1 **HIV-Tat immunization induces cross-clade neutralizing antibodies and $CD4^+$ T-cell increases**
2 **in antiretroviral-treated South African volunteers: a randomized phase II clinical trial**

3

4 Barbara Ensoli[1], Maphoshane Nchabeleng[2*], Fabrizio Ensoli[3], Antonella Tripiciano[1,3], Stefania
5 Bellino[1x], Orietta Picconi[1], Cecilia Sgadari[1], Olimpia Longo[1xx], Lara Tavoschi[4xxx], Daniel Joffe[4],
6 Aurelio Cafaro[1], Vittorio Francavilla[1,3], Sonia Moretti[1], Maria R Pavone Cossut[1], Barbara
7 Collacchi[1], Angela Arancio[1,3], Giovanni Paniccia[1,3], Anna Casabianca[5], Mauro Magnani[5], Stefano
8 Buttò[1], Elise Levendal[6xxxx], John Velaphi Ndimande[7], Bennett Asia[8], Yogan Pillay[8], Enrico
9 Garaci[9xxxxx], Paolo Monini[4].

10

11 [1]National AIDS Center, Istituto Superiore di Sanità, Rome, Italy; [2]Medunsa (MeCRU), Sefako
12 Makgatho Health Sciences University (SMHSU), Ga-Rankuwa, South Africa; [3]Laboratory of
13 Clinical Pathology and Microbiology, San Gallicano Institute, Istituti Fisioterapici Ospitalieri,
14 Rome, Italy; [4]Head Office, National AIDS Center, Istituto Superiore di Sanità, Cape Town, South
15 Africa; [5]Department of Biomolecular Science, University of Urbino, Urbino, Italy; [6]South African
16 Medical Research Council, Cape Town, South Africa; [7]Department of Health, Gauteng Province,
17 Marshall Town, South Africa; [8]National Department of Health, Pretoria, South Africa; [9]Istituto
18 Superiore di Sanità, Rome, Italy
19 [x]Present affiliation: National Center for Epidemiology, Surveillance and Health Promotion, Istituto
20 Superiore di Sanità, Rome, Italy.
21 [xx]Present affiliation: Italian Medicines Agency, Rome, Italy.
22 [xxx]Present affiliation: European Center for Disease Prevention and Control, Stockholm, Sweden.
23 [xxxx]Present affiliation: Health Systems Trust, Cape Town, South Africa.
24 [xxxxx]Present affiliation: University of Tor Vergata, Rome, Italy.
25 [*]For the SMHSU-MeCRU Team

26 Corresponding author: Dr. Barbara Ensoli, National AIDS Center, Istituto Superiore di Sanità, Viale
27 Regina Elena 299, 00161 Rome, Italy; e-mail: barbara.ensoli@iss.it
28

29 Barbara Ensoli: barbara.ensoli@iss.it; Maphoshane Nchabeleng:
30 Maphoshane.Nchabeleng@ul.ac.za; Fabrizio Ensoli: ensoli@ifo.it; Antonella Tripiciano:
31 antonella.tripiciano@iss.it; Stefania Bellino: stefania.bellino@iss.it; Orietta Picconi:
32 orietta.picconi@iss.it; Cecilia Sgadari: cecilia.sgadari@iss.it; Olimpia Longo: o.longo@aifa.gov.it;
33 Lara Tavoschi: lara.tavoschi@ecdc.europa.eu; Daniel Joffe: Daniel.Joffe@mrc.ac.za; Aurelio
34 Cafaro: aurelio.cafaro@iss.it; Vittorio Francavilla: vittorio.francavilla@iss.it; Sonia Moretti:
35 sonia.moretti@iss.it; Maria R Pavone Cossut: mariarosaria.pavonecossut@iss.it; Barbara Collacchi:
36 barbara.collacchi@iss.it; Angela Arancio: angela.arancio@iss.it; Giovanni Paniccia:
37 giovanni.paniccia@iss.it; Anna Casabianca: anna.casabianca@uniurb.it; Mauro Magnani:
38 mauro.magnani@uniurb.it; Stefano Buttò: stefano.butto@iss.it; Elise Levendal:
39 elise.levendal@hst.org.za; John Velaphi Ndimande: john.ndimande@gauteng.gov.za; Bennett Asia:
40 asiabe@health.gov.za; Yogan Pillay: PillaY@health.gov.za; Enrico Garaci:
41 garaci@med.uniroma2.it; Paolo Monini: Paolo.Monini@mrc.ac.za .

