19-, CD4-, and CD8-

459   specific monoclonal antibodies of peripheral blood prior to flow cytometry analysis at 3 weeks

460   before CTX treatment and 1 and 4 weeks post-CTX treatment. We developed this strategy to

461   augment negative selection results in animals lacking circulating NK cells and supportive IL-15,

462   as NK cells serve a critical role in CTX-mediated ADCC of huEGFRt+ cells (**Figure S7**).

463

464   **Analysis of tissue from transduced huBLT mice**

465   Humanized BLT mice were sacrificed at week 18-19 post-transplant, and the spleen and

466   BM tissues were harvested. Tissue samples were collected in MACS tissue storage solution

467   (Miltenyi Biotec, 130-100-008) at necropsy and processed immediately for single cell isolation

468   as described previously.[31,37] Isolated cells were stained for surface markers and analyzed by flow

469   cytometry or vector copy number was determined by digital PCR.

470   Single-cell suspensions prepared from peripheral blood, spleen, or BM of huBLT mice

471   were stained for surface markers and acquired on a LSRFortessa flow cytometer (BD

472   Biosciences). The following antibodies were used: CD45-eFluor 450 (HI30, eBioscience), CD3-

473   APC H7 (SK7, Pharmingen), CD4-APC (OKT4, eBioscience), CD8-PerCP Cy5.5 (SK1,

474   BioLegend), CD19-Brilliant Violet 605 (HIB19, BD Horizon), EGFR-PE (Hu1, R&D Systems),

475   and Countbright beads (Invitrogen). Red blood cells were lysed with RBC Lysis Buffer

476   (Biolegend) after cell surface marker staining. Stained cells were fixed with 2% formaldehyde in

477   PBS. The data were analyzed by FlowJo v.10 (Tree Star) software.

478

479   **Determination of vector copy number (VCN)**

480   Cell pellets from 25μL peripheral blood, spleen, or BM of huBLT mice were lysed with

481   5μL of 0.2M NaOH in a 75□ water bath for 5 min. Cell lysates were cooled in a 4°C refrigerator

482   for 5 min. 45μL Tri-HCl was added to neutralize the lysates. The lysate cells were directly used

483   in dPCR set at 96°C for 10 min, followed by 42 cycles of denaturation at 98°C for 15 s,

484   annealing at 60°C for 2 min, and a final extension at 60°C for 2 min. The primers and probe

485   specific to WPRE were customized by ThermoFisher, which are primer sequence 1, 5' -

486   CCTTTCCGGGACTTTCGCTTT-3', primer sequence 2, 5'-GCAGGCGGCGATGAGT-3', and

487   probe 5'-(FAM)-CCCCCTCCCTATTGCC-3'. The primers and probe specific to β-globin were

488   purchased from ThermoFisher (cat#4448489). Average VCN was determined by multiplex

489   dPCR of the WPRE sequence in the vector and normalized to the cell housekeeper gene β-

490   globin.

491

492   **Statistical Analysis**

493   Statistical analysis was performed using software Prism. Mann-Whitney U test was used

494   for nonparametric testing of independent groups, and student's t-test were used for parametric

495   testing of independent groups. Statistical significance was evaluated as *p <0.05. Other

496   significance levels are indicated as follows: **p <0.01, ***p <0.001, and ****p <0.0001.

497

498   **Data Availability Statement**

499   The data supporting the findings of this study are available from the corresponding author upon

500   reasonable request.

501

502   **Author contributions**

503   Authors QG, KP, and JZ contributed equally to this manuscript and are all considered co-first

504   authors. Authors AB, NJ, and GC contributed to experimental data collection and manuscript

505   revision. Authors AZ and DSA gave invaluable guidance and feedback throughout the

506   preparation of this manuscript. All authors had the opportunity to review the manuscript prior to

507   submission.

508

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519

Declaration of Interests

521   DSA has a financial interest in CSL Behring. No funding was provided by the company to

522   support this work. DSA holds a US patent for CCR5sh1005. All of the other authors declare no

523   competing interests.

