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1 **SLAMF6 enables efficient attachment, synapse formation, and killing of HIV-1-**
2 **infected CD4⁺ T cells by virus-specific CD8⁺ T cells¹**

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24
25 **Running title:** SLAMF6 drives CD8⁺ T-cell recognition of HIV-1⁺ CD4⁺ T cells

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33 **ABSTRACT**

34 Efficient recognition and elimination of HIV-1-infected CD4⁺ T cells by cytotoxic CD8⁺ T
35 cells (CTLs) require target cell engagement and the formation of a well-organized
36 immunological synapse. Surface proteins belonging to the SLAM family are known to be
37 crucial for stabilizing the immunological synapse and regulating antiviral responses
38 during lymphotropic viral infections. In the context of HIV-1, there have been reports of
39 SLAMF6 down-regulation in HIV-1-infected CD4⁺ T cells; however, the significance of
40 this modulation for CTL function remains unclear. In this investigation, we used CTL
41 lines from People living with HIV (PLWH) to examine the impact of SLAMF6 blockade
42 on three pivotal processes: (1) the formation of CD8⁺-CD4⁺ T-cell conjugates, (2) the
43 establishment of the immunological synapse, and (3) the killing and cytokine production
44 capacity of HIV-1-specific CTLs during HIV-1 infection. Our findings reveal that the
45 inability to form CD8⁺-CD4⁺ T-cell conjugates following incubation with an anti-SLAMF6
46 blocking antibody is primarily attributable to a defect in actin ring formation at the
47 immunological synapse. Furthermore, SLAMF6 blockade leads to a reduction in the
48 killing efficiency of HIV-1-infected CD4⁺ T cells by HIV-1-specific CTLs, underscoring
49 the critical role of SLAMF6 in cytolytic function. This study highlights the importance of
50 SLAMF6 receptors in modulating cytotoxic antiviral responses, shedding light on
51 potential avenues for manipulation and enhancement of this pathway in the context of
52 HIV and other lymphotropic viral infections.

53 **INTRODUCTION**

54 The emergence of HIV-1-specific cytotoxic CD8⁺ T lymphocytes (CTLs) during acute
55 HIV-1 infection plays a critical role in viral control (1–3). In the chronic phase of HIV
56 infection, CTLs are essential for controlling viral load (1–3), and viral control in a
57 monkey model of AIDS virus infection is enhanced by interventions that augment CTL
58 function (4)(1–3) CD8⁺ T-cell activation primarily occurs through the engagement of the
59 T cell receptor (TCR) by cognate peptide-MHC-I complexes, leading to the elimination
60 of HIV-infected cells. However, the precise mechanism that defines an effective
61 functional T cell response remains incompletely understood. Research has indicated
62 that, in addition to the TCR, several co-signaling molecules, such as CD28, LFA-1, LFA-
63 3, PD-1, and SLAMF4, expressed on the membranes of effector and target cells, are
64 crucial for facilitating cell-to-cell interactions required for effective recognition and killing
65 (5–7). These molecules either induce downstream signaling, mediate adhesion, or fulfill
66 both functions (6, 8–10). While other signaling receptors have been proposed as
67 essential elements for the effective activation of CTLs, their specific pathways have yet
68 to be elucidated.

69 SLAMF6, also known as Ly108 in mice and NTB-A in humans, belongs to the signaling
70 lymphocyte activation molecule (SLAM) family. This family comprises co-signaling
71 receptors expressed on the surfaces of hematopoietic cells (11). SLAMF6 engages in
72 homophilic interactions, meaning that the SLAMF6 receptor binds to another SLAMF6
73 molecule, leading to the recruitment of SH-2-containing signal-transducing molecules,
74 such as SLAM-associated protein (SAP) and Ewing's sarcoma-activated transcript 2
75 (EAT-2), to its intracellular tyrosine-based switch motif (ITSM) domain. This interaction
76 initiates a co-stimulatory signaling pathway.

77 SLAMF proteins have been shown to modulate the immune response, playing roles in
78 enhancing natural killer T (NKT) cell development (12), NK cell education (13) and co-
79 stimulating natural killer (NK) cell cytotoxicity when interacting with the influenza viral
80 hemagglutinin (14). Disruption of the SLAMF6 signaling pathway, such as through
81 mutations in SAP, can impede the ability of CTLs to clear Epstein-Barr virus (EBV)-
82 infected cells, underlining the significance of the SLAMF6 pathway in mounting an

83 effective antiviral immune response (11, 15–18). Studies in mice have demonstrated
84 that SLAMF6 can localize to the immunological synapse and modulate T cell signaling.
85 In SAP-deficient mice, engagement of SLAMF6 disrupts the T cell-B cell immunological
86 synapse, resulting in reduced cytolytic function (19). In the context of cancer, a soluble
87 SLAMF6 receptor has been shown to enhance CTL function and improve anti-
88 melanoma activity (20), while another study highlights the necessity of SLAMF6
89 clustering for enhanced T cell activation (21). Conversely, when overexpressed,
90 SLAMF6 may function as an immune checkpoint molecule, similar to PD-1, PD-L1, and
91 CTLA-4, thereby potentially suppressing the immune system's ability to target and
92 attack cancer cells (22). Collectively, these studies underscore the critical role of
93 SLAMF6 in immunological synapse formation and cytotoxicity.

94 In the context of HIV infection, the role of the SLAMF6 receptor and its associated
95 signaling pathway in CTL function remains incompletely characterized, although it holds
96 the potential to be a crucial regulatory pathway. Notably, both CD4⁺ T cells, which are
97 the primary targets of HIV, and CTLs express high levels of SLAMF6 on their cell
98 membranes, suggesting the possibility of interactions between SLAMF6 molecules on
99 these two cell types (23). Furthermore, Vpu, an HIV accessory protein, has been linked
100 to the downregulation of SLAMF6 expression in HIV-1-infected cells. However, the
101 extent and implications of this effect on HIV-1-specific cytotoxic immune responses
102 have yet to be thoroughly investigated (24).

103 In this study, we aimed to elucidate the role of SLAMF6 in HIV-1-specific CD8⁺ T cell
104 function. Our hypothesis posited that SLAMF6 plays a pivotal role in mediating the
105 attachment and synapse formation between effector CD8⁺ T cells and HIV-infected
106 CD4⁺ T cells. Our results show that SLAMF6 is indeed required for virus-specific CTLs
107 to efficiently attach to CD4⁺ T cells presenting viral peptides. Furthermore, we have
108 demonstrated that SLAMF6 is a crucial factor in the formation of synapses and the
109 subsequent elimination of HIV-infected CD4⁺ T cells by virus-specific CD8⁺ T cells.

110

111

112 **MATERIALS AND METHODS**

113 **Human subjects:** Peripheral blood was obtained by venipuncture with acid-citrate
114 dextrose anticoagulant from People Living with HIV (PLWH) at the Massachusetts
115 General Hospital (MGH), Boston. Written informed consent was obtained from all
116 volunteers prior to enrollment in the study. The study was approved by the MGH
117 Institutional Review Board (Protocol #2010-P-002463; Protocol #2010-P-002121). All
118 subjects were on antiretroviral therapy (ART) and aviremic at the time of blood draw.
119 Peripheral blood mononuclear cells (PBMC) from PLWH were isolated by Ficoll density
120 gradient centrifugation within six hours of blood draw and cryopreserved in the presence
121 of 10% DMSO in a liquid nitrogen freezer.

122 **Antibodies:** For blockade experiments, LEAF™ Purified anti-human CD352 (NTB-A)
123 (BioLegend, clone NT-7), anti-human CD48 functional grade purified antibody
124 (eBioscience eBio156-4H9), LEAF™ Purified anti-human CD58 antibody (LFA-3)
125 (BioLegend, clone: TS2/9) or mouse IgG1 K isotype control functional grade purified
126 antibody (eBioscience clone P3.6.2.8.1) were used. For intracellular cytokine staining
127 for flow cytometry, the following fluorochromes directly conjugated to mAbs were used:
128 CD3-Alexa Fluor 700 (BD; clone UCHT1), CD8-Qdot 605 (Invitrogen; clone 3B5),
129 SLAMF6-PE (BioLegend; clone NT-7), IFN γ -PE-Cy7 (BD; clone B27), CD4-APC-Cy7
130 (BD; clone SK3). For surface staining of human samples for flow cytometry, the
131 following dyes, and fluorochromes directly conjugated to mAbs were used: hCD45-
132 Alexa Fluor 700 (BioLegend; clone HI30), CD3-PerCP-Cy5.5 (BioLegend; clone
133 UCHT1), CD4-BV785 (BioLegend; clone RPA-T4), CD8-APC-Cy7 (BioLegend; clone
134 RPA-T8), CD45RA-PE-Cy7 (BioLegend; clone HI100), CCR7-BV421 (BD; clone
135 150503), CD38-BV605 (BioLegend; clone HIT2), HLA-DR-BV510 (BioLegend; clone
136 L243), PD-1-APC (BioLegend; clone EH12.2H7), SLAMF6-PE (BioLegend; clone NT-7).

137 **Cell dyes:** For flow cytometry CellTrace™ CFSE Cell Proliferation Kit (Life Science),
138 CellTracker™ Violet BMQC Dye (Life Science), and PKH26 Red Fluorescent Cell dye™
139 (Sigma Aldrich) were used. For confocal microscopy analysis CellTracker™ Blue
140 CMF2HC dye and CellTracker™ Orange CMTMR dye (Invitrogen) were used.

141 **Peptides:** Synthetic peptides were purchased from the MGH peptide core or Genscript
142 as follows: HIV-A*02-SL9, SLYNTVATL; HIV-B*57-KF11, KAFSPEVIPMF; Influenza-
143 A*02-GL9 GILGFVFTL; Epitope identification was facilitated by reference to the Immune
144 Epitope Database (www.iedb.org). HIV-1 Gag 15-mer overlapping peptide pool was
145 designed in-house and used for *ex vivo* conjugation experiments. CMV pp65 15-mer
146 peptide pool was purchased from StemCell Technology.

147 **T cell culture:** CTL lines specific for a given peptide were generated from
148 cryopreserved PBMC from A*02:01 and/or B*57:01⁺ donors living with HIV. PBMCs
149 were thawed and incubated with peptide for 10 days in R10 medium (RPMI
150 supplemented with 10% fetal bovine serum plus Hepes buffer, penicillin, streptomycin,
151 and L-glutamine). IL-2 (50 IU/mL) was added on day 3. Specificity for cognate peptide
152 was tested on day 10 by intracellular cytokine staining (ICS).

