

1 TAILORED DC INDUCE PROTECTIVE HIV-1 SPECIFIC POLYFUNCTIONAL CD8+  
2 T CELLS IN THE LYMPHOID TISSUE FROM HUMANIZED BLT MICE

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24

## 25 **Abstract**

26 Effective function of CD8<sup>+</sup> T cells and enhanced innate activation of dendritic cells (DC) in  
27 response to HIV-1 is linked to protective antiviral immunity in controllers. Manipulation of DC  
28 targeting the master regulator TANK-binding Kinase 1 (TBK1) might be useful to acquire  
29 controller-like properties. Here, we evaluated the impact of TBK1-primed DC inducing protective  
30 CD8<sup>+</sup> T cell responses in lymphoid tissue and peripheral blood and their association with reduced  
31 HIV-1 disease progression *in vivo* in the humanized bone marrow, liver and thymus (hBLT) mouse  
32 model. A higher proportion of hBLT-mice vaccinated with TBK1-primed DC exhibited less severe  
33 CD4<sup>+</sup> T cell depletion following HIV-1 infection compared to control groups. This was associated  
34 with infiltration of CD8<sup>+</sup> T cells in the white pulp from the spleen, reduced spread of infected p24<sup>+</sup>  
35 cells to secondary lymphoid organs and with preserved abilities of CD8<sup>+</sup> T cells from the spleen  
36 and blood of vaccinated animals to induce specific polyfunctional responses upon antigen  
37 stimulation. Therefore, TBK1-primed DC might be an useful tool for subsequent vaccine studies.

## 38 **Author summary**

39 Emulating protective immunological characteristics from individuals capable of spontaneously  
40 controlling HIV-1 infection might be useful for the development of a protective vaccine. Enhanced  
41 function of dendritic cells (DC) in these HIV-1 controllers depends on the activation of TANK-  
42 binding Kinase 1 (TBK1) and might associate with protective T cells. Our study evaluated the  
43 ability of DCs trained through TBK1 activation inducing protective adaptive immune responses  
44 against HIV-1 and reducing disease progression *in vivo*, using a humanized mouse model. Our  
45 data indicate that mice vaccinated with tailored DC exhibit delayed disease progression, increased  
46 induction of protective CD8<sup>+</sup> T lymphocyte subsets in the lymphoid tissue and blood upon antigen  
47 recognition. Therefore, trained-DC might be an useful tool for future HIV-1 vaccine designs.

48 **Introduction**

49

50 A remaining challenge to end the HIV-1 pandemic is the development of an effective vaccine  
51 capable of providing protective and long-lasting immunity against HIV-1 infection. While  
52 previous efforts to achieve this goal have failed (1, 2), the scientific community has come to  
53 understand that the induction of effective and durable HIV-1-specific T cell responses in different  
54 anatomical compartments will most likely require the targeting and fine-tuning of specific innate  
55 immune cell subsets, such as dendritic cells (DC). DC play a critical role during the priming of  
56 specific adaptive immune responses, since they are capable of both efficiently presenting antigens  
57 (Ags) to T cells and also mediating the polarization of effector lymphocytes (3-7). In fact, DC-  
58 based therapeutic vaccines have shown very promising results in clinical trials for cancer therapy  
59 (8). However, although encouraging, previous DC-based HIV-1 vaccination strategies have  
60 demonstrated limited abilities priming durable memory HIV-1-specific T cell responses (9-13). In  
61 addition, most vaccine studies used adjuvants systemically as a means to globally increase innate  
62 immune activation, without considering their individual impact on specific DC functional  
63 characteristics (14).

64 Previous studies showed that conventional DC (cDC) from HIV-1 elite controllers (EC) are  
65 capable of efficiently detecting HIV-1 reverse transcripts (15, 16) and inducing activation of the  
66 signal transducer TANK-binding Kinase 1 (TBK1) (17, 18). This mechanism leads to enhanced  
67 capabilities to prime polyfunctional HIV-1-specific CD8<sup>+</sup> T cell responses, which are associated  
68 with effective control of HIV-1 infection (19-22). Therefore, TBK1 may, in principle, represent a  
69 therapeutic target to improve DC maturation towards an EC-like phenotype and to more efficiently  
70 activate protective antiviral CD8<sup>+</sup> T cell responses in a broader population of individuals.  
71 Combined stimulation of DC with ligands to multiple intracellular sensors upstream TBK1 such

72 as cGAS, RIG-I, MDA5 or TLR3 (23), could synergistically act as TBK1 adjuvants and further  
73 improve the function of these cells. Supporting this possibility, initial studies suggested that the  
74 maturation of DC in the presence of the TLR3/RIG-I ligand Poly I:C boosts HIV-1-specific T cell  
75 responses from HIV-1-infected individuals *in vitro* (24). Multiple vaccine studies have mainly  
76 focused on analyzing activation patterns on circulating HIV-1-specific T cells, despite growing  
77 evidence of the critical role of lymphoid tissue-resident T cells controlling HIV-1 or simian  
78 immunodeficiency virus (SIV) (25, 26). Therefore, it is critical to determine the efficiency and  
79 relevance of potential novel DC-vaccine strategies inducing HIV-1-specific adaptive immune  
80 responses *in vivo* in different tissue locations.

81 The non-human primate model has been traditionally recognized as the gold standard *in vivo*  
82 model to test HIV-1 vaccine candidates (27). However, in addition to intrinsic differences with the  
83 human organism, this *in vivo* model might not always be accessible for initial phases of vaccine  
84 candidate evaluation. Immunodeficient NOD/SCID IL2R $\gamma^{-/-}$  (NSG) mice transplanted with human  
85 fetal hematopoietic stem cells, liver and thymus (here after referred to as hBLT-mouse) represent  
86 a more accessible humanized *in vivo* system that recapitulates the development of most human  
87 myeloid and lymphoid lineages (28-31). Importantly, hBLT-mice can be infected with HIV-1 and  
88 meet some aspects of HIV-1 disease progression, such as the depletion of CD4 $^{+}$  T cell lymphocytes  
89 and the induction of specific adaptive immune responses, including cytotoxic CD8 $^{+}$  T cells (CTL)  
90 (32-35). Moreover, the hBLT model supports the induction of effector memory HIV-1-specific  
91 CD8 $^{+}$  T cells similar to those observed in previous vaccine studies (12, 36, 37). Despite some  
92 limitations, the hBLT mouse represents a very attractive model for a proof-of-concept of HIV-1  
93 vaccine study. Recent data indicate that the immunization of hBLT mice with HIV-1 Gag protein  
94 potentiates the induction of Gag-specific T cells capable of reducing HIV-1 viremia and forcing

95 viral escape mutations (38). However, whether the hBLT model supports the induction of  
96 protective T cell responses in different lymphoid tissue compartments that could actively  
97 contribute to viral control after vaccination has not been studied in detail. In addition, little, if any,  
98 information on the polyfunctional characteristics of CD8<sup>+</sup> T cells, a critical hallmark of immune  
99 control of HIV-1 infection (39, 40), has been described in this system. Finally, the impact and  
100 potential benefit of a DC-based HIV-1 vaccine on the induction of HIV-1 specific T cells and  
101 disease progression have not been tested in the hBLT mouse model yet.

102 In this study, we assessed the ability of TBK1-primed DC to improve parameters of immune  
103 protection against HIV-1 in the lymphoid tissue and peripheral blood using the hBLT mouse  
104 model. Our data indicate that TBK1-primed DC potentiate the infiltration of CD8<sup>+</sup> T cells in the  
105 white pulp of spleen and the retention of infected HIV-1 p24<sup>+</sup> cells in these areas, preventing viral  
106 spread to secondary lymphoid organs. These histological parameters induced by TBK1 DC-  
107 vaccination correlated with preserved abilities to induce polyfunctional CD8<sup>+</sup> T cell responses in  
108 the spleen upon HIV-1 Gag stimulation and with less severe depletion of CD4<sup>+</sup> T cells at late time  
109 points of infection in vaccinated hBLT mice. Our study provides novel evidence of enhanced  
110 cellular immunity against HIV-1 in the lymphoid tissue induced by a tailored DC-based vaccine  
111 *in vivo*, which could be useful for the development of new vaccine strategies.

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118 **Results**

119 *Combination of TBK1 adjuvants improves maturation and functional properties of DC*

120 We first evaluated the efficacy of potential TBK1 adjuvants such as the TLR3 and STING agonists

121 Poly I:C and 2'3'-di-AM(PS) to enhance maturation and functional properties of DCs. To this end,

122 we stimulated Monocyte derived-DC (MDDC) and primary circulating CD1c<sup>+</sup> cDCs with these

123 molecules individually or in combination and monitored the phosphorylation of TBK1 and the

124 downstream effector IRF3 as a readout of activation. As shown in Figure 1A and Supplemental

125 Figure 1A, stimulation of both MDDC and cDC with a combination of the STING agonist and

126 Poly:IC led to a more significant increase in TBK1 and IRF3 phosphorylation compared to

127 individual treatments. Therefore, simultaneous stimulation with the STING agonist and Poly I:C

128 could have significant impact on the activation and subsequent maturation of DC. To test this, we

129 assessed expression of maturation markers and the transcription of inflammatory cytokines on

130 primary cDCs stimulated with TBK1 adjuvants. As shown in Supplemental Figure 1B, both

131 STING agonist and Poly I:C were able to significantly increase expression of CD40 and CD86

132 individually, and the combination of both TBK1 adjuvants led to limited but significant additional

133 increase in the expression of CD40. We observed that the combination of the STING agonist and

134 Poly I:C induced significantly higher mRNA levels of IFN $\beta$ , IL-12 and, to some extent, TNF $\alpha$ ,

135 suggesting an enhancement in the maturation program of cDC (Figure 1B). To determine whether

136 these changes in cDC could be translated into improved functional antigen presenting cell

137 properties, we first performed co-cultures of total T cells with allogeneic cDC pre-incubated in

138 media or in the presence of different combinations of TBK1 adjuvants. cDC treated with both Poly

139 I:C and the STING agonist were capable of inducing higher proportions of CD8<sup>+</sup>T cells co-

140 expressing IFN $\gamma$  and the degranulation marker CD107a (Figure 1C). Importantly, we observed that

141 treatment of PBMC from healthy donors with Poly I:C and STING agonist in the presence of a  
142 pool of HIV-1 Gag peptides and a subsequent boost with autologous Gag-peptide loaded cDC  
143 stimulated with both TBK1 adjuvants enhanced *de novo* induction of IFN $\gamma$ <sup>+</sup> HIV-1 Gag-specific  
144 CD8<sup>+</sup> T cells *in vitro* (Figure 1D, left). Moreover, significantly higher proportions of IFN $\gamma$ <sup>+</sup> CD8<sup>+</sup>  
145 T cells co-expressing CD107a were detected in the presence of Gag-peptide loaded cDC stimulated  
146 with TBK1 adjuvants (Figure 1D, right), suggesting enhanced polyfunctionality in HIV-1-specific  
147 cells. Importantly, these effects were dependent on the presence of the Ag, since no significant  
148 increase of T cell responses was observed after stimulation only with TBK1 adjuvants  
149 (Supplemental Figure 1C). Together, these data suggest that combination of of Poly I:C and  
150 STING agonists as effective TBK1 adjuvants potentiating the maturation and function of cDCs *in*  
151 *vitro*.

152

153 *Vaccination of hBLT-mice with TBK1-primed DC reduces HIV-1-mediated disease progression*

154 We next determined whether DC activated in the presence of the TBK1 adjuvant cocktail could  
155 also induce protective responses against HIV-1 infection *in vivo* using the hBLT mouse model. To  
156 ensure that only DC were manipulated with TBK1 adjuvants, we differentiated CD11c<sup>+</sup> CD14<sup>-</sup>  
157 HLA<sup>+</sup> cDC and CD11c<sup>+</sup> CD14<sup>+</sup> HLA<sup>+</sup> MoDC-like cells *in vitro* from a portion of the human  
158 fetal CD34<sup>+</sup> HSC precursors used to reconstitute the hBLT mice prior to vaccination  
159 (Supplemental Figure 2A). HSC-derived cDC and MoDC were sorted and cultured separately for  
160 24h in the presence of media alone (MED), a pool of peptides from HIV-1 Gag alone (GAG) or in  
161 combination with our TBK1 adjuvant cocktail (GAG-ADJ) (Supplemental Figure 2B-C). The  
162 individual addition of Gag peptides did not induce significant activation of sorted cells  
163 (Supplemental Figure 2C). However, despite differences in basal expression of activation markers,