524

23

Key words

Hematopoietic stem/progenitor cell (HSPC) based gene therapy, HIV-1, CCR5 short-hairpin

RNA, CD4-based anti-HIV-1 chimeric antigen receptor, C46 membrane anchored HIV-1 fusion

inhibitor, truncated human EGFR (huEGFRt), Cetuximab-mediated *in vivo* negative selection,

humanized mouse

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827

828   **Figure Legend**

829   **Figure 1. Development of a multi-pronged anti-HIV-1 lentiviral vector with safety kill**

830   **switch to defend against and attack HIV-1 infection**

831   (A) Design of novel lentiviral vectors expressing triple anti-HIV-1 genes (CCR5sh1005,

832   D1D2CAR 4-1BB, C46) and a selectable huEGFRt gene. The M1 vector uses an MNDU

833   promoter and the U1 vector uses a Ubc promoter for D1D2CAR 4-1BB and huEGFRt

834   expression, respectively. These vectors also includes ΔLTR: self-inactivating, U3 enhancer and

835   promoter deleted long terminal repeat, H1: H1 RNA polymerase III promoter, MNDU: murine

836   leukemia virus (MuLV) long terminal repeat promoter, UbC: Ubiquitin C RNA polymerase II

837   promoter, T2A: 2A self-cleaving peptide, huEGFRt: truncated nun-functional human epidermal

838   growth factor receptor, EF1α: human elongation factor 1 alpha promoter, WPRE: woodchuck

839   hepatitis virus posttranscriptional regulatory element. Non-CCR5sh1005 vector includes ΔLTR,

840   MNDU, D1D2CAR 4-1BB, T2A, huEGFRt, C46 and WPRE.

841   (B) Vector titer measured by infectious units/milliliter (IU/ml) determined based on percent

842   huEGFRt transgene expression in 293T cell line stained with CTX-PE mAb and measured by

843   flow cytometry at day 3 post-transduction.

844   (C) CCR5 downregulation by CCR5sh1005 in MT4-CCR5 cell lines. MT4-CCR5 cells were

845   transduced at MOI 1 with vectors M1 and U1. Untransduced and non-CCR5sh1005 vector

846   transduced MT4-CCR5 cells were used as a negative control. 4 days post-vector transduction,

37

847 CCR5 and huEGFRt were stained with mAbs and measured by flow cytometry. Normalized

848 CCR5 expression within huEGFRt positive population was calculated based on the following

849 formula: ([%CCR5+/huEGFRt+population]/([%CCR5+/huEGFRt+population] + [%CCR5-

850 /huEGFRt+population])×100%) and mean fluorescent intensity of CCR5 expression in huEGFRt

851 positive population (MFI) is indicated on the top of representative flow plot.

852 (D) C46 cell surface expression and MFI in MT4-CCR5 cell line at 4 days post-transduction.

853 MT4-CCR5 cells were transduced at MOI 1 with vectors M1 and U1. Mock is untransduced

854 negative control cells. C46 was stained with mAb and measured by flow cytometry.

855 (E) HuEGFRt and D1D2CAR 4-1BB surface protein expression in primary human CD8+ T cells

856 4 days post-transduction. CD8+ T cells isolated from healthy-donor PBMCs were transduced

857 with M1 and U1 vectors, respectively (MOI 10). Untransduced primary human CD8+ T cells

858 were used as a negative control. HuEGFRt and D1D2CAR 4-1BB expression was stained with

859 mAb and measured by flow cytometry.

860 (F) *In-vitro* HIV-1 inhibition by M1 and U1 vector-transduced MT4-CCR5 cells. Untransduced

861 MT4-CCR5 cells were used as a negative control. MT4-CCR5 cells were challenged with R5

862 tropic HIV-1$_{NFNSX}$ (MOI 1) or X4 tropic HIV-1$_{NL4-3}$ (MOI 0.005) virus. p24 capsid protein levels

863 in cell culture supernatant were determined by p24 ELISA assay 7 days post HIV-1 challenge

864 and used to assess inhibition ability. Data shows results from two independent experiments.