153 **Intracellular cytokine staining:** Cells were incubated with peptide (5ug/ml) for 30 min
154 in R10, and then overnight in R10 plus 10 µg/mL brefeldin A. Cells were stained for
155 surface markers (CD3, CD8, CD4, and SLAMF6), fixed, permeabilized, and stained for
156 internal IFN γ . Flow cytometry data were acquired using a BD LSR II cytometer and
157 were analyzed with FlowJo software (Treestar, Inc.).

158 **Surface staining of human samples for flow cytometry:** Frozen PBMC samples from 10
159 HIV-1-negative and 10 HIV-1-positive donors were thawed and resuspended in 1 mL of
160 medium consisting of Advanced RPMI (Corning), 10% FBS (Avantor), 1X primocin
161 (Invivogen), and 2 mM GlutaMAX (Life Technologies). These were transferred into a 24-
162 well plate for a 3-hour incubation at 37°C/5% CO₂ to allow rest and recovery from
163 freeze-thaw cycle. Post-incubation, each sample was collected and passed through a
164 70-µm Nylon filtered FACS tube followed by a wash with 2% FBS in PBS solution. Up to
165 10⁶ cells of each sample were transferred into new FACS tubes, washed with 2% FBS
166 in PBS, and resuspended in 100 µL of a master mix of 2% FBS in PBS containing 1X
167 Live/Dead Blue (Invitrogen) and the following antibodies: hCD45-Alexa Fluor 700, CD3-
168 PerCP-Cy5.5, CD4-BV785, CD8-APC-Cy7, CD45RA-PE-Cy7, CCR7-BV421, CD38-
169 BV605, HLA-DR-BV510, PD-1-APC, SLAMF6-PE. The cells were then incubated for 15
170 minutes at 4°C. The cells were subsequently washed with 2% FBS in PBS and

171 resuspended in 200 μ L of 2% FBS in PBS before proceeding to flow cytometry, which
172 was performed using a BD FACS Symphony (BD Biosciences) and analyzed using
173 FlowJo v10.7.1 and GraphPad Prism 9 software.

174 **Flow-based conjugation assay:** Preparation of target cells: 3 days prior to the
175 experiment, autologous CD4⁺ T cells were negatively Isolated from frozen PBMC by
176 magnetic isolation kit (StemSep™ Human CD4⁺ T Cell Enrichment Kit, STEMCELL
177 Technologies) and cultured in R10 supplemented with IL-2 (100 IU/ml). The day of the
178 experiment, cells were loaded with cognate peptide at the indicated concentration for
179 one hour at 37°C, 5% CO₂. The peptide was washed off and cells were stained with
180 Violet Dye following manufacturer's protocol (Life Technologies).

181 Preparation of effector cells: CD8⁺ T cells were negatively isolated from epitope-specific
182 CTL lines by magnetic separation (StemSep™ Human CD8⁺ T Cell Enrichment Kit,
183 STEMCELL Technologies) and stained with carboxyfluorescein succinimidyl ester
184 (CFSE).

185 Co-incubation: CD8⁺ and CD4⁺ T cells were co-cultured at a ratio 1:1 in a flat bottom 96-
186 well plate; the cells were centrifuged for 3 minutes at 25G and then incubated for 15 min
187 in a 37°C, 5% CO₂ incubator. Then, the plate was placed on ice, spun down at 4°C for 5
188 minutes at 750G. After, the supernatant was aspirated and cells were stained with a
189 viability dye 7-AAD (BD Pharmingen™), incubated for 10 min at 4°C, washed, and
190 gently resuspended in PBS-4% Paraformaldehyde (PFA) for fixation on ice. Samples
191 were analyzed by flow cytometry using a BD 5 Laser LSR2 Fortessa instrument. Flow
192 cytometry data were analyzed by FlowJo software (Treestar, Inc). The rate of antigen-
193 specific conjugation was determined by the frequency of double positive events
194 consisting of both green effector cells and violet loaded target cells.

195 **Confocal microscopy and analysis:** CD4⁺ T cells were stained with 5 μ M
196 CellTracker™ Blue CMF₂HC dye and CD8⁺ T cells with 5 μ M CellTracker™ Orange
197 CMTMR dye according to the manufacturer's instructions. CD4⁺ T cells were then
198 incubated with or without cognate peptides (5 μ g/ml) for 1h at 37°C. The peptide was
199 washed off, and LEAF™ Purified anti-human CD352 antibody or isotype IgG control

200 was added. CD4⁺ T cells and CD8⁺ cells were then mixed at a 1:1 ratio and were loaded
201 onto polylysine-coated coverslips (1x10⁶ cells in 160 µL) in 24 well plates. After 15 min
202 at 37°C, cells were fixed with 4% PFA for 15 min at room temperature, permeabilized
203 with triton 0.5% for 15 minutes at room temperature, blocked with 3% BSA in PBS for 1
204 hour at room temperature, and stained with AlexaFluor 488 Phalloidin (Molecular
205 Probes) overnight at 4°C. The coverslips were then mounted on slides using ProLong
206 Gold Antifade Reagent (Life Technology). Images were acquired with a Zeiss LSM 510
207 confocal microscope equipped with argon (488 nm) and Diode (405 nm, 561 nm) lasers,
208 100 X oil immersion objectives, and ZEN software. At least 10 contacts between CD4⁺ T
209 cells and CD8⁺ T cells were analyzed per condition with Z-stacks spaced by 0.33µm.
210 The 3-D images were then reconstituted using the IMARIS software and surfaces were
211 created for each channel after background deduction.

212 Actin polarization quantification: 3D reconstituted images of synapses were analyzed
213 with Imaris software. The fluorescent signal of the Phalloidin was quantified at the site of
214 contact between the effector and the target cell and compared to the Phalloidin signal
215 on the rest of the CD8 T cell. Ten immunological synapses were analyzed per condition
216 and the mean of the ratio of actin at synapse to actin in the rest of the CD8⁺ cell was
217 calculated for each coupling.

218 Actin ring quantification: Confocal Images were analyzed by Imaris Software, *en face*
219 views from each duplex were taken and the blue pixels (target cells) were removed to
220 allow an unobstructed view of the actin stain at the interface. Three blinded reviewers
221 were asked to score the actin network at the synapse, where 10 was the maximum
222 given to a perfect actin ring and 1 was the lowest score corresponding to the absolute
223 absence of actin rings. A total of 100 duplex images formed under different conditions
224 were randomized and quantified for the actin ring formation.

225 **HIV-1 infection:** Phytohemagglutinin-activated CD4⁺ T cells were harvested and
226 incubated at 1 × 10⁶ cells/mL in 200 µL of R10 in the presence of HIV-1 strain NL4.3 for
227 5 hours. Cells were then washed and plated at a concentration of 1× 10⁶ cells/ml in a 6-
228 well plate for 48 hours.

229 **Chromium-release assay:** Targets: CD4⁺ T cell isolated from PBMCs were loaded with
230 cognate peptide at the indicated concentration and incubated with ⁵¹Cr at a
231 concentration of 250 μCi /ml for one hour in a 37°C 5% CO₂ incubator. ⁵¹Cr-Labeled
232 cells were washed 3 times in R10 and resuspended at a concentration of 1 million
233 cells/ml. Target cells were plated in a flat bottom 96-well plate and incubated with an
234 isotype control or anti-SLAMF6 blocking antibodies for 15 min. Then, effector CD8⁺ T
235 cells isolated from epitope-specific CTL lines were added at the right concentration in
236 order to have an effector-target ratio of 1:1. Target cells treated with 5% Triton solution
237 were used to measure maximum chromium release and target CD4⁺ T cells incubated
238 without effectors were used for measuring the spontaneous ⁵¹Cr release. Cells, whether
239 loaded with peptide or infected, were plated in the absence of effector cells to determine
240 the level of spontaneous release (see **Supplemental Figure 4**). The supernatant was
241 collected after 4 hours of incubation at 37°C 5%CO₂. We used a Perkin Elmer
242 TopCount NXT Microplate Scintillation & Luminescence Counter to measure
243 radioactivity present in the supernatant. Quantification of specific killing was calculated
244 by specific killing =100* (sample release – spontaneous release) /(maximum release-
245 spontaneous release).

246 **Statistics:** Quantitative variables were compared by two-tailed Wilcoxon matched-pairs
247 signed rank test when quantitative variables were paired or Mann-Whitney test when
248 comparisons were made between independent quantitative variables. Correlations were
249 calculated using the Spearman correlation test. All calculations were performed using
250 GraphPad software version 6 and a difference or a correlation was considered
251 significant when $p < 0.05$. For every quantitative variable shown the error bar indicated
252 the SEM (Standard Error of the Mean).

253 RESULTS

254 *Epitope-specific attachment and conjugation of CTL lines and CD4⁺ T cell targets*

255 In order to assess the ability of CD8⁺ T cells to recognize and attach to viral peptides-
256 presenting CD4⁺ T cells, we developed a flow-based conjugation assay with cells from
257 people living with HIV (PLWH) that measures the rate of CD8⁺-CD4⁺ T cell doublets
258 between CTL lines specific for HLA-A*02:01-Flu-GL9 or HLA-B*57:01-HIV-KF11 and
259 autologous CD4⁺ T cell targets presenting MHC-class I epitopes (**Figure 1A**).

260 To validate the sensitivity of the assay, we analyzed in parallel the formation of
261 conjugates after 15 minutes and the frequency of CD8⁺ T cells producing IFN- γ after 6
262 hours of stimulation. We observed that the rate of CD8⁺-CD4⁺ T cell conjugate formation
263 after 15 minutes of co-culture predicted the rate of IFN- γ production by CD8⁺ T effector
264 cells 6 hours after the stimulation ($p = 0.0002$ and $R^2 = 0.76$ for HIV-KF11; $p = 0.05$ and
265 $R^2 = 0.34$ for Flu-GL9) (**Figure 1B**). There was a statistically significant correlation
266 between conjugate formation and IFN- γ production for the different tested peptides from
267 HIV-1 and influenza. This correlation was stronger for HIV-KF11-specific CTLs than for
268 Flu-GL9-specific CTLs. In addition, conjugate formation was proportional to the peptide
269 concentration used to pulse the target cells (**Figure 1C, 1D**) with the following linear
270 regression results: KF11 Donor 1 ($R^2 = 0.96$, $p = 0.02$), KF11 Donor 2 ($R^2 = 0.95$, $p =$
271 0.02), KF11 Donor 3 ($R^2 = 0.96$, $p = 0.02$), GL9 Donor 1 ($R^2 = 0.94$, $p = 0.03$), GL9
272 Donor 2 ($R^2 = 0.97$, $p = 0.01$), and GL9 Donor 3 ($R^2 = 0.99$, $p = 0.006$).