164 both sorted DC subsets responded to the adjuvant stimulation (Supplemental Figure 2C) and cDC  
165 and MoDC from each condition were pooled for vaccination (Supplemental Figure 2D). In two  
166 experiments performed with different batches of hBLT mice, a total of n=42 hBLT animals were  
167 subdivided in 3 groups of n=14 animals that were vaccinated intravenously with either MED, GAG  
168 or GAG-ADJ DC by injection in the tail vein (Supplemental Figure 2D). Two weeks after  
169 vaccination, mice were intravenously infected with 10,000 TCID<sub>50</sub> of JRCF HIV-1 strain. Prior  
170 pilot experiments indicated that HIV-1 plasma viremia begins to stabilize by 3 weeks post-  
171 infection (p.i.) and reaches a stable setpoint by 6 weeks p.i. in hBLT (Supplemental Figure 3A).  
172 In addition, it has been reported that at 6 weeks p.i. depletion of CD4<sup>+</sup> T cells and HIV disease  
173 progression reproducibly becomes more evident in hBLT mice infected with JRCSF HIV-1 (32)  
174 and is the peak time point of detection of HIV-1 specific T cell responses in the blood of these  
175 animals (38). Therefore, we analyzed clinical, histological and cellular parameters associated with  
176 protection or disease progression at three, five/six and six/seven weeks p.i. to cover these critical  
177 time points (Supplemental Figure 2D). As shown in Supplemental Figure 2E, no differences in  
178 weight were observed among the three hBLT mouse groups prior or after HIV-1 infection,  
179 suggesting vaccination did not have any significant impact on the induction of GvHD. Although  
180 all hBLT mouse groups experienced a significant reduction in circulating hCD4<sup>+</sup> T cells 3 weeks  
181 after infection with HIV-1 compared to baseline (Supplemental Figure 4A, C), we observed a  
182 noticeably less severe CD4<sup>+</sup> T cell depletion in the GAG-ADJ group (Figure 2A) at 5/6 weeks post  
183 infection (Figure 2A, Supplemental Figure 4A-C). Consistently, CD4<sup>+</sup>/CD8<sup>+</sup> T cell ratio in the  
184 blood tended to be higher in GAG-ADJ mice at later time points of infection (Supplemental Figure  
185 4B). Notably, the GAG-ADJ vaccinated group included a significantly higher proportion of  
186 animals experiencing less than 0.5-fold reduction in circulating CD4<sup>+</sup> T cell numbers (CD4Hi

187 phenotype) at these late time points (Figure 2A; Supplemental Figure 4A-C). In contrast, mice  
188 vaccinated with GAG DC experienced a dramatic depletion of CD4<sup>+</sup> T cells below 0.5-fold  
189 threshold (CD4Lo phenotype) in the majority of animals from this group (Figure 2A, Supplemental  
190 Figure 4A-C). Similar results were obtained at 6/7 weeks p.i., but differences between vaccinated  
191 groups were more pronounced at 5/6 weeks p.i. (Supplemental Figure 4A-C). These effects were  
192 consistently observed in the two independent hBLT mouse batches (Supplementary Figure 4A-C).  
193 Interestingly, mice vaccinated with MED DC that had not received adjuvant or Ag were  
194 characterized by an intermediate phenotype of 50% animals exhibiting dramatic (<0.5-fold  
195 decrease) and 50% less severe (>0.5-fold decrease) depletion of CD4<sup>+</sup> T cells, suggesting a partial  
196 and Ag-independent effect of vaccination with immature DC (Figure 2A, Supplemental Figure  
197 4A-B). Interestingly, while not significant differences in plasma viremia were observed at any time  
198 point between the total 3 groups of vaccinated animals (Supplemental Figure 3B), we observed an  
199 enrichment of lower viral loads at 3 weeks p.i. in those hBLT mice displaying a less severe CD4Hi  
200 phenotype at 5/6 weeks p.i., which again were more significantly enriched in the GAG-ADJ and  
201 MED animal groups (Figure 2B). The early control of viremia seemed to be transient and no  
202 significant differences were observed in plasma viral loads by 6/7 weeks p.i (Supplemental Figure  
203 3C). These data indicate that vaccination of hBLT mice with TBK1-trained DC is associated with  
204 less severe depletion of CD4<sup>+</sup> T cells and a concomitant partial early control of HIV-1 viremia,  
205 suggesting delayed progression of HIV-1 infection in these animals.

206

207 *Accumulation of CD8<sup>+</sup> T cells and HIV-1 infected cells in the white pulp after vaccination with*  
208 *TBK1-primed DC*

209 To better understand differences in HIV-1 disease progression in the three groups of vaccinated  
210 hBLT mice, we analyzed histological distribution of CD8<sup>+</sup> T cells and infected p24<sup>+</sup> cells by  
211 immunofluorescence in tissue sections from spleen and lymph nodes (LN) from the hBLT mice at  
212 6/7 weeks p.i. As shown in Figure 3A-B and 4A-B, p24<sup>+</sup> HIV-1-infected cells could be detected  
213 in the spleen and LN of all hBLT mice, consistent with previous observations (32). No differences  
214 were observed in total HIV-1 p24<sup>+</sup> cell counts in the spleen of infected animals (Supplemental  
215 Figure 5B, lower panel), and a weak enrichment on total splenic Granzyme B<sup>+</sup> CD8<sup>+</sup> T cells was  
216 observed in tissue sections from hBLT mice vaccinated with GAG-ADJ DC (Supplemental Figure  
217 5B, upper panel). However, infiltrated CD8<sup>+</sup> T cells were significantly higher in white pulp areas  
218 defined by hematoxylin/eosin staining, from the spleens from GAG-ADJ vaccinated hBLT mice  
219 at 6/7 weeks p.i. (Figure 3B left panel, Supplemental Figure 5A). Interestingly, increased  
220 infiltration of CD8<sup>+</sup> T cells in the white pulp was significantly associated with expression of  
221 Granzyme B in the spleen (Figure 3B, right). Additionally, we observed significantly increased  
222 proportions of Granzyme B<sup>+</sup> cytotoxic CD8<sup>+</sup> T cells in the surrounding red pulp areas in the spleen  
223 of GAG-ADJ mice, and higher frequencies of these cells correlated with infiltration of CD8<sup>+</sup> T  
224 cells in the white pulp (Supplemental Figure 5C-D). Interestingly, CD8<sup>+</sup> T cells infiltrated in the  
225 spleen white pulp also tended to express higher levels of Granzyme B and CXCR5 in GAG-ADJ  
226 mice (Supplemental Figure 5C,F). In contrast, opposite patterns were observed in GAG mice  
227 (Supplemental Figure 5C,F). Notably, we also observed a significantly higher accumulation of  
228 infected p24<sup>+</sup> cells in white pulp areas of spleen from these GAG-ADJ hBLT mice compared to  
229 those vaccinated with GAG, which was correlated with increased infiltration of CD8<sup>+</sup> T cells in  
230 this area (Figure 3C). These effects were more significantly appreciated in CD4Hi ADJ-GAG  
231 hBLT mice displaying less severe depletion of CD4<sup>+</sup> T cells compared to GAG-only hBLT mice

232 (Supplemental Figure 5E). In contrast, reduced frequencies of HIV-1 p24<sup>+</sup> cells and enrichment  
233 on CD8<sup>+</sup> T cells were observed in the LN of GAG-ADJ hBLT mice and more significantly in mice  
234 from this group experiencing less severe depletion of CD4<sup>+</sup> T cells (Figure 4 A-C; Supplemental  
235 Figure 6B.). In addition, numbers of p24<sup>+</sup> cells per area of the LN correlated with viral load  
236 detection either at early time-points (3 weeks p.i.) and late time-points (5-6 and 6-7 weeks p.i.)  
237 and were inversely associated with CD8<sup>+</sup> T cell recruitment in the spleen. (Supplemental Figure  
238 6C; Figure 4E). Moreover, CD8<sup>+</sup> T cells recruited in the LN from GAG-ADJ vaccinated hBLT  
239 animals distributed in significantly larger cell clusters (Figure 4D, Supplemental Figure 6A-D).  
240 Clustered CD8<sup>+</sup> T cells did not appear preferentially express Granzyme B (Figure 4A, right).  
241 Importantly, we observed that these histological CD8<sup>+</sup> T cell aggregation patterns were  
242 significantly inversely associated with less detection of p24<sup>+</sup> cells in the LN and positively  
243 correlated with increased recruitment of CD8<sup>+</sup> T cells to the spleen and infiltration in the white  
244 pulp areas and with less severe depletion of CD4<sup>+</sup> T cells at 5/6 weeks p.i. (Figure 4E-F). Our data  
245 clearly indicate that vaccination of hBLT mice with TBK1-trained DC induces specific and  
246 interconnected histological patterns of infiltrated CD8<sup>+</sup> T cell responses in the spleen that are  
247 associated with the retention of HIV-1 infected cells in this organ, preventing the spread and  
248 progression of HIV-1 infection in peripheral organs from these mice.

249

250 *Polyfunctional CD8<sup>+</sup> T cell responses in lymphoid tissue and blood from hBLT mice vaccinated*  
251 *with GAG-ADJ-DC*

252 We next addressed the polyfunctional profiles of splenic and circulating CD8<sup>+</sup> T cells from the  
253 three vaccinated hBLT mouse groups by analyzing expression of IFN $\gamma$ , IL-2, TNF $\alpha$  and CD107a  
254 *ex vivo* and after stimulation with a pool of HIV-1 Gag peptides at 3 and 6/7 weeks post-infection.

255 We analyzed the proportions of T cells co-expressing 2, 3 and 4 of the analyzed parameters as a  
256 readout for polyfunctionality and quantified all individual cell subsets by Boolean gating  
257 (Supplemental Figure 7). Overall, basal levels of cells displaying higher polyfunctionality tended  
258 to be increased on splenic and circulating CD8<sup>+</sup> T cells from GAG-ADJ DC vaccinated mice, but  
259 differences did not reach statistical significance in all cases (Supplemental Figure 7A). In fact,  
260 only polyfunctional cells co-expressing two-parameters were significantly higher in the blood at  
261 6/7 weeks p.i. of GAG-ADJ mice compared to GAG and MED control groups (Supplemental  
262 Figure 7C). In contrast, we observed a gradual increase in the induction of polyfunctional splenic  
263 CD8<sup>+</sup> T cells co-expressing 3 and 2 out of 4 analyzed parameters after Gag peptide stimulation in  
264 the hBLT mice groups receiving GAG and GAG-ADJ DC vaccines. These differences were only  
265 significant in the GAG-ADJ hBLT group compared to MED mice (Supplemental Figure 7B;  
266 Figure 5C, upper plot). In fact, the increase of 3-parameter polyfunctional CD8<sup>+</sup> T cells in GAG-  
267 ADJ hBLT mice after Gag peptide stimulation was driven by a more significant increase in the  
268 proportion of two specific subpopulations of CD107a<sup>+</sup>INF $\gamma$ <sup>+</sup>IL2<sup>-</sup>TNF $\alpha$ <sup>+</sup> and CD107a<sup>-</sup> INF $\gamma$ <sup>+</sup> IL2<sup>+</sup>  
269 TNF $\alpha$ <sup>+</sup> CD8<sup>+</sup> T cells, while the other combinations did not reach significance (Supplemental  
270 Figure 9A, Figure 5C bottom plot). To better determine whether the presence or the absence of  
271 total or specific polyfunctional populations was associated with reduced progression of HIV-1  
272 infection, we performed a correlation network between these cell subsets and the clinical and  
273 histological parameters previously observed (Supplemental Figure 8). This unbiased approach  
274 identified histological and T cell responses significantly associated with viral control (Figure 5A-  
275 B, D; Supplemental Figure 8A-B). In the spleen, we did not observe any significant association of  
276 histological or clinical parameters with basal polyfunctional CD8<sup>+</sup> T cell profiles differentially  
277 induced in the GAG-ADJ hBLT mice (Supplemental Figure 8A). However, significant

278 associations between proportions of antigen-mediated induction of splenic polyfunctional CD8<sup>+</sup> T  
279 cells co-expressing 3 parameters were found with less severe depletion of CD4<sup>+</sup> T cell counts,  
280 infiltration of CD8<sup>+</sup> T cells in the white pulp areas from the spleen and lower detection of infected  
281 p24<sup>+</sup> cells in the LN (Figure 5D, Supplemental Figure 9B). Interestingly, when we analyzed which  
282 of the two Ag-induced 3-parameter polyfunctional T cells were more associated with virological,  
283 immunological and histological patterns, we observed that the population of CD107a<sup>+</sup> IFN $\gamma$ <sup>+</sup> IL2<sup>-</sup>  
284 TNF $\alpha$ <sup>+</sup> CD8<sup>+</sup> T cells significantly induced in GAG-ADJ hBLT mice was more significantly  
285 correlated with these parameters than the other CD107a<sup>-</sup> INF $\gamma$ <sup>+</sup> IL2<sup>+</sup> TNF $\alpha$ <sup>+</sup> subset of CD8<sup>+</sup> T cells  
286 induced in these animals (Figure 5C bottom panel; Supplemental Figure 9A-C). In particular  
287 frequencies of these CD107a<sup>+</sup>IFN $\gamma$ <sup>+</sup>IL2<sup>-</sup>TNF $\alpha$ <sup>+</sup> CD8<sup>+</sup> T cells correlated more significantly with  
288 lower pVL at earlier time points (3 wk p.i; p=0.0081) and with higher CD4<sup>+</sup>/CD8<sup>+</sup> T cell ratios in  
289 the blood (p=0.0468), higher infiltration of CD8<sup>+</sup> T cells in the spleen (p=0.0010) and lower  
290 detection of p24<sup>+</sup> cells in the LN (p=0.0030) at the time of sacrifice (6/7wk p.i) (Supplemental  
291 Figure 9A-C). These data suggest that CD8<sup>+</sup> T cells from the spleen of GAG-ADJ hBLT mice  
292 display preserved abilities to induce specific patterns of polyfunctional cytotoxic and cytokine  
293 secreting cell subsets after antigen re-stimulation.