865 (G) *In-vitro* specific killing of HIV-1 envelope expressing ACH2 cells. PMA/ionomycin

866 stimulated (Env+) or unstimulated (Env-) ACH2 cells were co-cultured with vector-transduced

867 human primary CD8+ T cells at 1:1, 2:1, and 5:1 Effector:Target cell (E:T) ratio overnight.

868 %specific killing was calculated by (%live gag+ ACH2 cells with untransduced CD8+ cell—

869 %live gag+ ACH2 cells with vector transduced CD8+ cells)/ %live gag+ ACH2 cells with

38

870   untransduced CD8+ cell then normalized by %gag+ in ACH2 cells. Data shows Mean ± SEM

871   from a single experiment performed in triplicates. Mann-Whitney U test was performed to

872   calculate significance. *p <0.05 and **p <0.01.

873

874   **Figure 2. Efficient human HSPC vector-modification, transplantation and multi-lineage**

875   **human hematopoietic cell reconstitution in huBLT mice**

876   (A) Experimental design for the investigation of M1 and U1 vectors in NSG huBLT mice.

877   Human FL-CD34+ cells were transduced with M1 or U1 vectors at MOI 3 on Day -1. NSG

878   huBLT mice were conditioned with 270 cGy of sub-lethal body irradiation from a Cesium-137

879   source. Mice were transplanted with the vector transduced FL-CD34+ HSPC and human thymus

880   tissue on Day 0. Mice were challenged with R5-tropic HIV-1$_{NFNSXSL9}$ (200ng p24/ mouse) at 11

881   weeks post-transplant.

882   (B) HuEGFRt and D1D2CAR 4-1BB transgene expression in vector transduced FL-CD34+ cells

883   in *ex-vivo* culture. HuEGFRt and D1D2CAR 4-1BB co-expressing population was determined by

884   mAb staining and flow cytometry 4 days post-vector transduction.

885   (C) Human multilineage hematopoietic cell reconstitution in peripheral blood from 8 weeks post-

886   vector transduced HSPC transplant. Cell surface markers of human lymphocytes (CD45), T-cells

887   (CD3, CD4, and CD8) and B-cells (CD19) were determined by mAb staining and flow

888   cytometry. Dots and error bars show Mean ± S.E.M, respectively.

889   (D) Vector-marking levels were determined in peripheral blood cells from 8 weeks to 17 weeks

890   post-transplant by digital PCR. Average vector DNA copies were calculated by VCN = (WPRE

891   DNA copies in vector DNA/ul)/(human β-globin copies/ul/2). Dots and error bars show Mean ±

892   S.E.M, respectively.

893   (E) Human multilineage hematopoietic cell expansion within huEGFRt expressing population

894   after HIV-1 infection. Expression of huEGFRt was determined by mAb staining and flow

895   cytometry. Cell surface markers of human lymphocytes (CD45), T-cells (CD3, CD4, and CD8)

896   and B-cells (CD19) were also determined by mAb staining and flow cytometry and gated within

897   the huEGFRt positive population. Mice were challenged with HIV-1 at 11 weeks-post transplant

898   (not noted in this figure). Dots and error bars show Mean ± S.E.M, respectively. Mann-Whitney

899   U test was performed to calculate significance, *$p < 0.05$.

900   (F) E*x vivo* cytokine production measured by cytokine release assay. CD8+ T splenocytes from

901   M1 huBLT mice were co-cultured with Env+ target cells (PMA/ionomycin activated ACH2

902   cells) or unstimulated Env- cells (medium only) as a negative control *ex vivo*. Data was collected

903   from our replicate huBLT mice experiment (donor 2). Cells were collected at time of mouse

904   sacrifice at week 20 post-transplant. Cytokine expression was measured by flow cytometry. Dots

905   and error bars show Mean ± S.E.M, respectively. *t*-test with Holm-Šídák adjustment was

906   performed to calculate significance. *$p < 0.05$.