273 Having validated our experimental approach, we proceeded to assess and differentiate
274 epitope-specific and non-specific conjugation events. We set up an assay similar to the
275 cold-target competition assay used to measure cytotoxicity (25). In our setup, violet-
276 labeled non-pulsed CD4⁺ T cells ("cold targets"), red-labeled CD4⁺ T cells pulsed with
277 HIV-KF11 ("warm targets"), and green-labeled HIV-KF11-specific CD8⁺ T-cell effectors
278 were co-incubated for 15 minutes, and conjugate formation was assessed (**Figure 2A**).
279 Supplemental Figure 1 shows the gating strategy used for all the conjugation assays.
280 We demonstrated that blockade with anti-LFA-3 (also known as CD58) antibody, a
281 known adhesion receptor, dramatically reduced peptide-specific conjugation (**Figure**

282 **2B**). Importantly, LFA-3 was used as a positive control, as it is an adhesion molecule
283 necessary to create effective contact between T lymphocytes and antigen-presenting
284 cells (26, 27). Twenty CTL specific lines from 10 HIV-positive patients were tested, and
285 for each of them, we observed that LFA-3 blockade significantly reduced peptide-
286 specific conjugation compared to the isotype control antibody. This finding aligns with
287 the previously described role of LFA-3 as an adhesion molecule (26, 27). Furthermore,
288 although the blockade of LFA-3 resulted in a decrease in the rate of non-specific
289 conjugation, the overall rate of non-specific conjugation was minimal. This observation
290 further affirms the validity of our experimental approach.

291 ***Role of SLAMF6 in effector-target conjugate formation***

292 Prior studies have demonstrated that HIV-1 Vpu downmodulates SLAMF6 on HIV-1-
293 infected CD4⁺ T cells (24, 28) . We tested this by infecting *in vitro* activated primary
294 CD4⁺ T cells from healthy donors with GFP-expressing HIV-1 and measuring SLAMF6
295 expression 48 h post-infection. We observed that SLAMF6 is downmodulated on CD4^{lo}
296 GFP⁺ (i.e. infected) cells when compared to CD4^{hi} GFP⁻ (i.e. non-infected) cells, thus
297 confirming previous findings (**Supplemental figure 2**). In light of this finding and
298 previously published data demonstrating the importance of SLAMF6 signaling in
299 immunological synapse formation (17), we hypothesized that SLAMF6 plays a role in
300 virus-specific CD8⁺ T-cell attachment to target cells presenting viral peptides. To assess
301 the role of SLAMF6 in CD8⁺ T cell attachment to target cells we carried out the flow
302 cytometry-based conjugation assay in the presence of anti-SLAMF6 or an isotype
303 control antibody and determined the rate of specific conjugation of virus-specific CTL
304 lines specific for HIV-SL9, HIV-KF11, or Flu-GL9 to autologous CD4⁺ T cell targets
305 pulsed with cognate viral peptide. Therefore, twenty CTL lines were tested, and we
306 observed that SLAMF6 blockade reduced the rate of peptide-specific conjugation
307 (average reduction of 31.7 % \pm 5.1 %); $p < 0.001$; **Figure 3A**). However, SLAMF6
308 blockade did not significantly affect non-specific conjugation (**Figure 3B**). This was not
309 due to differences in SLAMF6 expression across CTL lines since SLAMF6 was found to
310 be highly expressed and at comparable levels across all CTL lines (MFI = 2455 \pm 93)
311 and *in vitro* cultured CD4⁺ T cells (MFI = 2301 \pm 82) (**Figure 3C and 3D**). Overall, these

312 results suggest that SLAMF6 plays an important role in the adhesion of virus-specific
313 CD8⁺ T cells to viral peptide-presenting CD4⁺ T cells.

314 The sensitivity of CTL lines to the SLAMF6 blockade varied depending on HLA class I
315 specificity. We observed that CTL lines that recognized the HIV-KF11 restricted by HLA-
316 B*57:01 haplotype were less sensitive to the SLAMF6 blockade than CTL lines
317 recognizing HIV-SL9 and Flu-GL9 restricted by HLA-A*02:01 haplotype (**Figure 4A**).
318 For HLA-A*02:01-restricted CTL lines, the SLAMF6 blockade reduced the frequency of
319 CD8⁺-CD4⁺ T cell conjugates by about 39.9 % ± 16.8 % relative to the isotype control,
320 whereas HLA-B*57:01-restricted CTL lines decreased conjugate formation in the
321 presence of SLAMF6 blockade by only 16.7 % ± 6.3%. In contrast, sensitivity towards
322 LFA-3 blockade was comparable for both specificities (**Figure 4B**).

323 To further investigate the role of SLAMF6 in antiviral human T cell responses in a more
324 physiological model, we utilized bulk PBMCs *ex vivo*. For this purpose, we did not limit
325 the stimulation to a single cognate peptide but used a mixture of overlapping peptides
326 from HIV-1 Gag protein and a mixture of peptides from CMV pp65 protein. Of note, we
327 chose CMV peptides, considering the high prevalence of this infection in the general
328 population (greater than 45%) (29).

329 Firstly, we measured SLAMF6 expression levels on peripheral blood CD4⁺ and CD8⁺ T
330 cells from HIV-1-negative (n = 10) and HIV-1-positive (n = 10) human donor PBMCs *ex*
331 *vivo* (**Supplemental Figure 3A**). Due to previous studies demonstrating differential
332 SLAMF6 expression across different T cell subsets (30), we subclassified CD4⁺ and
333 CD8⁺ T-cell subsets as naïve (CD45RA⁺CCR7⁺) or non-naïve (CD45RA⁻ or CCR7⁻),
334 which includes memory and effector T cells, and among non-naïve cells, we identified
335 activated (HLA-DR⁺CD38⁺) T cells and exhausted (PD-1⁺) T cells (**Supplemental**
336 **Figure 3B**). We found that among HIV-1-negative and HIV-1-positive donors, naïve
337 CD4⁺ and CD8⁺ T cells expressed SLAMF6 at similar levels (**Figure 5A**). However,
338 non-naïve CD4⁺ T cells and CD8⁺ T cells had significantly higher levels (approximately
339 2-fold higher relative to naïve), with non-naïve CD8⁺ T cells having significantly higher
340 levels of SLAMF6 than non-naïve CD4⁺ T cells in both donor cohorts (**Figure 5A**).
341 Furthermore, activated and exhausted CD8⁺ T cells exhibited similar SLAMF6

342 expression to non-naïve CD8+ T cells. In contrast, activated and exhausted CD4+ T
343 cells expressed significantly higher levels of SLAMF6 than bulk non-naïve CD4+ T cells,
344 with SLAMF6 levels more similar to those seen among non-naïve CD8+ T cells (**Figure**
345 **5A**). This suggests that non-naïve, activated, and exhausted CD8+ T cells constitutively
346 express similarly high levels of SLAMF6 at baseline, CD4+ T cells further increase
347 SLAMF6 expression levels upon activation and subsequent exhaustion. This is
348 congruent with our prior data demonstrating that *in vitro* activation and expansion of
349 CD4+ and CD8+ T cells with IL-2 results in similarly high expression levels of SLAMF6 (
350 **Figure 3C, 3D**). Regarding HIV-1 donor status, there was a trend for decreased
351 SLAMF6 expression among naïve, non-naïve and activated CD8⁺ T cells and increased
352 SLAMF6 expression on exhausted CD4+ T cells among HIV-1-positive donors, but this
353 was not statistically significant (**Figure 5A**). We did, however, find significant increases
354 of activated CD4+ and CD8+ T cells frequencies in HIV-1-positive donors
355 (**Supplemental Figure 3C**), which is characteristic of HIV-1 infection (30). Overall,
356 SLAMF6 is highly expressed among all CD4+ and CD8+ T-cell subsets, with even
357 higher expression among non-naïve, activated, and exhausted T cells, and no
358 significant differences between HIV-1-negative and HIV-1-positive individuals.

359 Having established the dynamics of SLAMF6 expression among peripheral blood CD8+
360 and CD4+ T cells, we obtained PBMCs from a cohort of HIV-1-positive patients who
361 were also seropositive for CMV. We then conducted a conjugation assay using target
362 CD4+ T cells loaded with overlapping synthetic peptides from HIV-1 Gag or CMV pp65
363 protein. The effect of LFA-3, SLAMF4, and SLAMF6 blockade on *ex vivo* T cells was
364 assessed in three different subjects co-infected with HIV-1 and CMV.

365 As shown for one representative patient (Figure 5B) and in the combined results (Figure
366 5C), the LFA-3 blockade abrogated specific conjugation for HIV-1 Gag-specific *ex-vivo*
367 CD8+ T cells confirming the role of LFA-3 in cell-cell interaction (26, 27), while SLAMF4
368 blockade had no significant effect, indicating that the SLAMF4 receptor does not
369 participate in the formation of conjugates and showing that the effect of SLAMF6 is
370 specific. However, our combined results demonstrated that SLAMF6 blockade
371 decreased specific conjugation between virus-specific CD8+ T cells and viral peptide-

372 presenting CD4⁺ T cells by approximately $46.17 \pm 4.7\%$ compared to the isotype control
373 (Figure 5C).

374 Overall, these findings indicate that SLAMF6 plays an important role in the formation of
375 stable conjugates between effector CD8⁺ T cells and CD4⁺ T cell targets, both in virus-
376 specific CTL lines and *ex-vivo* lymphocytes.

377 ***Effect of SLAMF6 blockade on immunological synapses formation***

378 Effective killing of target cells by CD8⁺ T cells requires peptide-MHC recognition by T
379 cell receptor (TCR), followed by the establishment of a cytolytic immunological synapse:
380 which involves polarization of actin towards the point of contact and rearrangement of
381 this actin to form a connecting membrane ring with a central clearance through which
382 cytolytic granules are released (31). Given the previously described role of co-signaling
383 molecules in this process, we investigated the role of SLAMF6 in immunological
384 synapse formation between CD8⁺-CD4⁺ T cell conjugates using high-resolution confocal
385 microscopy. To this end, we used HIV-1-specific CTL lines and HIV-peptide-pulsed
386 autologous CD4⁺ T cell targets and imaged immunological synapses formed in the
387 presence of anti-SLAMF6 or isotype control antibody.

388 Although SLAMF6 blockade decreased the number HLA-B*57:01-CTL conjugates,
389 because the HLA-B*57:01-restricted CTL lines were less sensitive to the SLAMF6
390 blockade we were able to locate under the microscope several CD8-CD4 contacts even
391 after blockade with SLAMF6. For this reason, we used B*57:01-restricted CTL lines
392 specific for KF11 peptide to analyze the actin cytoskeleton reorganization with or
393 without SLAMF6 blockade. Indeed, we showed an actin polarization with capping of the
394 actin cytoskeleton towards the zone of contact between autologous CD4⁺T cells pulsed
395 with KF11 peptide and the CTL cells. We observed that blockade of SLAMF6 decreased
396 actin polarization in CD8⁺ T cells towards the contact site with CD4⁺ T cells (**Figure 6A**
397 **and 6B**). In addition, central clearance of actin at the immunological synapse was
398 blunted in the presence of SLAMF6 blockade, which led to the absence of the actin ring
399 (**Figure 6C and 6D**). Altogether, these results show that SLAMF6 blockade disrupts

400 actin polarization and cytolytic immunological synapse formation in virus-specific CD8⁺
401 T cells contacting viral peptide-presenting CD4⁺T cells.