294 Our analyses also indicated that preserved polyfunctional responses after antigen stimulation from  
295 circulating cells were also associated with control of HIV-1 infection in hBLT mice. Interestingly,  
296 non-specific higher basal frequencies of polyfunctional CD107a<sup>+</sup> IFN $\gamma$ <sup>+</sup> IL2<sup>+</sup> TNF $\alpha$ <sup>+</sup> in the  
297 absence of Ag stimulation and increased induction after Gag-stimulation of CD107a<sup>-</sup> IFN $\gamma$ <sup>+</sup> IL2<sup>+</sup>  
298 TNF $\alpha$ <sup>+</sup> cells in circulating CD8<sup>+</sup> T cells were not associated with protection parameters but seemed  
299 to be indicative of pronounced disease progression (Figure 5A-B, Supplemental Figure 9D).  
300 However, we found that the polyfunctional CD107a<sup>+</sup>IFN $\gamma$ <sup>+</sup>IL2<sup>-</sup>TNF $\alpha$ <sup>-</sup> cell population induced by

301 Gag peptide stimulation from circulating CD8<sup>+</sup> T cells at 3 weeks p.i. which was more significantly  
302 increased in GAG-ADJ hBLT mice (Figure 5E, left plot). Although proportions of these  
303 polyfunctional CD107a<sup>+</sup>IFN $\gamma$ <sup>+</sup>IL2<sup>+</sup>TNF $\alpha$ <sup>-</sup> at 3 weeks p.i. was not directly associated with clinical  
304 and histological parameters (Figure 5A-B), this subset significantly correlates with subsequent  
305 increased proportions of protective Ag-induced CD107a<sup>+</sup>IFN $\gamma$ <sup>+</sup>IL2<sup>+</sup>TNF $\alpha$ <sup>+</sup> (Figure 5E, right  
306 plots). Together, our results indicate that vaccination of hBLT mice with TBK1 trained DC  
307 enhance Ag-inducible precursors of polyfunctional T cell responses on circulating cells that can  
308 serve as biomarkers of tissue polyfunctionality and reduced progression of HIV-1 infection.

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324 **Discussion**

325 Our study evaluates the efficacy of DC simultaneously matured with two TBK1 adjuvants, a  
326 STING agonist and Poly I:C, inducing parameters of immune control of HIV-1 infection *in vivo*.  
327 We demonstrate that vaccination with TBK1-tailored DC is associated with reduced progression  
328 of HIV-1 disease in hBLT mouse model. Previous clinical trials evaluated the benefit of systemic  
329 administration of Poly I:C to HIV-1 infected individuals, and demonstrated an increase of HIV-1-  
330 specific T cell responses but the therapeutic benefit of this format remains unclear (41-43). In  
331 addition, while previous studies in a murine model suggested that HIV-1 vaccines administered  
332 systemically targeting DC via CD40 or DEC205 and Poly I:C as an adjuvant could induce antigen-  
333 specific responses (44-46), our work provides new proof of concept of beneficial effects of the  
334 administration of a TBK1-tailored DC vaccine in an *in vivo* humanized model without systemic  
335 adjuvant addition, which can trigger other cell populations. This is particularly relevant since it  
336 has been shown that systemic Poly I:C administration can lead to HIV-1 reactivation on CD4<sup>+</sup> T  
337 cells (45, 47). Moreover, this study specifically explores the benefit of enhanced maturation of DC  
338 in the presence of a combination of Poly I:C and STING agonist, potentiating phosphorylation of  
339 TBK1 and IRF3 and more efficiently inducing the secretion of immunomodulatory cytokines such  
340 as IL-12 and IFN $\beta$ , which was associated with an increase of DC antigen presenting properties.  
341 However, we cannot completely rule out that in addition to activating TBK1, some of the adjuvants  
342 used in our study such Poly I:C could be also triggering additional pathways, which might also  
343 affect DC maturation. Despite this possibility, our data indicate that our combined adjuvant  
344 strategy is able to recapitulate some of the enhanced functional properties previously observed in  
345 DC from HIV-1 elite controllers (15, 48).

346 Importantly, while previous studies on HIV-1 vaccine prototypes have mainly focused on the  
347 phenotype or even polyfunctionality induced circulating T cells (49, 50), we were able to identify  
348 cellular and histological parameters associated with reduced spread of HIV-1 infection to  
349 secondary lymphoid organs, such as the spleen and the lymph nodes. Moreover, vaccination of  
350 hBLT mice with TBK1-tailored DC induced higher levels of infiltration of CD8<sup>+</sup> T cells in white  
351 pulp areas of spleen, which were associated with accumulation of infected HIV-1 p24<sup>+</sup> cells in  
352 these areas. This splenic phenotype was associated with higher volume of CD8<sup>+</sup> T cell clusters and  
353 lower detection of infected cells in the lymph node of hBLT mice. These histological patterns bear  
354 some resemblance to follicular CD8<sup>+</sup> T cell responses observed in primates able to control viral  
355 infection (26) and in HIV-1 controller patients (25). In fact, we observed expression of CXCR5  
356 preferentially on CD8<sup>+</sup> T cells infiltrating the white pulp areas from GAG-ADJ hBLT mice, which  
357 might support a follicular-like phenotype previously linked to viral control (51, 52). However,  
358 since deficiencies in lymphoid tissue architecture have been described in the hBLT model (32, 53),  
359 further characterization of white-pulp resident CD8<sup>+</sup> T cells in the hBLT mouse needs to be  
360 conducted in order to better understand these potential similarities. In fact, in our study we did not  
361 address the causal relationships between the enrichment in cytotoxic CD8<sup>+</sup> T cells in the red pulp  
362 and the infiltration of CXCR5<sup>+</sup> CD8<sup>+</sup> T cells in the white pulp and the differential accumulation  
363 of HIV-1 p24<sup>+</sup> cells observed in these areas. Furthermore, the relationship between the observed  
364 histological distribution of splenic CD8<sup>+</sup> T cells with inflammatory tissue fibrosis, previously  
365 linked to immunopathology of HIV-1 infection or the presence of CXCR5<sup>+</sup> CD4<sup>+</sup> T cells, was not  
366 addressed in our study and deserves further investigation (54, 55).

367 In addition to histological patterns, we identified in vaccinated hBLT mice preserved abilities of  
368 splenic CD8<sup>+</sup> T cells to induce a polyfunctional population of tissue resident CD107a<sup>+</sup> IFN $\gamma$ <sup>+</sup>

369 TNF $\alpha$ <sup>+</sup> IL2<sup>-</sup> cells upon Ag stimulation that was associated with less severe depletion of circulating  
370 CD4<sup>+</sup> T cells, higher infiltration of CD8<sup>+</sup> T cells in the white pulp areas and lower numbers of  
371 infected p24<sup>+</sup> cells in the lymph node, thus underscoring that these cells could display effective  
372 antiviral properties. Supporting this possibility, a number of studies have described that  
373 polyfunctional T cells co-expressing TNF $\alpha$  with other parameters correlate with protection against  
374 viral infections such as Zika and Cytomegalovirus (56, 57). However, future studies are needed to  
375 better understand the developmental kinetics and functional relationships of this particular subset  
376 of polyfunctional cells with other subpopulations that might also be present in the GAG-ADJ  
377 hBLT mice. While our data also indicate that TBK1-primed DC vaccination could induce control  
378 on plasma viral load, these effects could be mediated by HLA-variability or HIV-1 escape  
379 mutations induced in vaccinated hBLT mouse (33, 38). Although our study suggests that TBK-1  
380 DC can induce multiple histological and immunological parameters associated with immune  
381 control of HIV-1 infection, we focused on analyzing them at key time points previously described  
382 to mark HIV-1 pathogenesis and detection of HIV-1 responses in the blood. Thus, further  
383 longitudinal studies with a larger number of hBLT mice and a broader range of time point analyses  
384 are required to better establish the impact and evolution of the identified histological and  
385 immunological parameters during the course of HIV-1 infection and their relationship with  
386 protection.

387 Finally, an additional limitation of our study was the relatively high TCID<sub>50</sub> dose and the  
388 administration route of HIV-1 to the hBLT mice studied. The primary objective of our study was  
389 to address whether vaccination of mice with TBK1-tailored DC could induce some level of  
390 protection against progression of HIV-1 infection in a model in which we ensured infection of all  
391 mice. However, new studies evaluating the beneficial effect of TBK1-tailored DC under more

392 physiological conditions such as the use of lower viral titers and a mucosal administration route  
393 should be conducted. Despite these limitations, our study provides evidence of the beneficial  
394 effect of TBK1-tailored DC inducing more effective immune responses against HIV-1 at the  
395 histological, clinical, and cellular levels, and therefore it may be useful for the development of  
396 future vaccine strategies against HIV-1.

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415 **Materials and Methods**

416 *Isolation of human peripheral blood populations*

417 Human Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll (Pancoll, PAN  
418 Biotech) gradient centrifugation. Subsequently, conventional Dendritic Cells (cDC) and total T  
419 cells were purified from PBMC suspensions by negative immunomagnetic selection (purity >90%)  
420 using the Human Myeloid DC Enrichment Kit (STEMCELL) and the Untouched total human T  
421 cell (Invitrogen) kits, respectively. Monocyte-Derived Dendritic Cells (MDDC) were generated  
422 from adherent cells present in PBMCs and cultured in the presence of 100IU/ml of GM-CSF and  
423 IL-4 (Prepotech) for 5 days.

424

425 *In vitro functional assays*

426 Human PBMC or purified primary cDCs were cultured in RPMI 1640 media supplemented with  
427 10% Fetal Bovine Serum (HyClone) alone or in the presence of either 1 $\mu$ g/ml 2'3'-c'diAM(PS)2  
428 (Invivogen) or 5 $\mu$ g/ml Poly I:C (SIGMA) or a combination of both for 24h. For all functional  
429 assays, stimulated cDC were washed with 1X PBS prior to the experiment. For mixed leukocyte  
430 reaction (MLR) assays, activated DC were co-cultured with allogenic T cells at a DC:T ratio of  
431 1:2 in 96 round-bottom well plates for 5 days. At day 5, cultured lymphocytes were re-stimulated  
432 with 0.25 $\mu$ g/ml PMA (SIGMA) and ionomycin (SIGMA) for 1 h and cultured for 4 h in the  
433 presence of 0.5 $\mu$ g/ml Brefeldin A (SIGMA), 0.005mM Monensin and 0.2 $\mu$ g/ml anti-CD107a-APC  
434 antibody. Intracellular expression of INF $\gamma$  and CD107a on cultured CD8 $^{+}$  and CD4 $^{+}$  T cells was  
435 then analyzed by flow cytometry. For the experiments evaluating *de novo*-priming of HIV-1  
436 specific responses, total PBMCs from healthy donors were pre-stimulated with 5 $\mu$ g/ml of a pool  
437 of HIV-1 Gag peptides (NIH AIDS Reagent Program #11057) in the absence or the presence of

438 the adjuvant combinations previously mentioned and kept in culture in media supplemented with  
439 25 IU/ml IL-2 (Prepotech) for 2 weeks. Subsequently, cDC where isolated from PBMC from the  
440 same donor and activated under the same conditions in the absence or presence of a pool of HIV-1  
441 Gag peptides. After 16h, pre-cultured PBMC and stimulated autologous cDCs were co-cultured  
442 for additional 16h in the presence of Brefeldin A, Monensin and CD107a antibody and analyzed  
443 by flow cytometry as previously mentioned. All antibodies used for flow cytometry are listed in  
444 Table 1.

445

446 *Western blot analysis*

447 Total protein lysates from MDDC and cDC cultured for 1 h in the presence of media or individual  
448 or combined Poly I:C and 2'3'-di AM(PS) agonists were obtained using RIPA buffer containing  
449 1% phosphatase and protease inhibitors (Roche Diagnostics). Subsequently, protein lysates were  
450 resolved in a 10% agarose gel with SDS and transferred to a nitrocellulose membrane (Fisher  
451 Scientific). Membranes were blocked in 5% bovine serum albumin v (Sigma-Aldrich) in Tris  
452 buffered saline and incubated overnight with 1:100 dilution of primary anti-TBK1, anti-IRF3 or  
453 anti-GAPDH antibodies (Table 1). Then, membranes were incubated for 1 h with the appropriate  
454 anti-rabbit or anti-mouse secondary antibodies (Table 1). Protein band intensity was quantified by  
455 analyzing chemiluminescence detected using an ImageQuant 800 system (Amersham).

456

457 *Gene expression validation and RT-qPCR*

458 RNA was isolated from cDC cultured in the absence or the presence of TBK1 adjuvants using  
459 RNeasy Micro Kit (Qiagen) according to manufacturer instructions. Subsequently, cDNA was  
460 synthesized using the Reverse Transcription kit (Promega). Transcriptional levels of IFN- $\beta$ , IL-12

461 and TNF $\alpha$  were analyzed by semiquantitative PCR (SYBR Green assay Go Taq qPCR Master  
462 Mix; Promega) with specific primers (Metabion) on an Applied Biosystems StepOne Real-Time  
463 PCR system (Applied Biosystems). Relative gene expression was calculated after normalization  
464 to  $\beta$ -actin transcriptional levels.