907   (G) Viral loads were measured as HIV-1 RNA copies per mL in mouse plasma every 2 weeks

908   post- HIV-1 challenge by digital PCR in 2 different sets of experiments using 2 human CD34+

909   HSPC donors (Donor 1 and Donor 2). HuBLT mice groups were transplanted with either M1- (n

910   = 5 in both experiments) or U1-transduced (n = 7) HSPC. Untransduced huBLT mice were used

911   as a negative control in both experiments (n = 3 in first experiment, and n = 5 in replicate

912   experiment). Data were shown in Mean ± S.E.M. *t*-test with Holm-Šídák adjustment was

913   performed to calculate significance. NS = not significant, *$p < 0.05$, and ***$p < 0.001$.

914

915 **Figure 3. Cetuximab-mediated negative selection of huEGFRt expressing vector-modified**

916 **human hematopoietic cells in huBLT mice**

917 (A) Experimental design for the investigation of CTX-mediated negative selection of huEGFRt+

918 vector-modified human cells. CTX treatment group M1 huBLT mice (n=5) were injected with

919 1mg per mouse intraperitoneally for 11 consecutive days. 5×10^6 human natural killer (NK) cells

920 per mouse were injected retro-orbitally (RO) one day before first treatment (D0) and on D7

921 during CTX treatment. IL-15 expressing lentiviral vectors (2.5×10^6 IU) were injected RO on day

922 7 of CTX treatment (2.5×10^5 IU/mouse). CTX untreated group (n=4) served as a negative

923 control.

924 (B) HuEGFRt expression level in multilineage human peripheral blood cells (CD45+, CD3+,

925 CD19+, CD4+, and CD8+) in CTX treated and untreated M1 huBLT mice. Blood was collected

926 1 week before CTX treatment, and 1 and 4 weeks post-onset of CTX treatment. Samples were

927 stained with mAbs and measured by flow cytometry. HuEGFRt expression was measured by

928 flow cytometry using mAb CTX-PE. Dots and error bars show Mean ± S.E.M, respectively.

929 Mann-Whitney U test was performed to calculate significance. NS = not significant, and **p
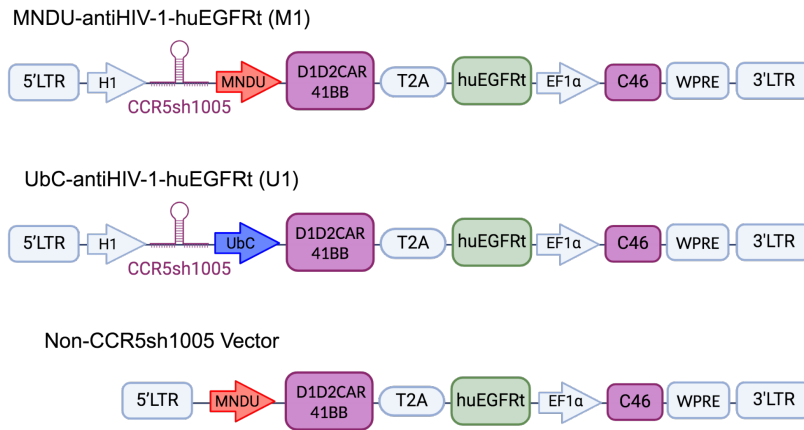
930 <0.01.

931 (C) Absolute multilineage huEGFRt+ cell count in CTX-treated and untreated M1 huBLT mice

932 in peripheral blood. Blood was collected at weeks 13, 15, and 18 post-transplantation (1 week

933 pre-CTX treatment, and 1 and 4 weeks post-CTX treatment, respectively). HuEGFRt and surface

934 markers of human lymphocytes (CD45), T-cells (CD3, CD4, and CD8) and B-cells (CD19),

935 were stained by mAbs and measured by flow cytometry. Mann-Whitney U test was performed to

936 calculate significance. NS = not significant, *p <0.05, **p <0.01, and ***p <0.001.