42

43

44 **ABSTRACT**

45

46 **BACKGROUND.** Although combined antiretroviral therapy (cART) has saved millions of lives, it
47 is incapable of full immune reconstitution and virus eradication. The Transactivator of transcription
48 (Tat) protein is a key human immunodeficiency virus (HIV) virulence factor required for virus
49 replication and transmission. Tat is expressed and released extracellularly by infected cells also
50 under cART and in this form induces immune dysregulation, and promotes virus reactivation, entry
51 and spreading. Of note, anti-Tat antibodies are rare in natural infection and, when present, correlate

52 with asymptomatic state and reduced disease progression. This suggested that induction of anti-Tat
53 antibodies represents a pathogenesis-driven intervention to block progression and to intensify
54 cART. Indeed Tat-based vaccination was safe, immunogenic and capable of immune restoration in
55 an open-label, randomized phase II clinical trial conducted in 168 cART-treated volunteers in Italy.
56 To assess whether B-clade Tat immunization would be effective also in patients with different
57 genetic background and infecting virus, a phase II trial was conducted in South Africa.

58 **METHODS**. The ISS T-003 was a 48-week randomised, double-blinded, placebo-controlled trial to
59 evaluate immunogenicity (primary endpoint) and safety (secondary endpoint) of B-clade Tat (30
60 μg) given intradermally, 3 times at 4-week intervals, in 200 HIV-infected adults on effective cART
61 (randomised 1:1) with $CD4^+$ T-cell counts ≥200 cells/μL. Study outcomes also included cross-clade
62 anti-Tat antibodies, neutralization, $CD4^+$ T-cell counts and therapy compliance.

63 **RESULTS**. Immunization was safe and well-tolerated and induced durable, high titers anti-Tat B-
64 clade antibodies in 97% vaccinees. Anti-Tat antibodies were cross-clade (all vaccinees tested) and
65 neutralized Tat-mediated entry of oligomeric B-clade and C-clade Envelope (Env) in dendritic cells
66 (24 participants tested). Anti-Tat antibody titers correlated positively with neutralization. Tat
67 vaccination increased $CD4^+$ T-cell numbers (all participants tested), particularly when baseline
68 levels were still low after years of therapy, and this had a positive correlation with HIV
69 neutralization. Finally, in cART non-compliant patients (24 participants), vaccination contained
70 viral load rebound and maintained $CD4^+$ T-cell numbers over study entry levels as compared to
71 placebo.

72 **CONCLUSIONS**. The data indicate that Tat vaccination can restore the immune system and
73 induces cross-clade neutralizing anti-Tat antibodies in patients with different genetic backgrounds
74 and infecting viruses, supporting the conduct of phase III studies in South Africa.

75 **TRIAL REGISTRATION.** ClinicalTrials.gov NCT01513135, 01/23/2012.

76 **KEY WORDS**: Tat, HIV, AIDS, clinical trials, vaccine, cross-clade antibodies, neutralization,
77 $CD4^+$ T cells, cART, therapy intensification.