465

466 *Generation of humanized BLT-mice*

467 NOD/SCID IL2Ry $^{-/-}$  (NSG) mice transplanted with human bone marrow, fetal liver and thymus  
468 (BLT-mouse) were generated as previously described (32) at the Human Immune System Core  
469 from the Ragon Institute and Massachussets General Hospital in collaboration with Dr. Vladimir  
470 Vrbanac, Dr. Maud Deruaz and Dr. Alejandro Balazs. Mice were housed in microisolator cages  
471 and fed autoclaved food and water at a pathogen-free facility. Human immune reconstitution was  
472 monitored for 17 weeks and mice were considered reconstituted with proportions of human CD45 $^{+}$   
473 lymphocytes superior to 30%.

474

475 *DC vaccination and HIV-1 infection of hBLT mice*

476 Dendritic cells (DC) were generated from the same CD34 $^{+}$  HSC precursors used to reconstitute  
477 the corresponding batch of hBLT mice in the presence of 100IU/ml FLT3L, SCF, IL-7 and GM-  
478 CSF (Prepotech). After 10 days, cDC (CD14 $^{+}$  HLA-DR $^{-}$ ) and MoDC (CD14 $^{+}$  HLA-DR $^{+}$ ) present  
479 in cultures were sorted and incubated in media in the absence (MED mice group) or in the presence  
480 of 5ug/ml of a Gag pool of peptides (GAG mice group) alone or in combination with 1 $\mu$ g/ml of  
481 2'3'-c'diAM(PS) and 5  $\mu$ g/ml Poly I:C adjuvants (ADJ mice group). After 24 h, cDC and MoDC  
482 from each culture condition were pooled and hBLT mice were intravenously vaccinated in the tail  
483 vein with approximately 10 $^{5}$  total DC per animal. Two weeks after vaccination, hBLT mice were

484 infected intravenously with a dose of 10,000 TCID<sub>50</sub> of HIV-1<sub>JR-CSF</sub>. For histological analyses  
485 some unvaccinated uninfected mice were included as controls.

486 Plasma HIV-1 viral loads were assessed at 3 and 5/6 and 6/7 weeks post-infection by isolating  
487 viral RNA from plasma and quantified by RT-qPCR as previously described (38). Circulating  
488 CD4<sup>+</sup> T cell counts were assessed at day 0, 3 weeks, 5/6 weeks and 6/7 weeks post-infection by  
489 flow cytometry using counting beads (CountBright, ThermoFisher).

490

491 *Histological analysis of tissue sections from hBLT mice*

492 Lymph nodes and spleens were paraffin-embedded and segmented in fragments of 2  $\mu$ m of  
493 thickness in a Leica microtome. Tissue sections deparaffinization, hydration and target retroviral  
494 were performed with a PT-LINK (Dako) previous to staining.

495 For paraffin-preserved tissue, we used rabbit anti-human CD8 (abcam), rabbit anti-human CXCR5  
496 (GeneTex), rat anti-human CD8 (Bio-Rad), rat anti-human Granzyme B (eBioscience), mouse  
497 anti-human CD3 (Dako) and mouse anti-HIV-1 P24 (Dako), as primary antibodies; and goat anti-  
498 rabbit AF488 (Invitrogen), donkey anti-rat AF594 (Jackson ImmunoResearch) and donkey anti-  
499 mouse AF647 (ThermoFisher) as secondary antibodies. Images were taken with a Leica TCS SP5  
500 confocal and processed with the LAS AF software. 3-D CD8<sup>+</sup> T cell aggregations were analyzed  
501 with Imaris 9.1 software. CD8<sup>+</sup> T cell, Granzyme B and HIV-1 P24 cell counts, co-localization  
502 and distance 2-Dimensions maps were analyzed with ImageJ software. In some cases, spleen tissue  
503 sections were also stained with hematoxylin and eosin to discriminate white (no eosin staining)  
504 and red pulp (intense eosin staining due to enrichment in erythrocytes) areas containing nucleated  
505 cells (hematoxylin stained).

506

507 *Analysis of polyfunctional T cell responses*

508 Blood was extracted from hBLT mice at 3 and 5-6 weeks post-infection and lysed with Red Blood  
509 Cell Lysis Buffer (SIGMA). T cells were activated for 1.5 h with 5 $\mu$ l/ml of anti-CD28 and anti-  
510 CD49d in the presence or absence of 6.4 $\mu$ g/ml of a Gag pool of peptides in the presence of  
511 0.5 $\mu$ g/ml Brefeldin A, Golgi Plug and CD107a antibody (see Table 1). After 5 h of incubation,  
512 polyfunctionality of T cell response was assessed by INF $\gamma$ , IL2, TNF $\alpha$  and CD107a expression by  
513 multicolor flow cytometry panel (all antibodies used are listed in Table 1) in a BD LSR Fortessa  
514 Instrument (BD Biosciences). Polyfunctionality was evaluated using Boolean gating obtained with  
515 FlowJo v10 software.

516

517 *Statistics*

518 Significance of phenotypical and functional differences between paired conditions or different  
519 animals were assessed using a Wilcoxon matched-pairs signed-rank test or Mann-Whitney U test,  
520 or using a Kruskal-wallis or Friedman test followed by a Dunn's post-hoc multiple comparison  
521 test, as appropriate. Dependence of contingency tables values were calculated with Chi-square  
522 statistic. Association between clinical, histological and phenotypical parameters were calculated  
523 using non-parametric Spearman correlation individually between two parameters or using a  
524 correlation network. All statistical analyses were performed using the GrapPad Prism 8 Sofware.

525

526 *Ethics statement*

527 This study was conducted following ethical standards for the treated animals specified in the  
528 IACUC protocol of the Human Immune System Core led by Dr. Vladimir Vrbanac and approved

529 by the Research Ethics Committee from Massachusetts General Hospital and Universidad  
530 Autónoma de Madrid and the Bioethical committees.

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545

546 **Author contributions**

547 E.M.G., V.V., D.C., M.D., A.B., M.J.B. developed the research idea and study concept, designed  
548 the study and wrote the manuscript;  
549 E.M.G., V.V. supervised the study;  
550 M.C.M., D.C. M.D and S.T. designed and conducted most experiments and equally contributed to  
551 the study;

552 T.A. and D.C. provided longitudinal VL data evolution in BLT mice from a pilot experiment  
553 M.C.M. performed the histology staining and the image analysis of tissue sections from the study.  
554 M.D and D.C. provided critical feedback during experimental design and execution phases of the  
555 studies and were directly involved in the experiments.  
556 M.J.B. and C.S. provided reagents and support for the histological analyses performed in the study.  
557 F.S.M., A.A., M.A.MF, I.D.S, L.G.F and J.S. provided peripheral blood, reagents and participated  
558 on the analysis of the data.

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560 **Declarations of Interests:** The authors declare no competing interests.

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747 **Figure legends**

748

749 **Figure 1. Impact of combined TBK1 adjuvants on maturation and function of DC *in vitro*.**

750 (A): Representative Western blot images of analysis of phosphorylated and total TBK1 and IRF3

751 proteins in Monocyte-Derived DC (MDDC) cultured in the absence or the presence of individual

752 or combined TBK1 adjuvants (left panel). Activation of TBK1 (left plot) and IRF3 (right plot)

753 proteins was determined by calculating the ratio of phosphorylated vs total protein and normalized

754 to GAPDH as housekeeping protein for DCs. Data shown in the right represent ratios normalized

755 to values from the control condition (MDDC alone) of each experiment (n=5 experiments).

756 Statistical significance was calculated using a Kruskal-Wallis multiple comparison test with

757 Dunn's correction (\*p<0.05; \*\*p<0.01; \*\*\*p<0.001). (B): RT-qPCR analysis of IFN $\gamma$ , IL-12 and

758 TNF $\alpha$  mRNA expression normalized to  $\beta$ -actin levels in cDC cultured for 16 h hours with media

759 alone or in the presence of 2'3'-c-di-AM(PS)2 and/or Poly I:C. (n=8 experiments). Statistical

760 significance was calculated using a two-tailed matched-pairs Wilcoxon test (\*p<0.05; \*\*p<0.01).

761 (C): Proportions of polyfunctional IFN $\gamma$  $^+$  CD107a $^+$  CD8 $^+$  T cells detected by flow cytometry after

762 culture of total T cells with allogeneic cDCs pre-treated with media or in the presence of individual

763 or combined TBK1 adjuvants. Significance was calculated using a two-tailed Wilcoxon test

764 (\*p<0.05). (D): Proportions of *de novo* induced total (IFN $\gamma$  $^+$ , left) and polyfunctional (IFN $\gamma$  $^+$

765 CD107a $^+$ , right) HIV-1-Gag-specific T cells from healthy donors cultured for 2 weeks in the

766 absence or the presence of a pool of HIV-1 Gag peptides alone or combined with the indicated

767 TBK1 adjuvants and restimulated with autologous cDC pre-treated in the same mentioned culture

768 conditions. Significance was calculated using a two-tailed Wilcoxon test (\*p<0.05; \*\*p<0.01).

769

770 **Figure 2. hBLT mice vaccinated with GAG-ADJ DC display less severe progression of HIV-**  
771 **1 infection.** (A): Fold-change in circulating hCD4<sup>+</sup> T cell counts in infected hBLT mice at 5-6  
772 weeks post-infection with HIV-1 relative to basal counts present on each mouse at day 0 (upper  
773 panels). Significance was calculated using a two-tailed Wilcoxon test (\*p<0.05; \*\*p<0.01;  
774 \*\*\*p<0.001). Pie charts showing percentage of mice displaying less severe decrease of hCD4<sup>+</sup> T  
775 cell counts (hCD4<sup>+</sup> T cell fold change  $\geq$  0.5; CD4 Hi) and those animals with severe depletion  
776 (hCD4<sup>+</sup> T cell fold change < 0.5; CD4 Low). Statistical significance of differences was calculated  
777 using a Chi-square test with Yates correction (\*\*p<0.01; \*\*\*\*p<0.0001). (B): HIV-1 plasma viral  
778 loads (upper panels) quantified by RT-qPCR from the plasma of hBLT-mice vaccinated with  
779 MED, GAG and GAG-ADJ treated DCs at 3 weeks post infection, stratified by CD4 Hi and CD4  
780 Low phenotypes within each indicated hBLT mouse subgroup. Pie charts (lower panels)  
781 representing mice with VL either equal or higher than  $10^5$  copies/ml (dark color) or lower than  $10^5$   
782 copies/ml (light color) per treatment group and CD4<sup>+</sup> T cell fold-change stratification. Statistical  
783 significance of differences was calculated using a Chi-square test with Yates correction (\*\*p<0.01;  
784 \*\*\*p<0.0001).

785  
786 **Figure 3. Histological analysis of CD8<sup>+</sup> T cell and HIV-1-infected cell distribution in spleen**  
787 **from vaccinated hBLT mice.** (A): Representative confocal microscopy image (magnification  
788 40X) of a whole transversal splenic section showing staining of cell nuclei (DAPI; blue), human  
789 CD8<sup>+</sup> T cells (green), Granzyme B<sup>+</sup> (gray) and HIV-1 p24<sup>+</sup> infected cells (red). Zoomed images  
790 (40X magnification) from selected white pulp (i) and red pulp (ii) areas highlighted by dashed  
791 lines and defined as in Supplemental Figure 4, are displayed on the right to appreciate cellular  
792 patterns. Green arrows CD8<sup>+</sup> T cells; white arrow Granzyme B<sup>+</sup> cell; dashed white arrow

793 Granzyme B<sup>+</sup> CD8<sup>+</sup> T cells; red arrow HIV-1 p24<sup>+</sup> cells. (B-C): Analysis of hCD8<sup>+</sup> T cells (B,  
794 left) and HIV-1 p24<sup>+</sup> cells (C, left) infiltrated in the white pulp areas from spleen of the indicated  
795 groups of hBLT mice. Significance was calculated using a Kruskal-Wallis multiple comparison  
796 test with Dunn's correction (\*\*p<0.01; \*\*\*p<0.001). Spearman correlation analysis of association  
797 of frequencies of CD8<sup>+</sup> T cells in the white pulp and proportions of Granzyme B<sup>+</sup> CD8<sup>+</sup> T cells  
798 (B, right) and p24<sup>+</sup> in the white pulp (C, right) are also shown. Spearman R and P values are  
799 highlighted on the upper right areas of each plot.