41

937   (D) HuEGFRt expression level across multilineage human T-cell cell populations (CD45+,

938   CD3+, CD4+, CD8+, and CD4+CD8+) in spleen and bone marrow tissue collected from CTX

939   treated and untreated M1 huBLT mice. Samples were stained with mAb CTX-PE. Dots and error

940   bars show Mean ± S.E.M, respectively. *t*-test with Holm-Šídák adjustment was performed to

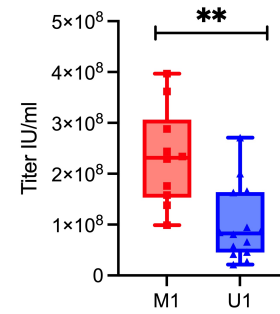941   calculate significance. **p <0.01, ***p <0.001 and ****p <0.0001.

942   (E) Representative flow cytometry data showing huEGFRt expression within

943   CD34+/CD90+/CD38- population of bone marrow cells in CTX treated and untreated M1

944   huBLT mice. Samples were stained with mAb CTX-PE and measured by flow cytometry.

945   (F) Cumulative data showing huEGFRt expression within CD34+/CD90+/CD38- population of

946   bone marrow cells collected from CTX treated and untreated M1 huBLT mice. Samples were

947   stained with mAb CTX-PE and measured by flow cytometry. % huEGFRt was normalized to

948   background levels from mock transduced HSPC transplanted huBLT mice. Dots and error bars

949   show Mean ± S.E.M, respectively. Student's t-test was performed to calculate significance. *p

950   <0.05.
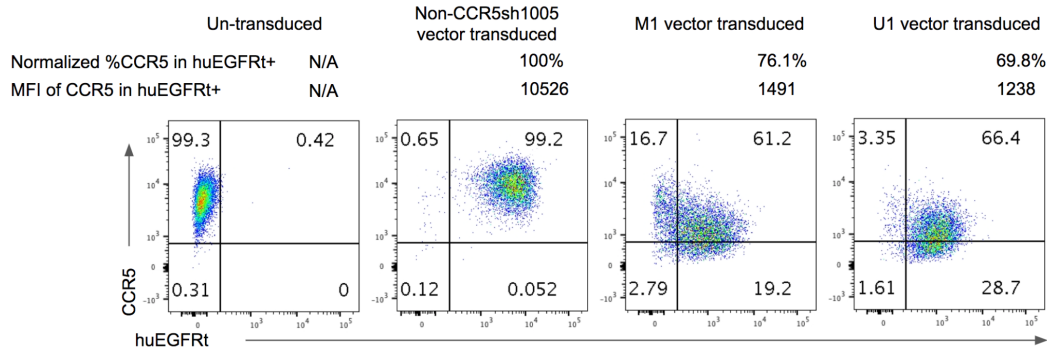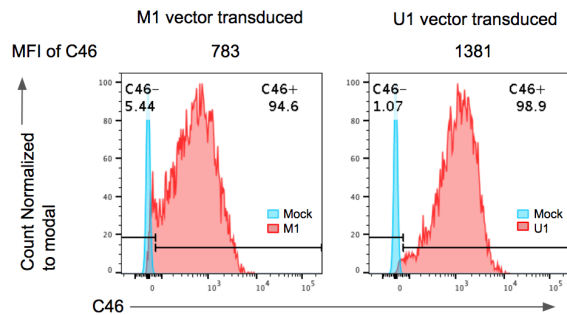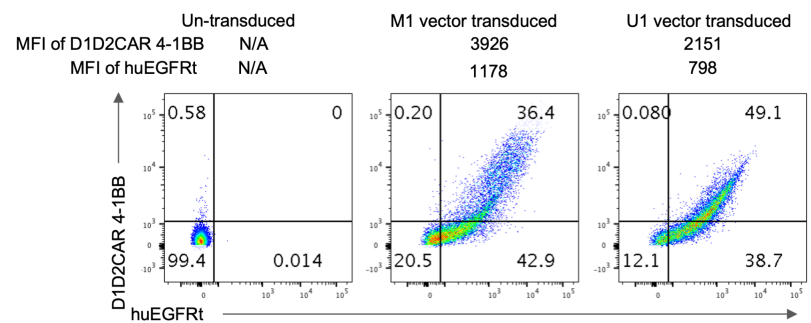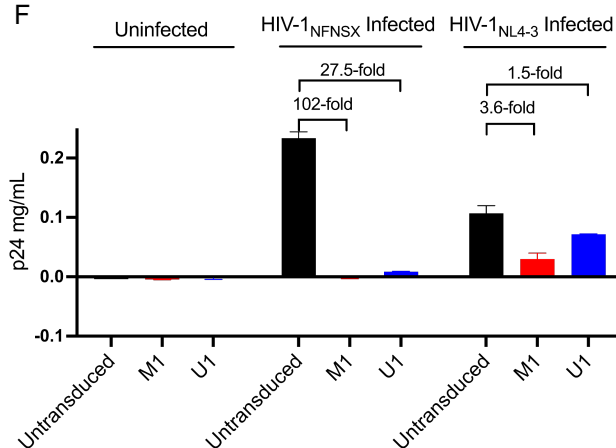
A