402 ***SLAMF6 blockade decreases CTL cytotoxicity***

403 To assess the functional consequences of decreased conjugate formation by SLAMF6
404 blockade, we measured the cytotoxic activity of CTL lines specific for HIV-KF11 and
405 HIV-SL9 by chromium-51 (⁵¹Cr) release assay in the presence of anti-SLAMF6 or
406 isotype control antibodies. We found specific killing of CTL lines against peptide loaded
407 and against HIV-infected target cells but no killing against targets in the absence of
408 peptide or infection. (**Supplemental Figure 4**). We observed a decrease in specific
409 killing in the presence of anti-SLAMF6 antibody for CTL lines restricted by both HLA-
410 A*02:01 (41.8%±17.95 reduction in the presence of SLAMF6 antibody) and HLA-
411 B*57:01 (51.7.8%± 20.32 reduction in the presence of SLAMF6 antibody) alleles
412 (**Figure 7A**). To further investigate the role of SLAMF6 in a more biologically-relevant
413 model, we compared the killing of HIV-infected CD4⁺ T cells by autologous specific CTL
414 lines in presence of an isotype control or an anti-SLAMF6 blockade antibody. We
415 showed that specific killing was reduced from 11.18 %±8.45 to 4.2%±1.92
416 (35.59%±6.41 rate of reduction) in the presence of SLAMF6 blockade antibody (**Figure**
417 **7B**). These data show that SLAMF6 plays a role in the cytolytic function of CD8⁺ T cells
418 against HIV-infected CD4⁺ T target cells by participating to the establishment of
419 functional immune synapses .

420

421 **DISCUSSION**

422 While the pivotal role of SLAMF6 in stabilizing immunological synapses and regulating
423 anti-viral responses has been demonstrated in mice (19, 32), its involvement in human
424 antiviral immune responses has been understudied. Here we delved into the specific
425 role of SLAMF6 in the cytotoxic response against CD4⁺ T cells presenting HIV-1 and
426 CMV-derived viral peptides, as well as HIV-1-infected CD4⁺ T cells.

427 In line with the previous studies in mice, our findings underscore the importance of
428 SLAMF6 for the efficient attachment of CTLs to CD4⁺ T cell targets. Blocking SLAMF6
429 with anti-SLAMF6 antibodies led to a significant reduction in conjugate formation.
430 Interestingly, CTLs recognizing HIV-1 Gag epitopes restricted by HLA-B57:01 exhibited
431 less sensitivity to SLAMF6 blockade compared to CTLs restricted by HLA-A02:01.
432 Given that the HLA-A*02:01-restricted CTL lines are more sensitive to blocking by
433 SLAMF6, it was challenging to study the CD8-CD4 interaction in the context of SLAMF6
434 blockade by confocal microscopy as we were unable to find adequate CD8-CD4
435 contacts to study under the microscope. For HLA-B57:01 restricted CTL, confocal
436 microscopy unveiled that the remaining CD8⁺-CD4⁺ T cell conjugates, after anti-
437 SLAMF6 antibody incubation, primarily stemmed from defects in actin ring formation at
438 the immunological synapse. Moreover, SLAMF6 blockade resulted in decreased killing
439 of HIV-1-infected CD4⁺ T cells by HIV-1-specific CTLs restricted by both HLA alleles,
440 underscoring the broad dependence of cytolytic function on SLAMF6.

441 Firstly, our study reveals the important role of SLAMF6 in the formation of antigen-
442 specific conjugates between antiviral CD8⁺ T cells and CD4⁺ T cell targets. It is
443 consistent with previous research highlighting SLAMF6's significance in cell-cell
444 interactions. For instance, in mice, the absence of intracellular SLAM-associated protein
445 (SAP) disrupts contacts between CD4⁺ T helper cells and cognate B cells, resulting in
446 defective germinal center maintenance and reduced B cell stimulation (33–35). Notably,
447 deletion of SLAMF6 in CD4⁺ T cells in a mouse model reverses germinal center
448 formation defects in SAP knockout mice, demonstrating the role of SLAMF6
449 engagement in SAP-mediated T cell-B cell adhesion (32). Furthermore, Dragovich et al.
450 illustrated that SLAMF6 clustering is essential for the formation of a mature

451 immunological synapse between T cells and antigen-presenting cells, with SLAMF6
452 engagement triggering an activating signal (21). Additionally, bispecific anti-
453 CD3/SLAMF6 antibodies designed to promote SLAMF6 clustering with CD3 enhance T
454 cell activation (36).

455 Our study extends these findings, demonstrating that SLAMF6, in humans, is not only
456 pivotal in regulating cell contacts in the humoral immune response but also in the
457 cytotoxic antiviral immune response by mediating CTL-target cell adhesion in the
458 context of lymphotropic infections like HIV-1.

459 Regarding conjugate formation, we observed differential sensitivity to SLAMF6 blockade
460 between A02:01-restricted CTL lines and B57:01-restricted ones. Of particular interest
461 in HIV-1 infection is HLA-B57:01, strongly associated with spontaneous control (37–40).
462 In our study, we tested an HIV-peptide presented in the HLA-B57:01 context (KF11),
463 known for its immune protective role (37) and high-avidity class-I restricted TCRs (41).
464 High-avidity TCRs, as suggested by previous studies, may be less reliant on accessory
465 molecules in the immunological synapse compared to low-avidity TCRs (42, 43). Thus,
466 we propose that, for SLAMF6-expressing lymphocyte targets, low-avidity CD8⁺ T cell
467 responses are more dependent on SLAM receptor-mediated attachment than high-
468 avidity responses. The TCR-KF11-MHC interaction may suffice to support stable
469 conjugation between CD8⁺-CD4⁺ T cells. However, for HLA-A02-restricted peptides
470 tested in our study, the TCR-pMHC interaction likely lacked the strength, making CTL
471 lines more reliant on the co-signaling molecule SLAMF6. This observation is in line with
472 a previous finding from our laboratory (44), which demonstrated that SLAMF2-mediated
473 modulation of the IFN γ response from CD8⁺ T cells was more pronounced in HLA-A02-
474 restricted specificities compared to those restricted by HLA-B57. The mechanisms
475 underlying why HLA-A02-restricted T cells are more sensitive to SLAMF6 blockade than
476 HLA-B57-expressing cells require further investigation. It is worth noting that, in our
477 study, HLA-B57:01-restricted CTLs, although less sensitive to SLAMF6 in terms of
478 attachment, still depended on SLAMF6 for both immunological synapse formation and
479 cytolytic activity. Our results suggest that HLA-B*57:01-restricted cells might establish
480 effector-target cell contacts differently, with the TCR-pMHC interaction being dominant,

481 while SLAM receptors fine-tune the CTL response to lymphocyte targets. However,
482 further experiments with various peptides presented by the same HLA alleles are
483 needed to explore the relationship between TCR-restricted avidity, synapse formation,
484 and sensitivity to SLAMF6. Furthermore, testing different T cell clones with the same
485 peptide-MHC specificity but with different avidities to SLAMF6 blockade would be
486 helpful in answering this question. It is also important to consider that SLAMF6 may
487 have a role in immune responses among elite controllers, individuals who can control
488 HIV-1 infection below detectable levels without treatment. A comparative study between
489 elite controllers and progressors might provide insights into this hypothesis.

490 Our study also investigated the role of SLAMF6 in immunological synapse formation. A
491 mature immunological synapse involves actin polarization towards the interface (45, 46)
492 followed by the formation of an actin ring at the contact surface between effector and
493 target cells (31), where adhesion molecules like LFA-1 and LFA-3 accumulate (6, 7, 10,
494 47). This facilitates contact stabilization and directional secretion of cytotoxic perforin
495 and granzyme (48). Our study revealed that blocking the SLAMF6 pathway disrupted
496 synaptic architecture, as observed through confocal imaging, and impaired the killing of
497 CD4⁺ T cells presenting peptides and HIV-infected CD4⁺ T cells by specific CD8⁺ T
498 cells. This suggests that SLAMF6 blockade leads to the formation of a "weak synapse,"
499 preventing the secretion of cytotoxic granules into target cells, ultimately reducing
500 specific killing by effector T cells. This observation aligns with previous research in a
501 mouse model, which showed that structurally fragile synapses were associated with
502 impaired killing responses, while well-organized synapses effectively eliminated target
503 cells (49). Furthermore, a recent study demonstrated that SLAMF1 activation enhances
504 the anti-tumoral CD8⁺ T cell response by forming a "corolla" of SLAMF1 around the
505 immune synapse, amplifying TCR signaling. However, engagement with PD-1
506 abrogates SLAMF1-mediated TCR signaling amplification (50). Building upon these
507 results, we hypothesize that the SLAMF6 regulatory pathway is essential for
508 establishing a mature immunological synapse between peptide-pulsed CD4⁺ T cells and
509 autologous peptide-specific CTLs. Given that both recognition and killing of infected
510 CD4⁺ T cells are crucial for controlling HIV-1 infection (1), SLAMF6 may play a

511 significant role in this context. Further investigations are needed to explore the
512 interaction between SLAMF6 and inhibitory coreceptors like PD-1.

513 In mice, the absence of SAP signaling through SLAM family members SLAMF6 and
514 SLAMF4 disrupted synapse formation exclusively between cytotoxic T cells and OVA-
515 pulsed B cells upon SLAMF6 ligand engagement. SHP-1 phosphatase binds to the
516 phosphorylated intracellular tail of SLAMF6, rendering it incapable of forming a proper
517 actin ring (19). In our *in vitro* human cell model, SLAMF6 blockade resulted in a more
518 pronounced phenotype than that observed in SAP knock-out mice. In addition to defects
519 in actin ring formation, we noted a failure of actin polarization towards the contact zone.
520 The underlying mechanism remains to be elucidated, but we hypothesize that basal
521 interaction between SHP-1 phosphatase and actin may exist in cytotoxic effector cells.
522 Thus, SLAMF6 signaling might be necessary to recruit SAP to the synapse and clear
523 actin from its center, as suggested by Chu et al. (51). Their research indicated that the
524 interaction between SHP-1 and the SLAMF6 intracellular tail is constitutive and requires
525 SLAMF6 crosslinking to recruit SAP. In humans, further signaling studies are warranted
526 to validate this hypothesis.