800

801 **Figure 4. Histological CD8<sup>+</sup> T cell and HIV-1 p24<sup>+</sup> cell characterization in lymph nodes from**  
802 **vaccinated hBLT mice.** (A): Representative confocal microscopy image (40x magnification)  
803 example of whole lymph node section showing staining of nuclei (DAPI, blue), hCD8<sup>+</sup> T cells  
804 (green), Granzyme B<sup>+</sup> (grey) and infected HIV-1 p24<sup>+</sup> cells (red). A zoom of an (i) area from the  
805 same original image is shown on the right. Dashed lines highlight CD8<sup>+</sup> T cell cluster areas. Green  
806 arrows CD8<sup>+</sup> T cells; white arrow Granzyme B<sup>+</sup> cell; dashed white arrow Granzyme B<sup>+</sup> CD8<sup>+</sup> T  
807 cells; red arrow HIV-1 p24<sup>+</sup> cells. (B): Quantification of number of infected HIV-1 p24<sup>+</sup> cells per  
808 lymph node area from the indicated hBLT mice groups. Pie charts shown below represent the  
809 percentage of mice displaying high density of infected cells per area ( $\geq 0.00003$  p24<sup>+</sup> cells/square  
810 micron) or low density of infected cells per area ( $< 0.00003$  p24<sup>+</sup> cells/square micron) within each  
811 hBLT mouse subgroup. Statistical significance of differences was calculated using a Chi-square  
812 test with Yates correction (\*\*p<0.001). (C): Number of hCD8<sup>+</sup> T cells per lymph node area  
813 (upper panel) from the indicated hBLT mice groups. Pie charts showing the percentage of mice  
814 per group displaying high density of CD8<sup>+</sup> T cells per lymph node area ( $\geq 0.002$  hCD8<sup>+</sup> T  
815 cells/square micron) or low density of CD8<sup>+</sup> T cells per lymph node area ( $< 0.002$  hCD8<sup>+</sup> T

816 cells/square micron) are shown below. Statistical significance of differences in proportions of mice  
817 with enrichment of CD8<sup>+</sup> T cells among groups was calculated using a Chi-square test with Yates  
818 correction (\*\*p<0.01; \*\*\*p<0.001). (D): Percentage of mice presenting CD8<sup>+</sup> T cells large volume  
819 clusters ( $\geq$  6000 cubic microns) in the lymph nodes corresponding to the quantifications shown in  
820 Supplemental Figure 5. Statistical significance of differences was calculated using a Chi-square  
821 test with Yates correction (\*\*\*\*p<0.0001). (E): Two tailed Spearman correlation network  
822 showing R (left heatmap) and p values (right heatmap) between selected histological parameters  
823 and plasma viral loads and fold change in PB CD4<sup>+</sup> T cell count and CD4<sup>+</sup>/CD8<sup>+</sup> T cell ratios at  
824 different times post infection. (F): Individual Spearman correlations between numbers (Upper row)  
825 and median volume (lower row) of large CD8<sup>+</sup> T cell clusters ( $\geq$  6000 cubic microns) versus the  
826 indicated spleen histological patterns (upper row) and clinical parameters (lower row). Spearman  
827 R and p values for all animals (black) and GAG-ADJ group (red) are shown on each plot.

828

829 **Figure 5. Identification of CD8<sup>+</sup> T cell polyfunctional patterns associated with histological**  
830 **and clinical parameters of progression of HIV-1 infection in hBLT mice.** (A-B): Correlation  
831 networks showing Spearman R (A) and p values (B) between selected clinical and histological  
832 parameters and basal and antigen-induced polyfunctional phenotype of splenic and circulating  
833 CD8<sup>+</sup> T cell populations analyzed by flow cytometry at different times post-infection (B). Positive  
834 and negative correlations are highlighted in red and blue respectively; Significant p values for each  
835 correlation are highlighted in brown scale. (C): Proportion of splenic CD8<sup>+</sup> T cells either co-  
836 expressing 3 out of 4 tested cytokine/degranulation parameters upon stimulation with a pool of  
837 HIV-1 Gag peptides (upper plot) or a splenic population of polyfunctional cells defined as  
838 CD107a<sup>+</sup> INF $\gamma$ <sup>+</sup> TNF $\alpha$ <sup>+</sup> IL-2<sup>-</sup> detected under these conditions (lower plot). (D): Individual

839 Spearman correlations between proportions of Gag-peptide induced splenic CD8<sup>+</sup> T cells co-  
840 expressing 3 cytokine/degranulation parameters and the indicated clinical and histological  
841 parameters. Spearman p and r values are highlighted in each plot. \*p<0.05; \*\*p<0.01. (E):  
842 Proportions of CD107a<sup>+</sup> INF $\gamma$ <sup>+</sup> TNF $\alpha$ <sup>-</sup> IL-2<sup>-</sup> included in circulating CD8<sup>+</sup> T cells at 3 weeks p.i.  
843 after HIV-1 Gag-peptide stimulation. Individual Spearman correlations between proportions of  
844 this population and Ag-induced 3 parameter polyfunctional cells and p24<sup>+</sup> cell No. in LN are  
845 shown in the right. Significance for (C, E) was calculated using a Kruskal-Wallis multiple  
846 comparison test with Dunn's correction (\*p<0.05; \*\*p<0.01).

847 **Table 1. List of commercial antibodies used in the study**

Antibody	Vendor	Application	Dilution
CD107a APC	Biolegend	FACS	1ul/ml
CD107a PE-Cy7	Biolegend	FACS	1ul/ml
CD3 Pacific Blue	Immunostep	FACS	1:50
CD3 V605	Biolegend	FACS	2:50
CD4 APC-Cy7	Biolegend	FACS	2:50
CD45 PerCP	Biolegend	FACS	2:50
CD8 APC	Biolegend	FACS	2:50
CD8 PerCP	Biolegend	FACS	1:50
IL-2 PE	Biolegend	FACS	3:50
INF $\gamma$ FITC	Biolegend	FACS	3:50
INF $\gamma$ FITC	BD Pharmigen	FACS	1:50
TNF $\alpha$ Pacific Blue	Biolegend	FACS	2:50
Ghost Dye Red 780	TONBO Bioscience	FACS	1:1000
Live/Dead Fixable Blue Dead Cell Stain Kit	Thermo Fisher	FACS	1:1000
phosphoTBK1	Cell Signaling	WB	1:1000
TBK1	Cell Signaling	WB	1:1000
phosphoIRF3	Cell Signaling	WB	1:1000
IRF3	Cell Signaling	WB	1:1000
GAPDH	Biolegend	WB	1:1000
anti-rabbit	Invitrogen	WB	1:5000
anti-mouse	Invitrogen	WB	1:2000
CD8	abcam	IF	1:100
Granzyme B	eBioscience	IF	1:100
HIV P24	Dako	IF	1:10
CD3	Dako	IF	1:25
CXCR5	GeneTex	IF	1:100
anti-rabbit AF488	Invitrogen	IF	1:200
anti-rat AF594	Jackson ImmunoResearch	IF	1:200
anti-mouse AF647	Thermo Fisher	IF	1:200

848 **Supplemental figure legends**

849

850 **Supplemental figure 1. Impact of TBK1 adjuvants in activation and function of cDC *in vitro*.**

851 (A): Representative Western blot analysis of TBK1 and IRF3 phosphorylation in primary cDCs  
852 cultured for 1 h in the presence of media alone or with 2'3'-c-di-AM(PS)2 and/or Poly I:C. Ratios  
853 for phosphorylated vs total TBK1 and IRF3 proteins are shown on the right. Significance was  
854 calculated using a Kruskal-Wallis multiple comparison test with Dunn's correction (\*p<0.05). (B):  
855 Flow cytometry analysis of Mean Fluorescence Intensity (MFI) of CD40 (left) and CD86 (right)  
856 in cDC following culture in the absence or the presence of different indicated adjuvant  
857 combinations (n=8 experiments). Significance was calculated using a two-tailed Wilcoxon test  
858 (\*p<0.05). (C): Flow cytometry dot plots showing analysis of IFN $\gamma$  versus CD8 on gated CD8 $^{+}$  T  
859 cells from healthy individuals exposed to autologous cDCs pre-cultured in media alone or activated  
860 with 2'3'-c-di-AM(PS)2 and Poly I:C in the absence or the presence of a pool of HIV-1 Gag  
861 peptides. Dot plots from three representative experiments are shown. Number below gates  
862 represent proportion of positive cells.

863

864 **Supplemental figure 2. *In vitro* generation and isolation of HSC-derived DC and**  
865 **experimental design for *in vivo* hBLT vaccination and analysis.** (A): Representative pre-sorting  
866 gating strategy showing cell populations derived from human fetal CD34 $^{+}$  HSC cultured *in vitro*  
867 for 2 weeks (see methods). Conventional dendritic cells (cDC) and monocyte derived DC-like  
868 (MoDC-like) derived from HSC were defined as live CD33 $^{+}$  HLA-DR $^{+}$  myeloid cells differing on  
869 CD14 expression, respectively. (B): Flow cytometry analysis of CD14 vs HLA-DR expression on  
870 sorted cDC (upper plots) and MoDC-like cells (lower plots). Proportion of cells included on each

871 gate are highlighted. Levels of CD11c expression overlayed with FMO controls (blue histograms)  
872 for each of these two populations (red histograms) is also shown on the right. (C): Flow cytometry  
873 analysis of expression of CD40 versus CD86 on sorted CD34<sup>+</sup> HSC-derived cDC and MoDC-like  
874 cultured in just media (MED) or in the presence of a pool of HIV-1 Gag peptides alone (GAG) or  
875 in combination with the TBK1 adjuvant cocktail (GAG-ADJ). Numbers in quadrants indicate  
876 proportions of positive cells. (D): Schematic representation of the experimental generation of  
877 hBLT mice, *in vivo* vaccination regime and analysis design. (E): Analysis of hBLT mice weight  
878 during the course of the experiment. Individual weights of hBLT mice are shown in a lighter color  
879 and median for each hBLT mouse subgroup is highlighted in a darker color and thicker lines  
880 (yellow for Media (MED), blue for Gag pool (GAG) and red for Gag pool + adjuvants (GAG  
881 AGJ)).

882

883 **Supplemental figure 3. Evolution of plasma HIV-1 viral loads in vaccinated hBLT mice**  
884 **infected with HIV-1.** (A): Pilot experiment showing RT-qPCR analysis of the evolution of plasma  
885 HIV-1 (RNA copies/ml) in n= 7 hBLT mice at different weeks after infection with JRCSF HIV-1  
886 virus. (B-C): RT-qPCR analysis of plasma viral load in hBLT-mice vaccinated with MED, GAG  
887 and GAG-ADJ treated DCs at 3, 5/6 and 6/7 weeks post infection (B) and stratified in CD4 Hi and  
888 CD4 Low animals included within each hBLT mouse subgroup at 6/7 weeks post infection (C).  
889 Pie charts (lower panel C) represent the proportions of mice presenting plasma viral load either  
890 equal or higher than 10<sup>5</sup> copies/ml (dark color) or lower than 10<sup>5</sup> copies/ml (light color). Statistical  
891 significance of differences was calculated using a Chi-square test with Yates correction  
892 (\*\*\*\*p<0.0001).

893 **Supplemental figure 4. Depletion of circulating CD4+ T cells in vaccinated hBLT mice**  
894 **infected with HIV-1.** (A-C): Fold-change in peripheral hCD4<sup>+</sup> T cell counts at 3, 5-6 and 6-7  
895 weeks post infection with HIV-1 in the study (A) or shown individually in two separate  
896 experiments performed with different batches of hBLT mice (C; experiment 1, n=24, left and  
897 experiment 2, n=18, right). Individual data for each mouse was normalized to the corresponding  
898 baseline hCD4<sup>+</sup> T count values present at day 0. (B): Fold change in CD4<sup>+</sup>/CD8<sup>+</sup> T cell ratios in  
899 the blood at 5/6 and 6/7 weeks post-infection from the values observed at 3 weeks post-infection  
900 in the indicated groups of vaccinated animals. Statistical significance was calculated using a two-  
901 tailed matched-pairs Wilcoxon test (\*p<0.05; \*\*p<0.01).

902  
903 **Supplemental figure 5. Histological analysis of hBLT mice splenic architecture and**  
904 **association with CD8<sup>+</sup> T cell activation.** (A): Representative image of a hematoxylin-eosin  
905 staining of a full spleen section from a hBLT mouse used for the study and defining white and red  
906 pulp areas (magnification 5x). Dashed areas include white pulp and exclude red pulp and (i) and  
907 (ii) sections from these regions are further zoomed in the lower panels (magnification 20x). (B):  
908 Quantification of percentage of cytotoxic Granzyme B<sup>+</sup> hCD8<sup>+</sup> T cell from total splenic hCD8<sup>+</sup> T  
909 cells (upper panel) and number of HIV p24<sup>+</sup>-infected cells per square microns (lower panel)  
910 detected per splenic section of the hBLT mice from the indicated subgroups. Significance was  
911 calculated using a Kruskal-Wallis multiple comparison test with Dunn's correction. (C): Analysis  
912 of the percentages of cytotoxic Granzyme B<sup>+</sup> cells from total CD8<sup>+</sup> T cells found in the white pulp  
913 (WP, upper plot) and in the red pulp (RP, lower plot) areas in the spleen of the indicated hBLT  
914 mouse subgroups. Significance between white and red pulp paired values was calculated using a  
915 two-tailed Wilcoxon test (\*p<0.05; \*\*p<0.01; \*\*\*p<0.001). Intergroup significance was

916 calculated using a Kruskal-Wallis multiple comparison test with Dunn's correction (\*p<0.05). (D):  
917 Spearman correlation between proportions of Granzyme B<sup>+</sup> CD8<sup>+</sup> T cells present in the red versus  
918 the white pulp of the spleen of vaccinated hBLT mice. Spearman R and p values are highlighted  
919 in the upper right area of the plot. (E): Frequencies of CD8<sup>+</sup> T cells and p24<sup>+</sup> cells per spleen area  
920 in vaccinated MED (yellow), GAG (blue) and GAG-ADJ (red) hBLT mice stratified by less severe  
921 (CD4Hi) and marked (CD4Lo) depletion of circulating CD4<sup>+</sup> T cell counts at 5/6 wk p.i. Statistical  
922 significance between values from CD4Hi ADJ-GAG mice and the indicated subgroups were  
923 performed using a two-tailed Mann Whitney test. (F): Representative confocal microscopy images  
924 (magnification 40x) from white pulp areas of the spleen of a GAG (left panel) and a GAG-ADJ  
925 (right panel) spleen section stained with anti-CD3 (yellow), anti-CD8 (green), CXCR5 (magenta);  
926 Nuclei were stained with DAPI (blue). White arrows highlight CXCR5<sup>+</sup> CD8<sup>+</sup> T cells in the white  
927 pulp areas from the spleen.