MNDU-antiHIV-1-huEGFRt (M1)

5'LTR — H1 — CCR5sh1005 — MNDU — D1D2CAR 41BB — T2A — huEGFRt — EF1α — C46 — WPRE — 3'LTR

UbC-antiHIV-1-huEGFRt (U1)

5'LTR — H1 — CCR5sh1005 — UbC — D1D2CAR 41BB — T2A — huEGFRt — EF1α — C46 — WPRE — 3'LTR

Non-CCR5sh1005 Vector

5'LTR — MNDU — D1D2CAR 41BB — T2A — huEGFRt — EF1α — C46 — WPRE — 3'LTR

B

Titer IU/ml — M1 vs U1 — **

C

	Un-transduced	Non-CCR5sh1005 vector transduced	M1 vector transduced	U1 vector transduced
Normalized %CCR5 in huEGFRt+	N/A	100%	76.1%	69.8%
MFI of CCR5 in huEGFRt+	N/A	10526	1491	1238

CCR5 vs huEGFRt

Un-transduced: 99.3 / 0.42 / 0.31 / 0
Non-CCR5sh1005: 0.65 / 99.2 / 0.12 / 0.052
M1: 16.7 / 61.2 / 2.79 / 19.2
U1: 3.35 / 66.4 / 1.61 / 28.7

D

	M1 vector transduced	U1 vector transduced
MFI of C46	783	1381

M1: C46− 5.44 / C46+ 94.6 — Mock, M1
U1: C46− 1.07 / C46+ 98.9 — Mock, U1

Count Normalized to modal vs C46

E

	Un-transduced	M1 vector transduced	U1 vector transduced
MFI of D1D2CAR 4-1BB	N/A	3926	2151
MFI of huEGFRt	N/A	1178	798

D1D2CAR 4-1BB vs huEGFRt

Un-transduced: 0.58 / 0 / 99.4 / 0.014
M1: 0.20 / 36.4 / 20.5 / 42.9
U1: 0.080 / 49.1 / 12.1 / 38.7

F

p24 mg/mL

Uninfected: Untransduced, M1, U1
HIV-1_NFNSX Infected: Untransduced, M1, U1 — 102-fold, 27.5-fold
HIV-1_NL4-3 Infected: Untransduced, M1, U1 — 3.6-fold, 1.5-fold

G

% Specific Killing vs E:T ratio (1:1, 2:1, 5:1)

M1 Env⁺ — M1 Env⁻ — p
U1 Env⁺ — U1 Env⁻ — p