527 In the context of HIV-1 infection, the viral accessory protein Vpu has been shown to
528 downmodulate SLAMF6 on the surface of HIV-1-infected CD4⁺ T cells (24, 52). Our
529 work uncovers a novel evasion mechanism whereby viral protein-mediated
530 downregulation (**supplemental Figure 4**) of SLAMF6 leads to fewer CD8⁺-CD4⁺ T cell
531 contacts, reduced formation of mature immunological synapses, and ultimately,
532 decreased killing of HIV-infected cells. Investigating the effects of Vpu-blocking drugs
533 on the cytotoxic response against HIV-1 mediated by SLAMF6 could yield valuable
534 insights.

535 In summary, our results highlight the significance of SLAMF6 signaling in effector-target
536 cell adhesion, synapse formation, and cytolytic killing of CD4⁺ T cells presenting HIV-1
537 antigens to CD8⁺ T cells. This study sheds light on the critical role of SLAMF6 in
538 combating lymphotropic infections like HIV-1 and suggests potential interventions that
539 could enhance the effects of therapeutic vaccines and T-cell-based immunotherapies
540 for infectious diseases. For instance, exploring the addition of a SLAMF6 cross-linking

541 antibody as an adjuvant to activate its signaling pathway may increase the number of
542 effector-target cell interactions and stabilize immune synapses between HIV-infected
543 cells and HIV-1-specific CD8⁺ T cells.

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706 **FIGURE LEGENDS**

707 **Figure 1: Conjugation of virus-specific CD8⁺ CTLs to CD4⁺ T-cell targets loaded**
708 **with cognate peptide can be accurately measured via flow cytometry and predict**
709 **CTL functionality. (A)** Experimental two-color design for quantifying antigen-specific
710 CD8⁺ and CD4⁺ T-cell Interactions is shown. In this experiment, CTLs were labeled with
711 green fluorescence (CFSE) and autologous CD4⁺ T cells were loaded with varying
712 doses of cognate peptide and stained with a violet dye. Effector and target cells were
713 mixed at a 1:1 ratio of CD8⁺ to CD4⁺ T cells and co-cultured at 37°C for 15 minutes.
714 Subsequently, the cells were fixed and subjected to flow cytometry analysis. The rate of
715 antigen-specific conjugations was determined by calculating the frequency of double-
716 positive cells, where green effector cells overlapped with violet target cells, located in
717 the upper right quadrant of the cytometry plot. **(B)** We investigated the relationship
718 between the frequency of CD8⁺-CD4⁺ T cell conjugate formation occurring within a 15-
719 minute timeframe and subsequent cytokine production by CD8⁺ CTLs at six hours of co-
720 culture. This was done for HIV-1 KF11 peptide (HIV-KF11)-specific CTLs (left) and
721 influenza GL9 peptide (Flu-GL9)-specific CTLs (right) at varying concentration of
722 cognate peptide loaded onto autologous CD4⁺ T-cell targets ($n = 12$ for each). The X-
723 axis represents the frequency of specific conjugate formation observed after 15
724 minutes, while the Y-axis illustrates the frequency of IFN- γ ⁺ CTLs after 6 hours of co-
725 culture. The graphs presented here serve as representatives from a total of three
726 independent experiments, and the correlation between these variables was assessed
727 using a simple linear regression (p values and R^2 shown on graphs). **(C)** CD4⁺ T cell
728 targets were loaded with the HIV-KF11 epitope at the indicated concentrations and then
729 washed to remove excess peptide. HIV-KF11-specific CD8⁺ CTLs were incubated with
730 autologous peptide-pulsed CD4⁺ T cell targets for 15 minutes. Each flow plot represents
731 a different concentration of the loaded peptide, and the conjugate formation is depicted
732 in orange (identified as the double-positive green and violet population) and the
733 conjugate frequency is indicated for each plot. This experiment is representative of
734 three independent experiments. **(D)** The effect of cognate peptide concentration on
735 conjugates formation was measured by co-incubating HIV-KF11-specific (left) or Flu-
736 GL9-specific (right) CTLs with autologous CD4⁺ T-cell targets loaded with different

737 concentration of HIV-KF11 or Flu-GL9 peptide. A simple linear regression between the
738 log-transformed peptide concentration (*X-axis*) and percent of conjugates (*Y-axis*) was
739 assessed, with results as follows: KF11 Donor 1 ($R^2 = 0.96$, $p = 0.02$), KF11 Donor 2
740 ($R^2 = 0.95$, $p = 0.02$), KF11 Donor 3 ($R^2 = 0.96$, $p = 0.02$), GL9 Donor 1 ($R^2 = 0.94$, $p =$
741 0.03), GL9 Donor 2 ($R^2 = 0.97$, $p = 0.01$), and GL9 Donor 3 ($R^2 = 0.99$, $p = 0.006$).

742 **Figure 2: Blockade of LFA-3 inhibits both antigen-specific and non-specific**
743 **attachment and conjugation of CD8⁺ CTLs to autologous CD4⁺ T cells. (A)** We
744 developed a three-colors experimental design for measuring antigen specific and non-
745 specific attachment and conjugation of CD8⁺ CTLs to autologous CD4⁺ T cells. CTLs
746 were labeled with green fluorescence (CFSE) and autologous CD4⁺ T cells isolated
747 from donor PBMCs were divided into two samples: peptide-pulsed targets cells stained
748 in violet and non-peptide-pulsed target cells stained in red. Peptide-pulsed target cells
749 and non-pulsed target cells were co-incubated at a ratio 1:1:1 for 15 minutes at 37°C.
750 Cells were fixed and analyzed by flow cytometry. Within the CFSE+ cells (i.e., CD8⁺
751 CTLs) the rate of antigen-specific conjugation is determined by the frequency of violet-
752 positive conjugates and the rate of non-specific conjugation is determined by the
753 frequency of red-positive conjugates. **(B)** CD8⁺-CD4⁺ conjugate formation sensitive to
754 LFA-3 blockade was explored. CD8⁺ T cells were co-cultured with CD4⁺ T target cells
755 for 15 minutes. Conjugate formation was analyzed by flow cytometry, gating on cells
756 that were double positive for CD8 and CD4. Two conditions were assessed: (i) Percent
757 of conjugation of CD8⁺ T cells (CFSE⁺) and CD4⁺ T cells loaded with cognate peptide
758 (Violet+) (left panel), and (ii) Percent of conjugation of CD8⁺ T cells (CFSE⁺) and CD4⁺
759 T cells not loaded with cognate peptide (Red⁺) (right panel). The analysis was
760 performed using either an isotype control antibody or a blocking antibody against LFA-3
761 (CD58). Co-cultures were analyzed to determine the percentage of specific and non-
762 specific conjugates formed under these conditions. Data represents the conjugation rate
763 from 20 independent experiments ($n = 20$).

764 **Figure 3: SLAMF6 is required for efficient formation of primary human antiviral**
765 **CD8⁺-CD4⁺ T cell conjugates. (A-B)** The conjugation assay was performed with
766 primary T cell lines from a cohort of HIV1-positive subjects ($n = 10$). Multiple CTL lines

767 were generated for each donor and were specific for any of the following viral epitopes:
768 HIV-SL9; HIV-KF11; Flu-GL9. Compiled results from a total of 20 CTL lines are shown.
769 The rate of specific conjugation in the presence of SLAMF6 blockade or isotype control
770 is shown in **(A)** and the rate of non-specific conjugation is shown in **(B)** The effect of the
771 blockade was compared to the IgG isotype control by a two-tailed Wilcoxon matched-
772 pairs signed rank test; p values are shown on graph, with n.s. denoting non-significant
773 ($p > 0.05$). **(C)** Representative flow cytometry histograms measuring SLAMF6
774 expression on T cells used for the conjugation assay is shown for virus-specific CD8⁺
775 CTLs (left) and autologous primary CD4⁺ T cells isolated from PBMC and maintained for
776 3 days in medium containing IL-2 (50 U/ml) (right). The black-filled histogram represents
777 SLAMF6 staining and the dotted histogram represents the FMO (fluorescence minus
778 one). **(D)** Combined results of SLAMF6 expression on CTLs and IL-2-cultured CD4⁺ T
779 cells from 10 different HIV-1-positive subjects are shown. Two-tailed Wilcoxon matched-
780 pairs signed rank test showed no statistically significant differences (n.s.).

781 **Figure 4: HLA-B57:01-restricted CTL lines are less sensitive to SLAMF6 blockade**
782 **than HLA-A*02:01-restricted lines.** For each CTL line studied, the "percent conjugate
783 inhibition" was calculated as: $100\% - (\text{Percent specific conjugation in the presence of}$
784 $\text{the blockade} / \text{Percent of specific conjugation in the presence of the IgG Isotype}$
785 $\text{control})$. The mean "specific-conjugate inhibition" in the presence of blockade of **(A)**
786 SLAMF6 or **(B)** LFA-3 was assessed for HLA-B57:01-restricted CTL lines ($n = 7$)
787 compared to HLA-A*02:01-restricted CTL lines ($n = 13$) and compared by the Mann-
788 Whitney test. The p -value is indicated for each graph.

789 **Figure 5: SLAMF6 is highly but differentially expressed on CD8⁺ and CD4⁺ T-cell**
790 **subsets and is required for efficient CD8⁺-CD4⁺ T cell conjugate formation directly**
791 **ex vivo.** **(A)** SLAMF6 expression, measured as median fluorescence intensity (MdFI),
792 was assessed on different CD8⁺ T and CD4⁺ T-cell subsets in PBMCs from HIV-1-
793 positive ($n = 10$) and HIV-1-negative ($n = 10$) donors *ex vivo* via flow cytometry;
794 representative histograms of SLAMF6 expression on different T-cell subsets is shown,
795 with aggregate data plotted below as violin plots. One-way ANOVA with correction for
796 multiple comparisons was performed; statistical significance is denoted as follows: * $p <$

797 0.05, ** $p < 0.01$, **** $p < 0.0001$. **(B-C)**. Conjugation assay on CD8⁺ T cells from *ex*
798 *vivo* PBMCs and autologous CD4⁺ T target cells in the presence of SLAMF6, SLAMF4
799 or LFA-3 blocking antibodies, or isotype IgG control. The peptides used in this assay
800 were pools of overlapping synthetic peptides representing the HIV-1 Gag protein or the
801 CMV pp65 protein. Specific conjugation is shown in the upper left quadrant. All subjects
802 studied were HIV-1 and CMV co-infected. The flow plots in **B** are representative of six
803 additional conjugation assays performed on *ex vivo* T cells. The rate of specific
804 conjugation in the presence of a SLAMF6, SLAMF4 and LFA-3 blockade was assessed
805 and shown in **C** ($n = 6$). The effects of the blockade were compared to an IgG isotype
806 control using a two-tailed Wilcoxon matched-pairs signed rank test with p -values shown
807 on graphs.