928

929 **Supplemental figure 6. Analysis of CD8<sup>+</sup> T cell clusters in the lymph node from hBLT mice.**  
930 (A): Analysis of the volume of CD8<sup>+</sup> T cell clusters detected on hBLT LN tissue sections using  
931 the Imaris 9.2 software. CD8<sup>+</sup> clusters are colored with a gradient from higher volumes (red and  
932 orange) to lower volumes (purple and dark blue). (B): Proportion of infected HIV-1 p24<sup>+</sup> cells per  
933 lymph node area stratified by CD4 Hi and CD4 Low animals included on each hBLT mouse  
934 subgroup. (C): Individual Spearman correlations of p24<sup>+</sup> cells per LN area versus plasma viral  
935 loads at different time points and CD8<sup>+</sup> T cell per spleen area at 6/7 weeks p.i. Values of r and p  
936 in Total (black) and GAG-ADJ (red) hBLT mice are highlighted on each plot. \*p<0.05; \*\*p<0.01;  
937 \*\*\*p<0.001; \*\*\*\*p<0.0001. (D): Quantification of numbers of large (500-3000000  $\mu\text{m}^3$ , upper  
938 pot; red line showing high-volume elements cut-off) and low (0-500  $\mu\text{m}^3$ , lower pot) volume CD8<sup>+</sup>

939 T cell clusters obtained with the Imaris 9.2 software for every single lymph node and per hBLT  
940 mouse.

941  
942 **Supplemental figure 7. Quantification of basal and HIV-1 peptide induced polyfunctional**  
943 **profiles of splenic and circulating CD8<sup>+</sup> T cells from hBLT mice.** (A, B): Percentages of  
944 polyfunctional splenic CD8<sup>+</sup> T cells at 6/7 weeks post-infection defined as lymphocytes co-  
945 expressing either 4, 3 or 2 analyzed cytokine and degranulation parameters on gated CD8<sup>+</sup> T cells  
946 either basally (A) or upon HIV-1 Gag peptide stimulation (B). (C-F): Percentage of polyfunctional  
947 cells, as previously defined, included on circulating CD8<sup>+</sup> T cells at 3 and 6/7 weeks post-infection  
948 either basally (C for 6/7 wk p.i., and E for 3 wk p.i.) and after HIV-1 Gag peptide-stimulation (D  
949 for 6/7 wk p.i., and F for 3 wk p.i.). Statistical significance was calculated using a Kruskal-Wallis  
950 multiple comparison test with Dunn's correction (\*p<0.05; \*\*p<0.01; \*\*\*p<0.001).

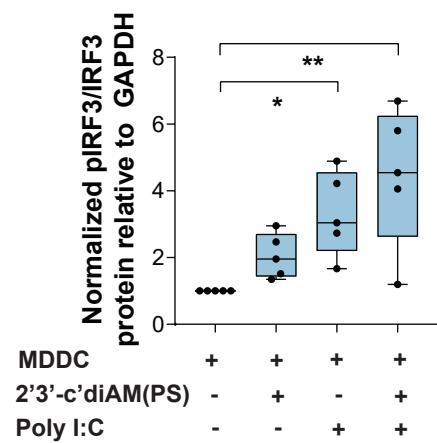
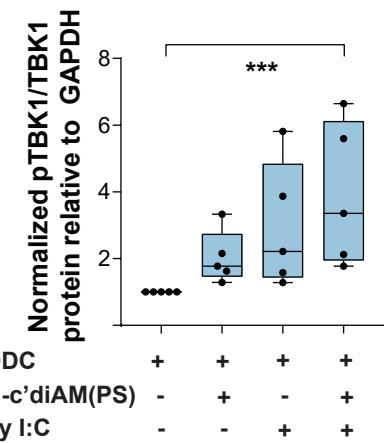
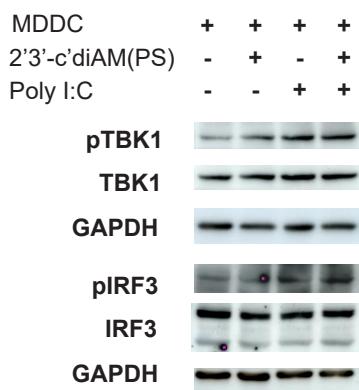
951  
952 **Supplemental figure 8. Association of HIV-1 Gag-peptide induced splenic CD107a<sup>+</sup> INF $\gamma$ <sup>+</sup>**  
953 **TNF $\alpha$ <sup>+</sup> CD8<sup>+</sup> T cells and HIV-1 disease progression parameters.** (A-B): Correlation network  
954 showing Spearman R (A) and p values (B) between the indicated clinical, histological, and basal  
955 and antigen-induced polyfunctional phenotype of splenic and circulating CD8<sup>+</sup> T cell populations  
956 analyzed by flow cytometry at different times post-infection. Positive and negative correlations  
957 are highlighted in red and blue respectively; Significant p values for each correlation are  
958 highlighted in brown scale

959  
960 **Supplemental Figure 9. Association of basal and HIV-1 Gag-peptide induced polyfunctional**  
961 **CD8<sup>+</sup> T cells populations and histological and HIV-1 disease progression parameters.** A):

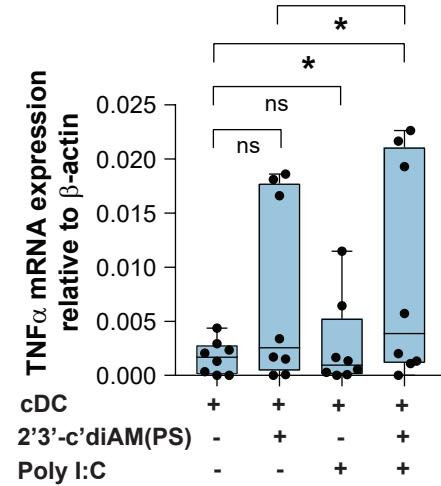
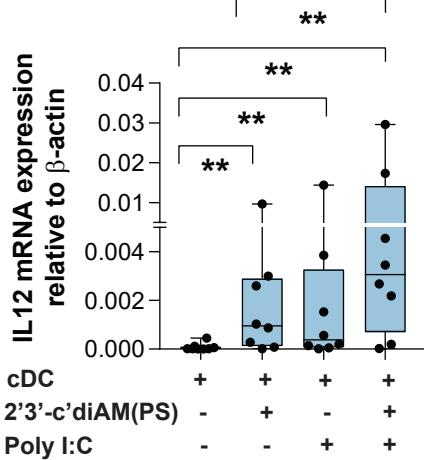
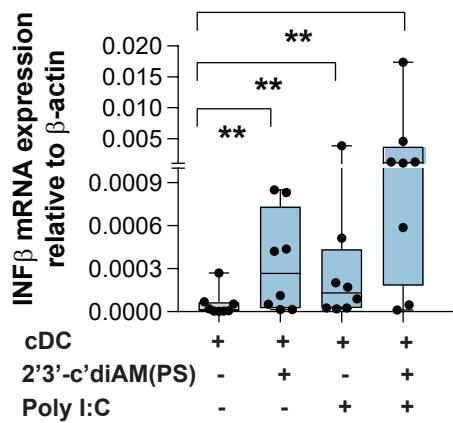
962 Proportions of CD107a<sup>+</sup>IFN $\gamma$ <sup>+</sup>IL2<sup>+</sup>TNF $\alpha$ <sup>-</sup>, CD107a<sup>+</sup>IFN $\gamma$ <sup>-</sup>IL2<sup>+</sup>TNF $\alpha$ <sup>+</sup> and CD107a<sup>-</sup>  
963 IFN $\gamma$ <sup>+</sup>IL2<sup>+</sup>TNF $\alpha$ <sup>+</sup> 3-parameter polyfunctional subpopulations from splenic CD8<sup>+</sup> T cells induced  
964 after HIV-1 Gag peptide stimulation CD8<sup>+</sup> T cells. Statistical significance was calculated using a  
965 two-tailed Mann Whitney test, \*\*p<0.01 (B-D): Individual Spearman correlation between  
966 proportions of CD107a<sup>+</sup> INF $\gamma$ <sup>+</sup> IL2<sup>-</sup> TNF $\alpha$ <sup>+</sup> (Upper rows) and CD107a<sup>-</sup> INF $\gamma$ <sup>+</sup> IL2<sup>+</sup> TNF $\alpha$ <sup>+</sup> (bottom  
967 rows) from splenic CD8<sup>+</sup> T cell detected after HIV-1 Gag-peptide stimulation and the indicated  
968 virological (B) and immunological (C) parameters. Correlations between indicated histological  
969 and immunological parameters and proportions of CD107a<sup>+</sup>IFN $\gamma$ <sup>+</sup>IL2<sup>+</sup>TNF $\alpha$ <sup>+</sup> cells basally present  
970 in circulating CD8<sup>+</sup> T cells at 3 weeks post-infection are shown in panel D. Spearman R and p  
971 values of all and ADJ-GAG hBLT mice groups are highlighted in black and red, respectively.

**Figure 1**

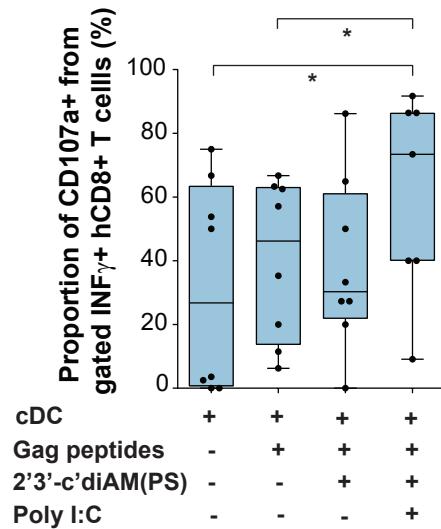
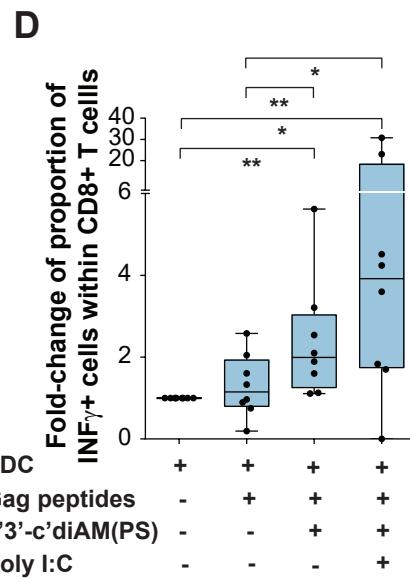
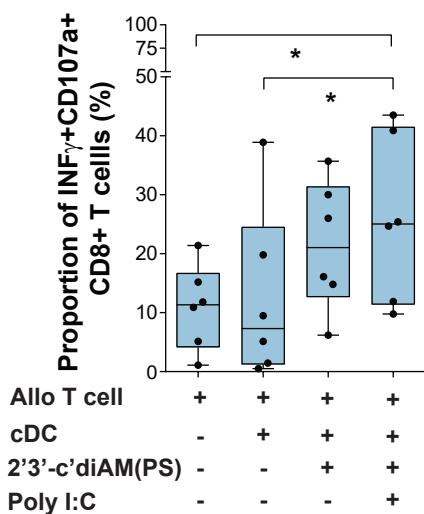
**A**