808 **Figure 6: SLAMF6 is necessary for immunological synapse formation between**
809 **CD8⁺ T effector cells and CD4⁺ T target cells.** **(A)** HIV-KF11-specific CD8⁺ T cells
810 (red) were co-cultured for fifteen minutes with autologous antigen-loaded CD4⁺ T cell
811 targets (blue) in the absence or presence of a SLAMF6 blockade, stained for actin
812 localization with phalloidin (green), and analyzed by confocal microscopy. Four different
813 images with a magnification of X100 are shown, representative of 100 different pictures
814 taken. **(B)** Antigen-specific actin polarization in CD8⁺-CD4⁺ T cell contacts is observed
815 in the presence of the SLAMF6 blockade (right) and isotype control (left). The ratio of
816 actin polarization in the presence or absence of the SLAMF6 blockade was compared
817 using the Mann-Whitney test; the p -value is indicated in the graph. **(C)** The 3D plots
818 show *en face* views of the CD4⁺-CD8⁺ T cell contacts with a magnification of 100X. The
819 blue pixels were removed to allow an unobstructed view of the synapse. Four
820 representative images are shown, both in the presence of the isotype control (left) and
821 the SLAMF6 blockade (right). **(D)** The ring formation was scored for all 100 images
822 analyzed from 5 independent experiments, and SLAMF6 vs. isotype control were
823 compared for each experiment and analyzed by the Mann-Whitney test; the p -value is
824 indicated in the graph.

825 **Figure 7: SLAMF6 is necessary for CD8⁺ T cell killing function.** **(A)** Cr⁵¹-labeled
826 CD4⁺ T cells were loaded with two different peptides, either HIV-KF11 (black squares)

827 or HIV-SL9 (open circles), at 5 $\mu\text{g}/\text{mL}$ and co-cultured with autologous peptide-specific
828 CD8^+ T cells. Cr^{51} release from effectors was assessed in the presence of a SLAMF6
829 blocking antibody or an isotype control. Seven different CTL lines were tested. **(B)**
830 Activated primary CD4^+ T cells were infected with HIV-1 (clone NL4-3) virus for 48
831 hours, then labeled with Cr^{51} and incubated with autologous peptide-specific CD8^+ T
832 effector cells for 4 hours at a ratio of 1:1 in the presence of a SLAMF6 blockade or an
833 isotype control. Five different CTL lines were tested. Black squares represent the HIV-
834 KF11-specific CTL lines, and open circles represent the HIV-SL9-specific CTL lines
835 tested. Specific killing of target cells in the Cr^{51} release assay was calculated as $100 \times$
836 $(\text{sample release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$.
837 Statistical differences were measured using the Wilcoxon matched-pairs test with p -
838 values shown on graph.

839 **Supplemental Figure 1: Gating strategy flow cytometry showing a representative**
840 **gating strategy.** After gating on lymphocytes gate, 7AAD negative cells were selected
841 (viable cells) and, within those the percent of specific conjugation rate (CFSE^+ and Violet
842 Cell Tracker⁺) or Unspecific conjugation rate (CFSE^+ PK26H Red⁺) was assessed.

843 **Supplemental Figure 2: SLAMF6 surface expression is reduced in HIV-infected**
844 **CD4^+ T-cells.** **(A)** Activated primary CD4^+ T-cells from two HIV-negative subjects were
845 infected with NL4.3-GFP HIV and were stained for CD4^+ T cells expression. Infected
846 cells were gated on CD4^+ T cells low and GFP^+ cells. **(B)** Expression of SLAMF6 at the
847 surfaces of infected GFP^+ cells (filled) was compared to the uninfected GFP^- cells (dotted
848 line).

849 **Supplemental Figure 3: Characterization of SLAMF6 expression in CD4^+ and**
850 **CD8^+ T-cell subsets among HIV-1-positive and HIV-1-negative donors.** **(A)**
851 Demographics and relevant clinical data are shown for HIV-1-positive donors ($n = 10$);
852 HIV-1-negative donors were healthy anonymous adults. **(B)** Flow cytometry gating
853 scheme used to differentiate different CD4^+ and CD8^+ T-cell subsets is demonstrated.
854 **(C)** The frequency of the different T-cell subsets among HIV-1-positive and HIV-1-
855 negative donors is shown. One-way ANOVA with correction for multiple comparisons
856 was performed; statistical significance is denoted as follows: **** $p < 0.0001$

857 **Supplemental Figure 4: Set-up of the chromium release killing assay.** We tested
858 various effector-to-target (E:T) cell ratios. Target cells were either loaded with 5 µg/ml of
859 peptide or left unloaded before the addition of CTL effector cells. The percentage of
860 killing was determined using the formula: $100 \times (\text{average of the two duplicates} - \text{average}$
861 $\text{of spontaneous release}) / (\text{average duplicates of maximum release} - \text{average of}$
862 $\text{spontaneous release})$. **(A)** Raw data obtained from a Chromium release assay. **(B)** The
863 percentages of killing under different experimental conditions are presented below.

864

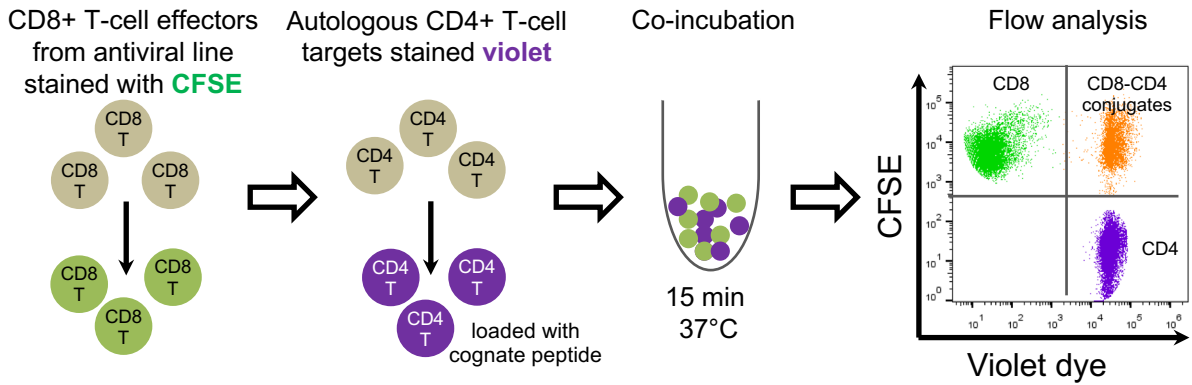
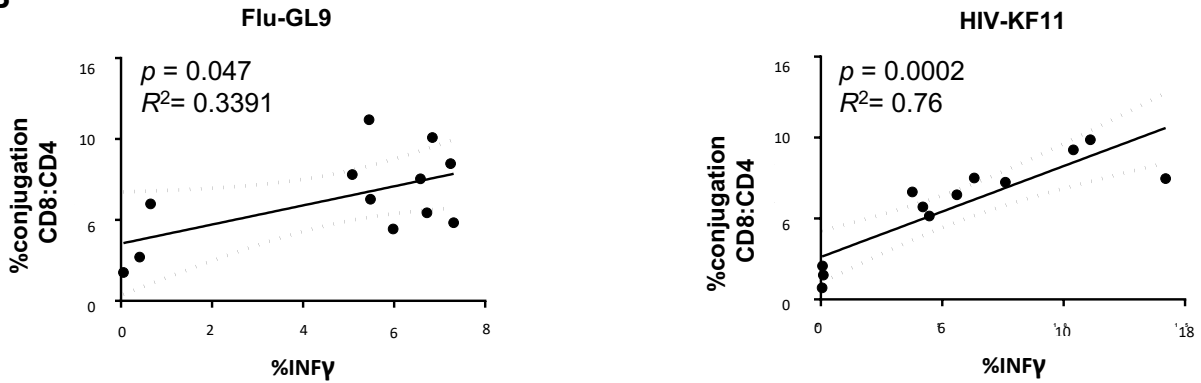
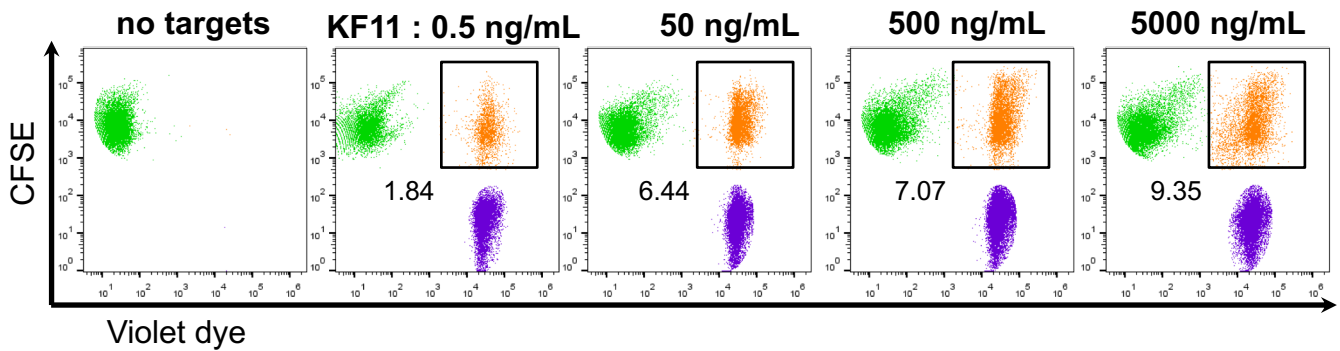
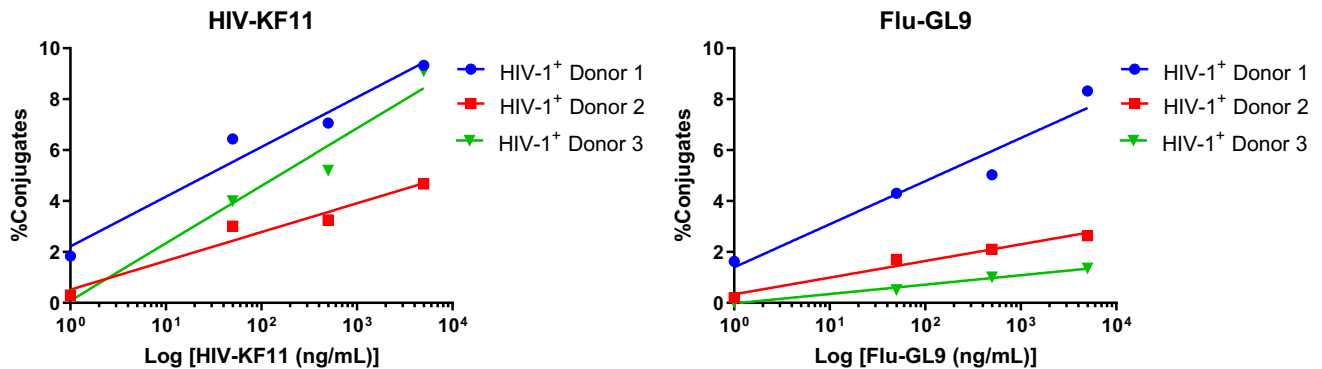
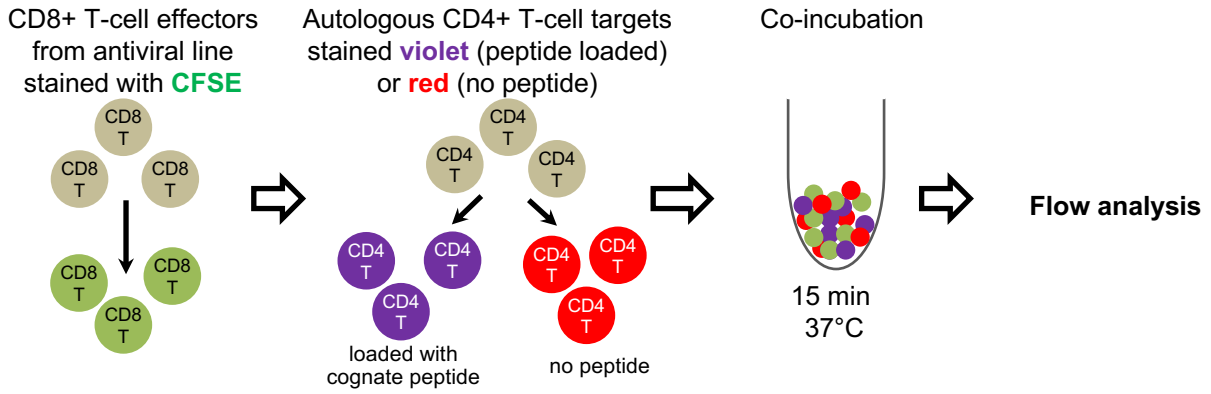
Figure 1**A****B****C****D**