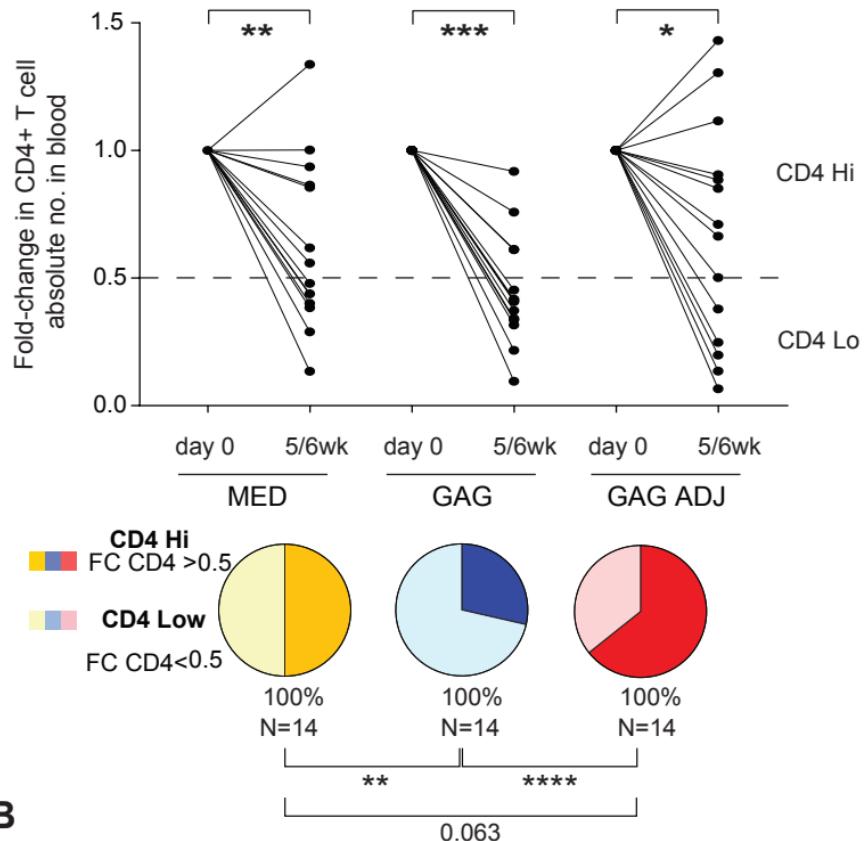
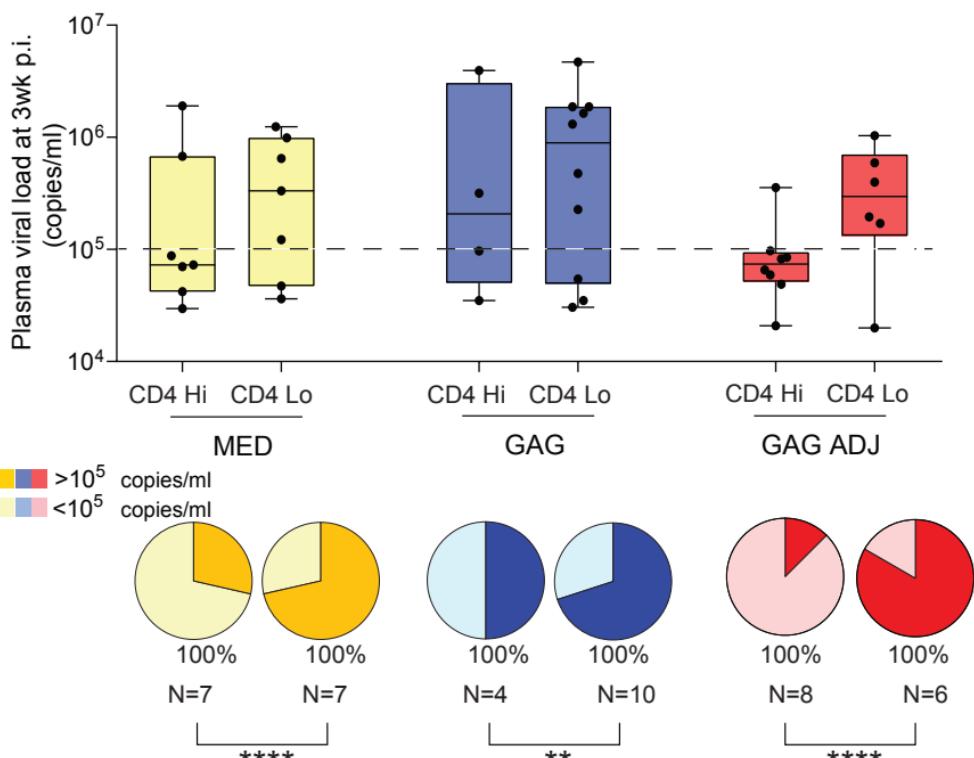


**B**



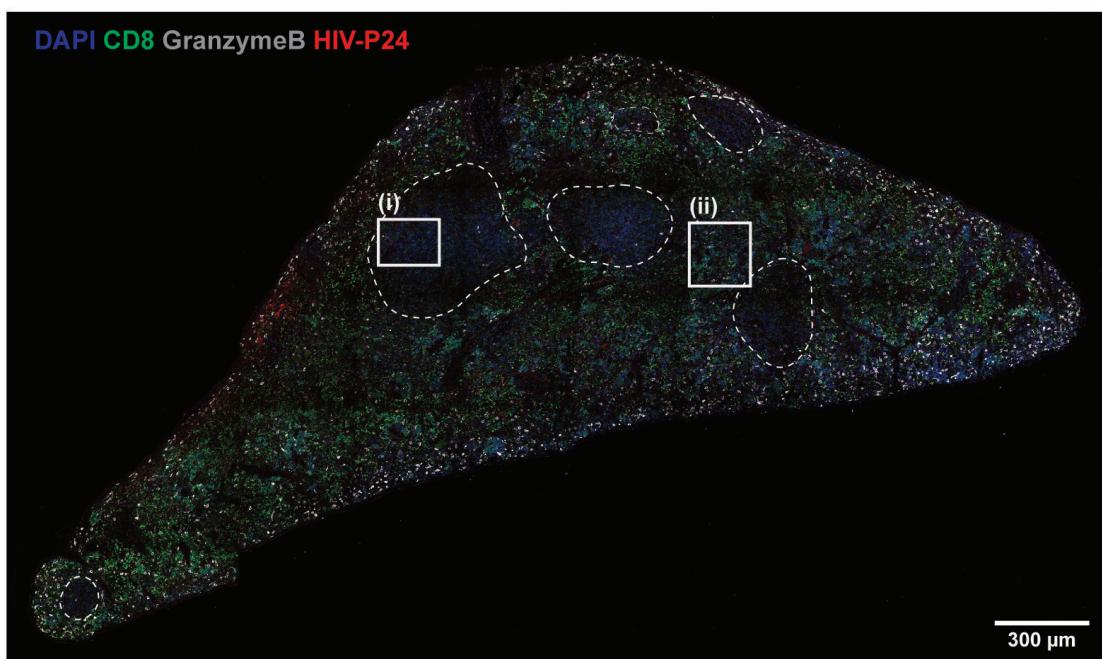
**C**



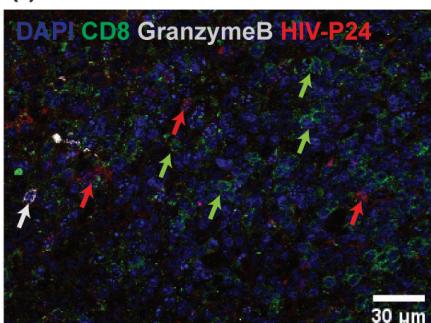
**Figure 2****A****B**

# Figure 3

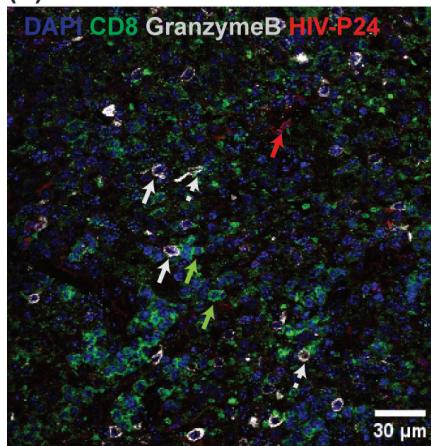
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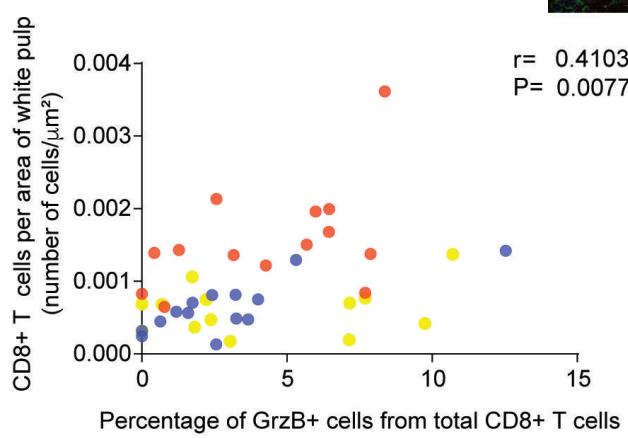
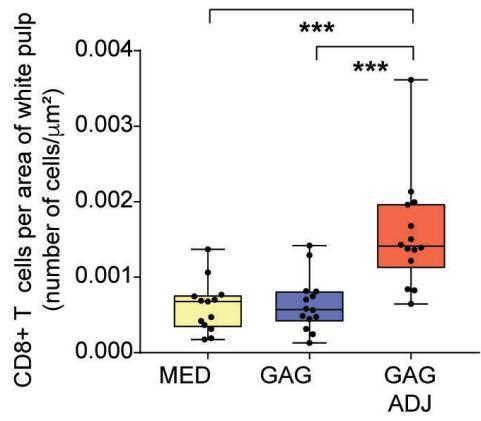
(i) WHITE PULP



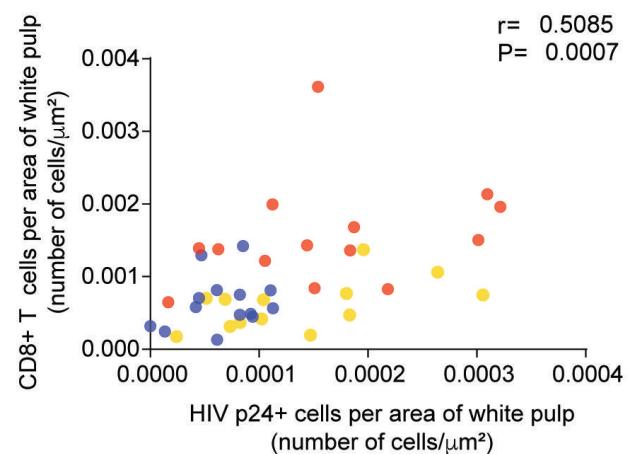
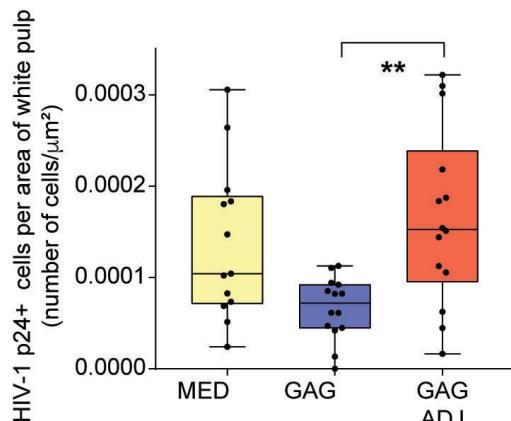
(ii) RED PULP



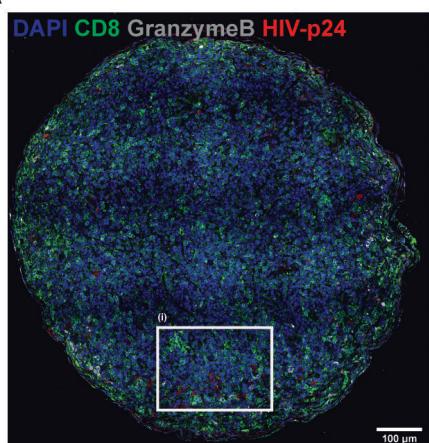
B



C



A



DAPI CD8 GranzymeB HIV-p24

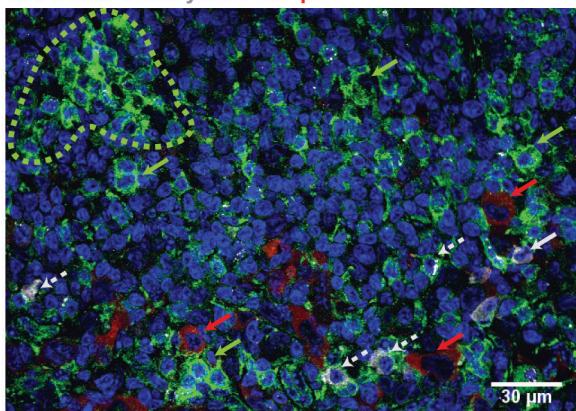
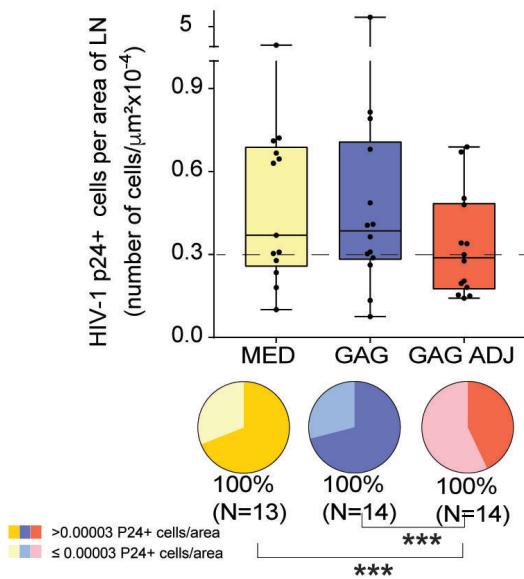
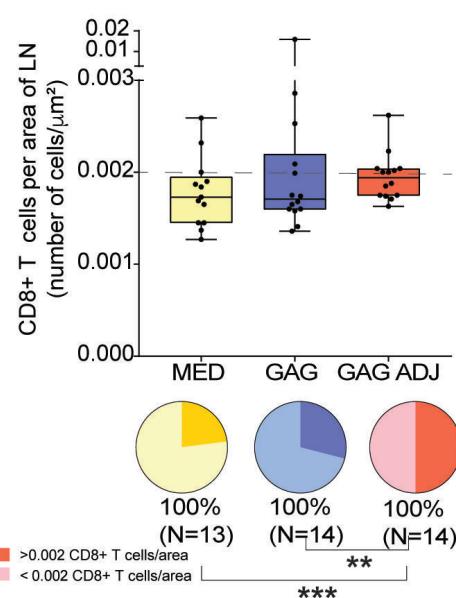