Figure 2

A



B

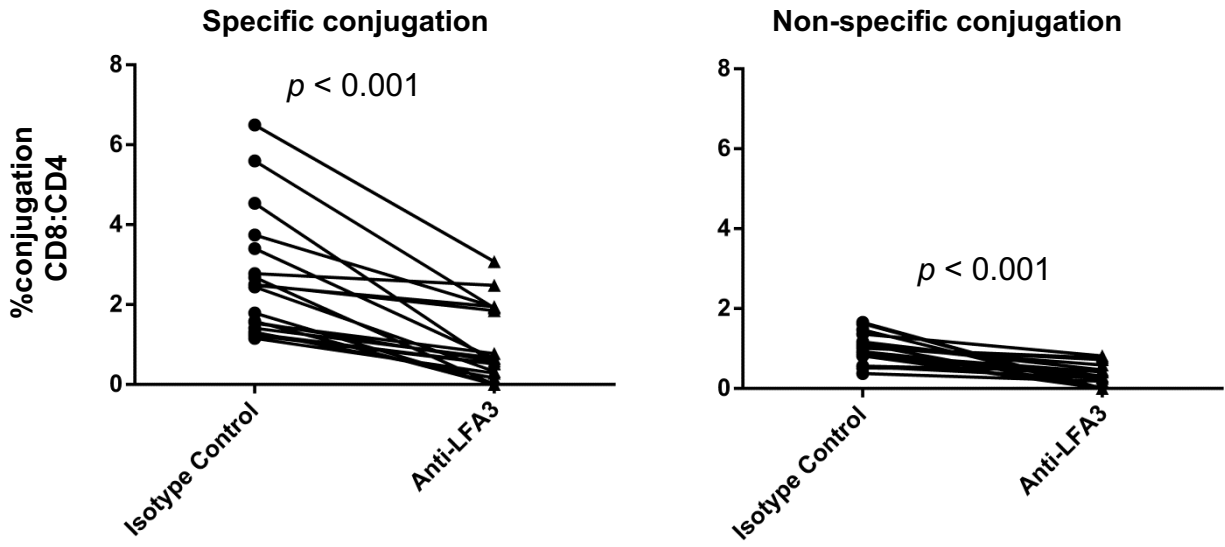


Figure 3

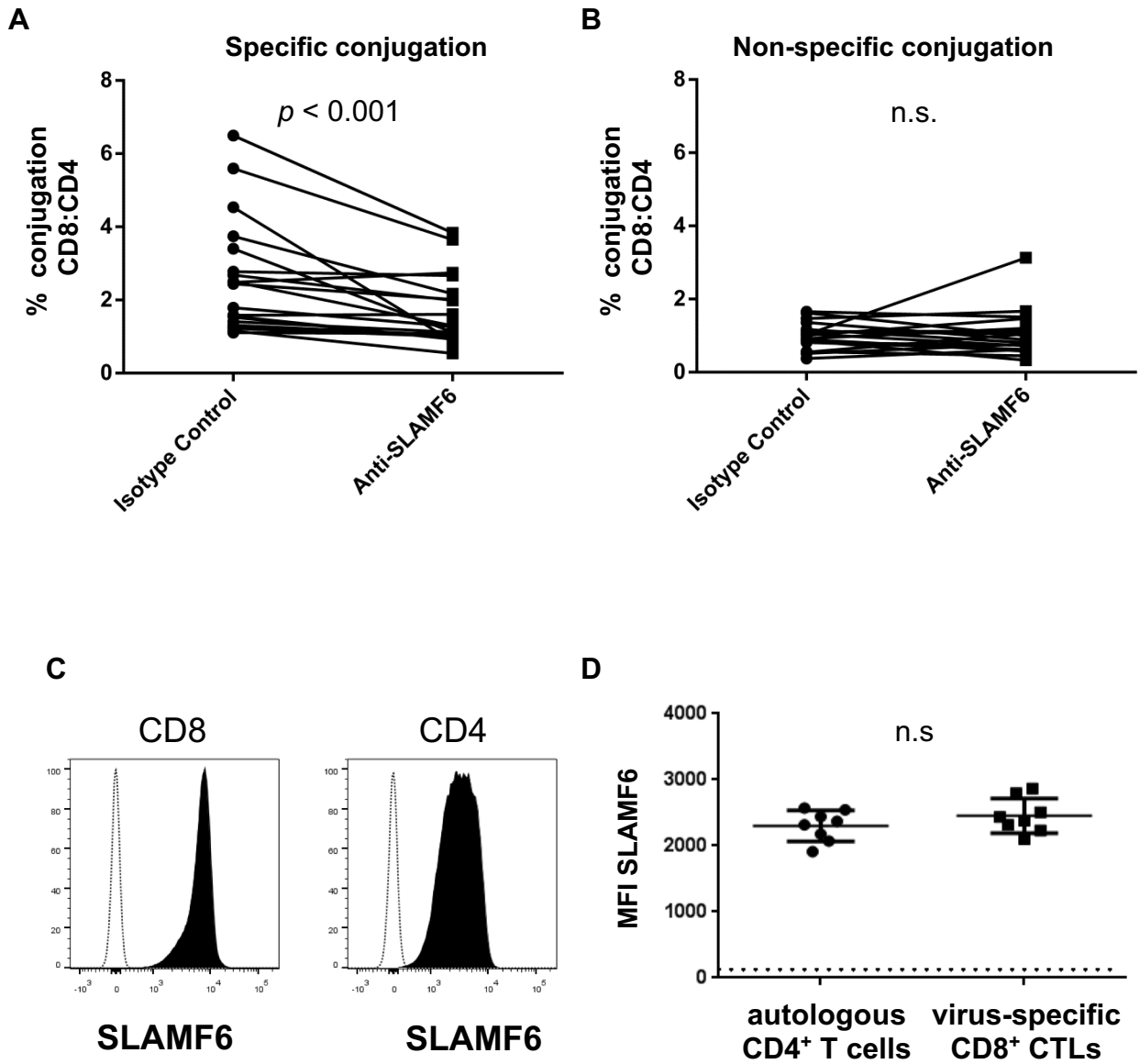
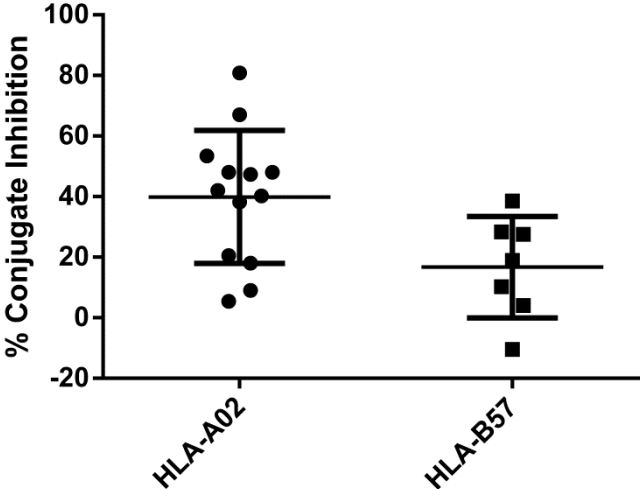