Figure 4

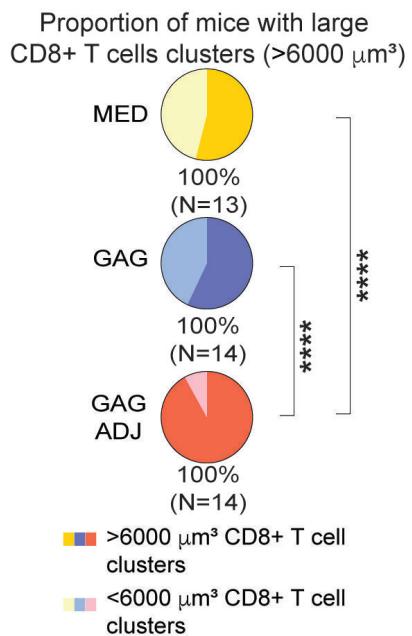
B



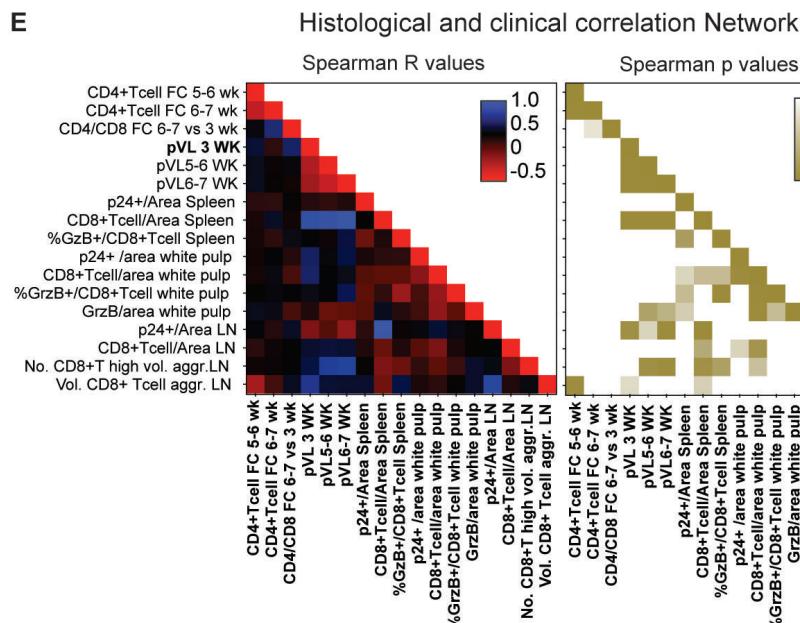
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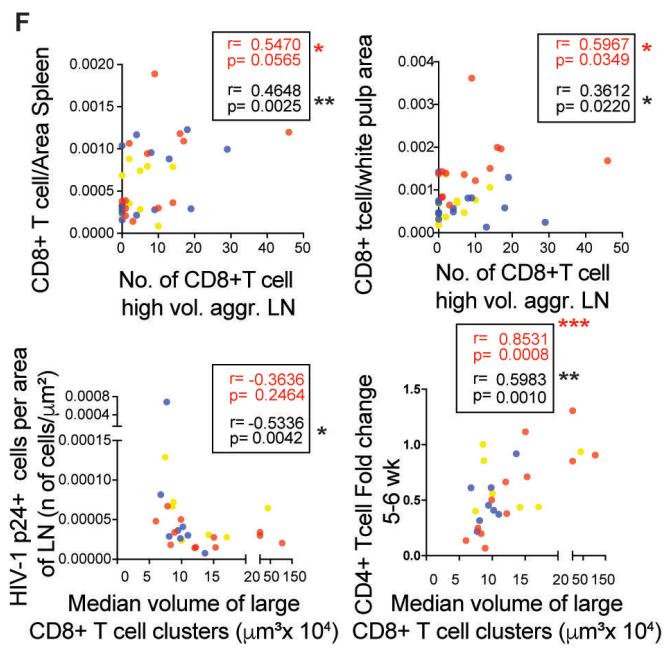
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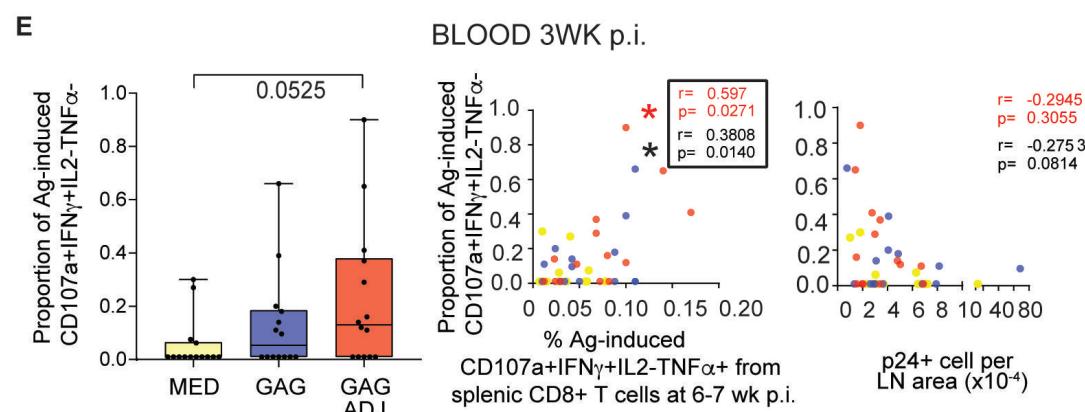
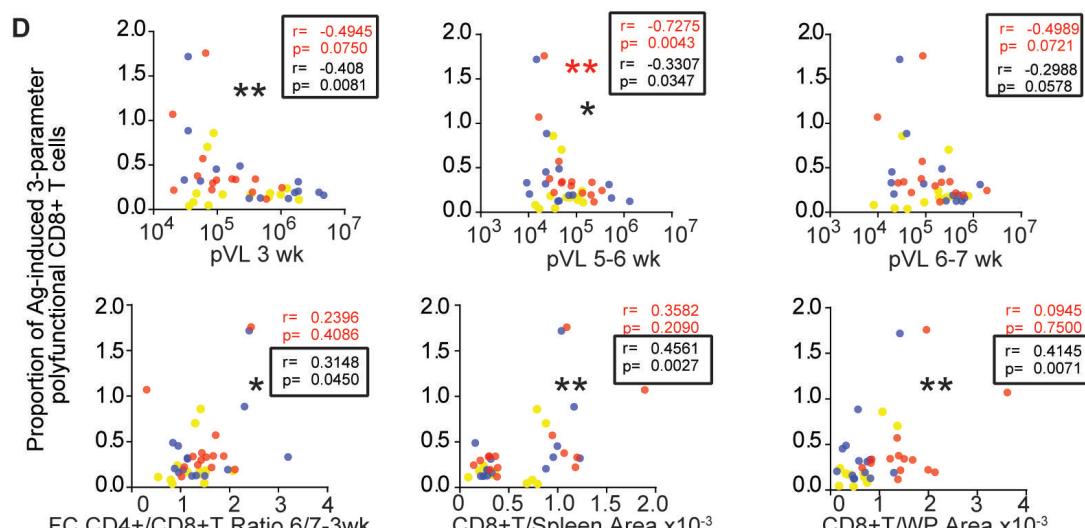
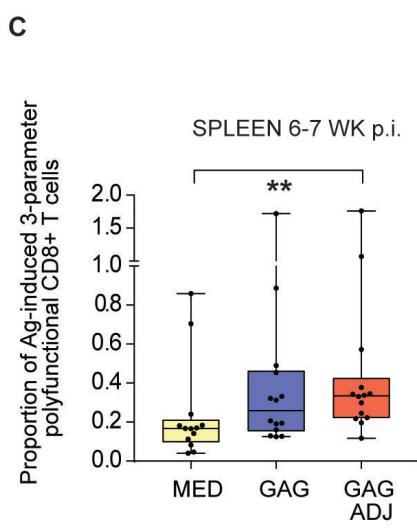
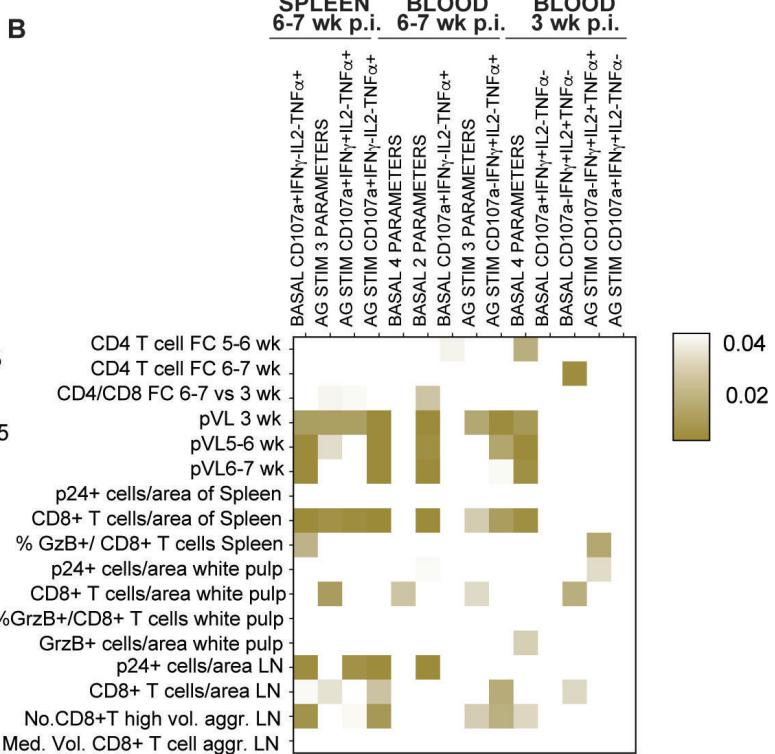
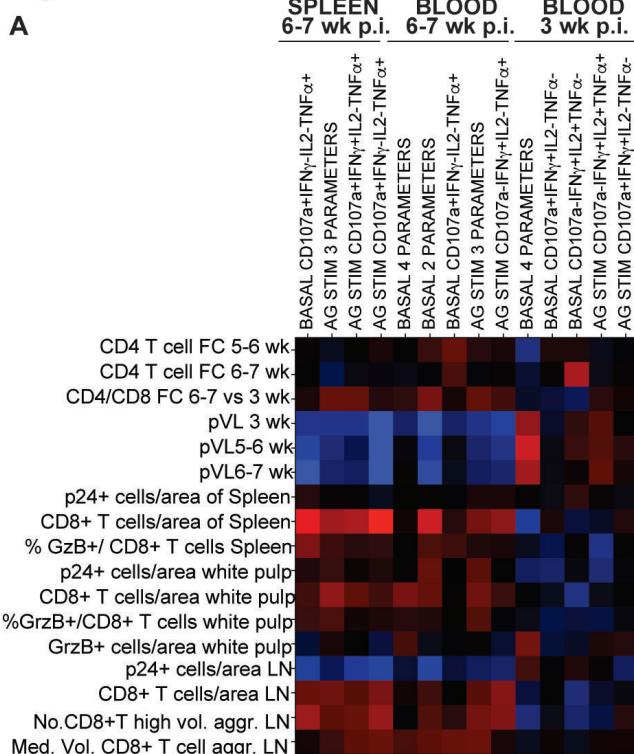
E

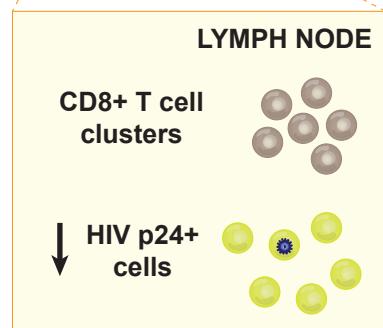
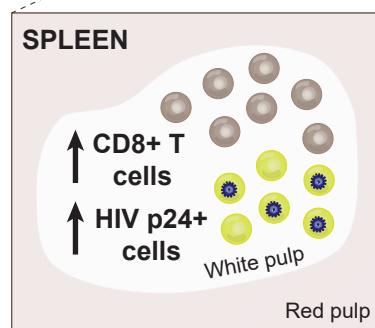
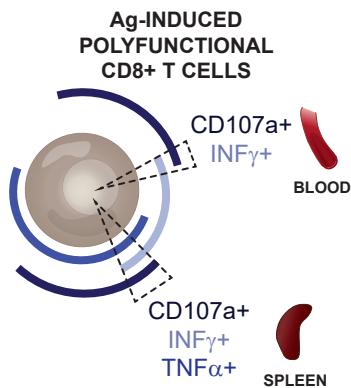
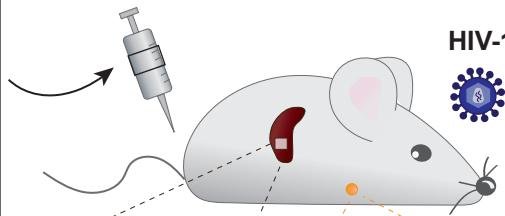
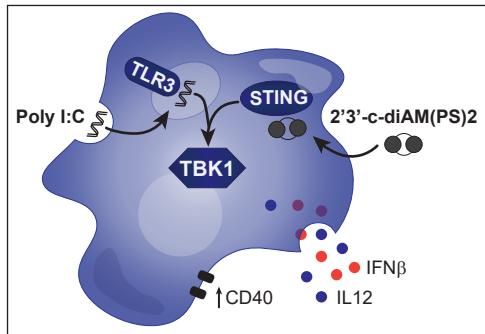


F



**Figure 5**





Less severe CD4+ T cell depletion