Figure 4

A Anti-SLAMF6 blockade

$p = 0.03$



B Anti-LFA-3 blockade

$p = 0.4$

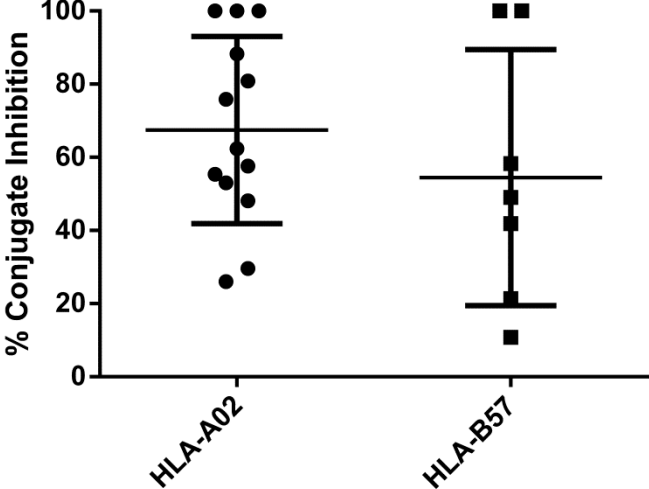


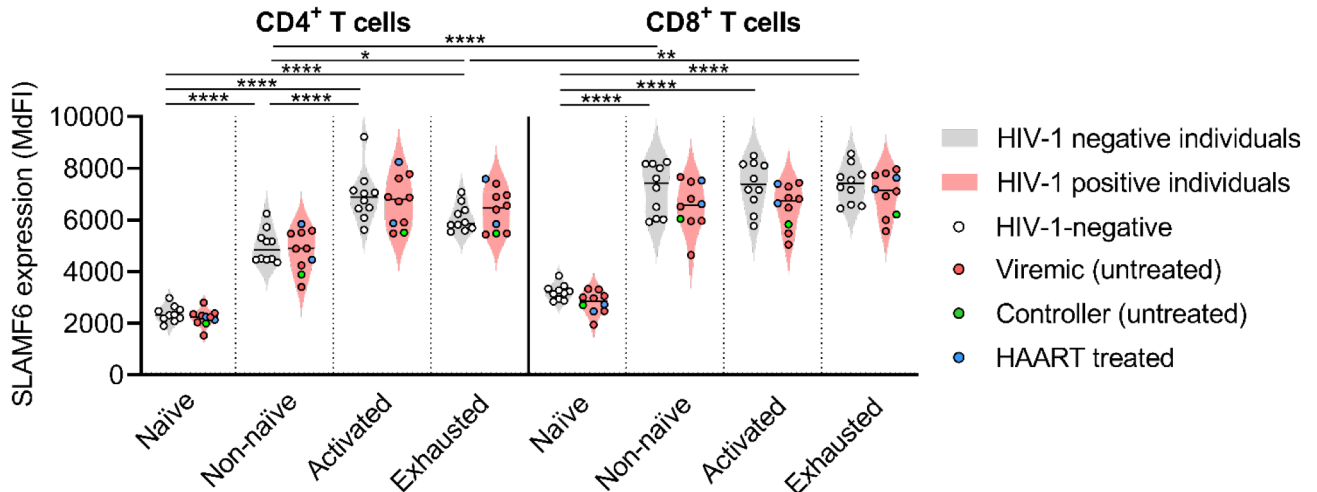
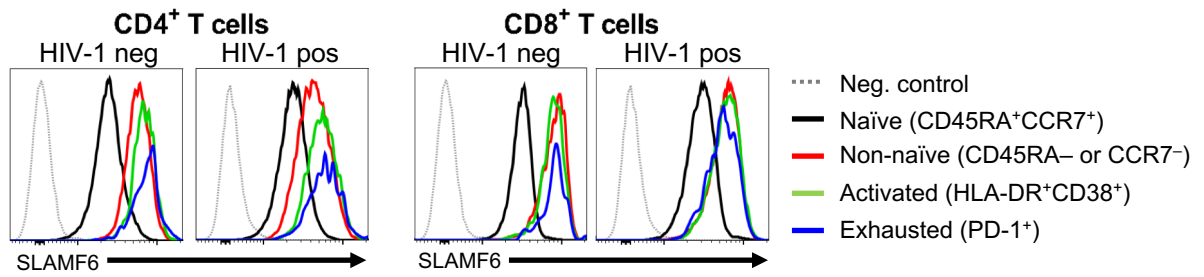
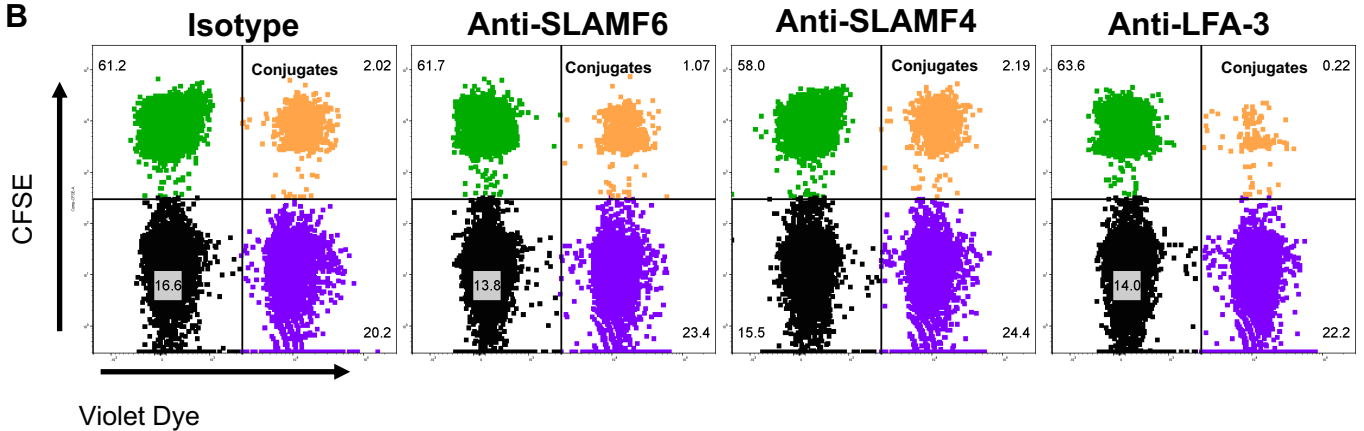
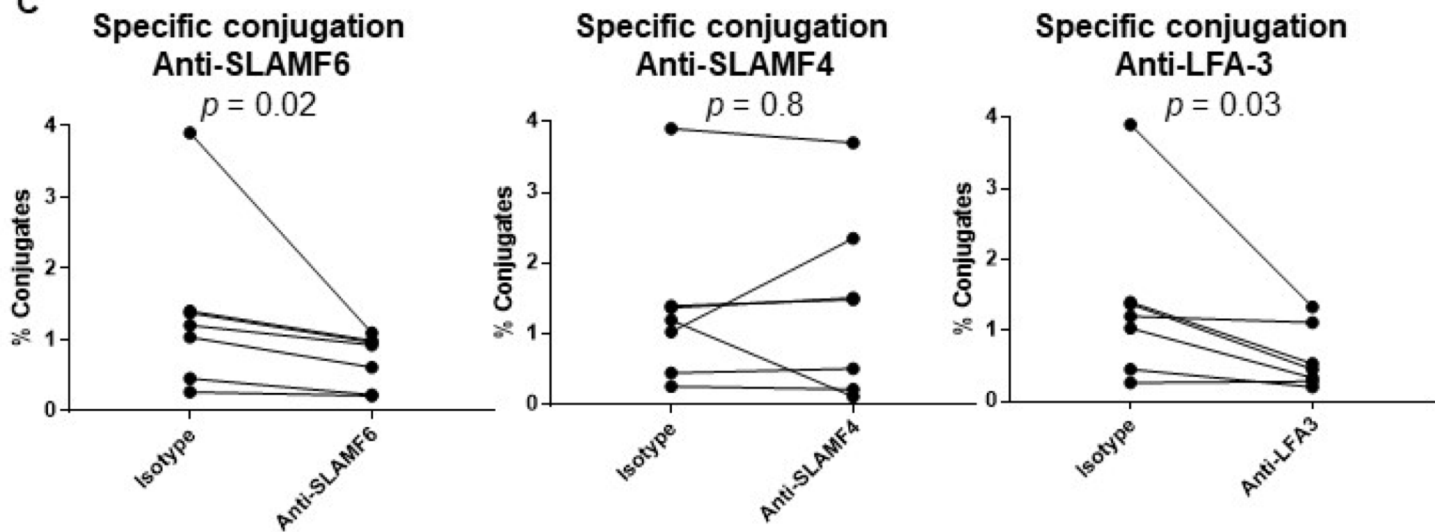
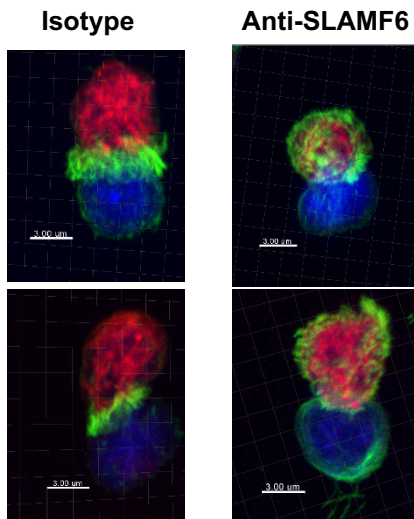
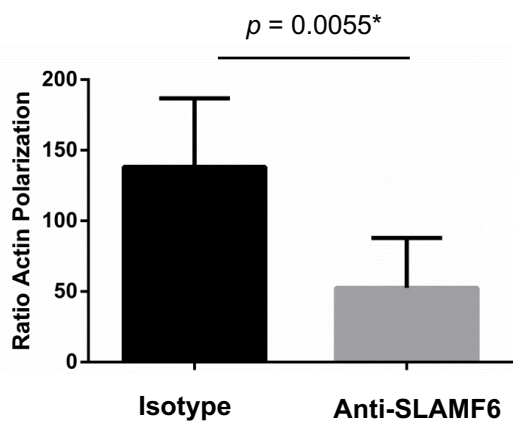
Figure 5**A****B****C**

Figure 6

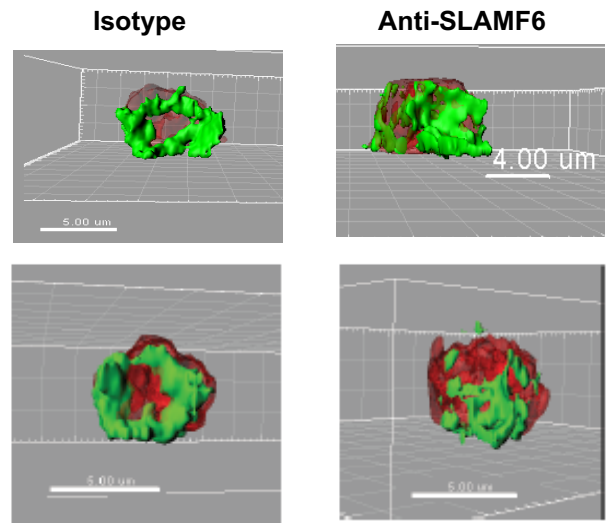
A



B



C



D

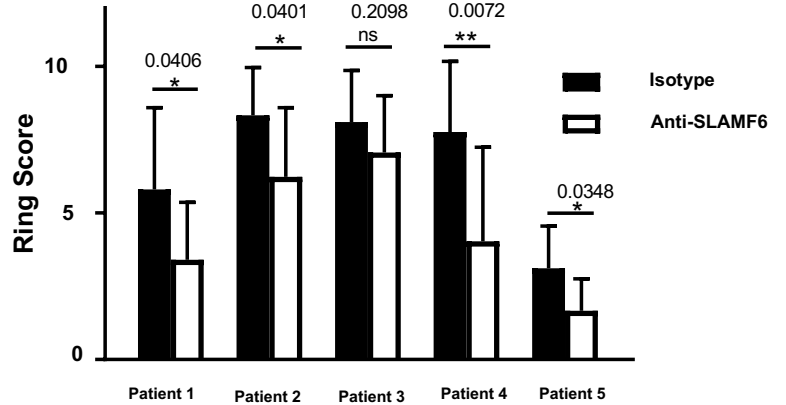


Figure 7