78

BACKGROUND

South Africa is severely affected by human immunodeficiency virus (HIV) infection (1). The HIV counselling and testing campaign (HCT) launched by the National Department of Health has steadily increased the proportion of HIV-infected patients on combined antiretroviral therapy (cART). However, access to therapy and care of millions of people living with HIV is posing an enormous challenge to the public health system by means of a growing work overload and economic burden. This is going to be further complicated by the expected implementation of the new World Health Organization (WHO) guidelines that recommend starting therapy at the time of the first positive HIV testing ("test and treat") (2). However, despite vast access to cART, the rates of HIV morbidity/mortality are still high, with a 14% annual increase of HIV drug resistance related to insufficient treatment compliance, which hampers an effective suppression of virus replication, a prerequisite to reduce virus transmission (3). Further, late therapy initiation is still frequent in South Africa limiting the extent of $CD4^+$ T cell recovery and immune restoration (4,5). Similarly, persistent immune activation, particularly when associated with poor immunological response to therapy, leads to disease progression even under HIV suppression (6-8). These are causes of increasing co-morbidities, hospitalization, deaths and costs for the National Health Systems. In this context, an effective therapeutic vaccine, in conjunction with existing strategies, may represent a relevant, cost-effective intervention to intensify cART (9).

The Transactivator of transcription (Tat) is a key HIV virulence factor playing pivotal roles in virus gene expression, replication, transmission and disease progression (reviewed in 10 and 11). Tat is produced very early upon infection (12-16) and continues to be expressed under cART (17, 18), is released extracellularly (19-21), accumulates in tissues (22, 23), and exerts effects on both the virus and the immune system (17, 24-52) that make it an optimal candidate for therapeutic immunization and cART intensification (53-58). In particular, by promoting an excessive and improper immune stimulation, Tat prepares target cells for virus propagation, while disabling an effective immune

104 control (17, 24-52). This leads to the chronic loss of immune homeostasis observed in HIV-infected
105 patients, which is only partially reverted by cART (59-63). Further, extracellular Tat, which is
106 present on virions (64), binds the Envelope (Env) spikes forming a virus entry complex that favors
107 infection of dendritic cell (DC) and T cells, key components of the virus reservoir (65). Of note, by
108 binding the Env C-C chemokine receptor 5 (CCR5) co-receptor binding sites, Tat shields Env from
109 anti-HIV antibodies (Abs), thus inhibiting virus neutralization, which, however, is restored by anti-
110 Tat Abs (65). Notably, anti-Tat Abs are uncommon in natural infection and, when present, correlate
111 with the asymptomatic state, higher $CD4^+$ T-cell number, lower viral load, and reduced disease
112 progression (66-70). This suggested that the induction of effective anti-Tat Abs represents a
113 pathogenesis-driven intervention to block progression and to intensify cART efficacy.
114 After completion of randomised, placebo-controlled, double-blinded phase I trials with the
115 biologically active HIV-1 B-clade Tat protein in HIV-infected and uninfected individuals in Italy
116 (54-56), an open-label randomised exploratory phase II trial with Tat was conducted in 168 HIV-
117 infected anti-Tat Abs negative, virologically suppressed cART-treated (mean of 6 years) adult
118 subjects in Italy (ISS T-002, ClinicalTrials.gov NCT00751595) (53, 57). The endpoints were to
119 evaluate immunogenicity and safety of B-clade Tat protein administered at 7.5 or 30 μg, given 3 or
120 5 times monthly, and to investigate immunological and virological disease biomarkers. The vaccine
121 was safe and well tolerated and induced anti-Tat Abs in most patients (79%), with the highest
122 frequency and durability in the Tat 30 μg groups (89%), particularly when given 3 times (92%).
123 Vaccination promoted a durable and significant restoration of T, B, and natural killer (NK) cell
124 numbers, increased $CD4^+$ and $CD8^+$ central memory subsets, and upregulated the expression of
125 human leukocyte antigen-D related ($HLA\text{-}DR^+$) on $CD8^+$ killer T cells, a phenotype found to be
126 increased in elite controllers and to contribute to HIV containment (71, 72). Moreover, a significant
127 reduction of blood proviral DNA was seen after 3 years from the first immunisation, particularly
128 under protease inhibitor (PI)-based regimens and with Tat 30 μg given 3 times (30 μg, 3x), reaching
129 a predicted 70% decay with a half-life of 88 weeks (57). This decay was significantly associated

130 with anti-Tat immunoglobulin (Ig) M and IgG Ab titers and neutralization of Tat-mediated entry of
131 oligomeric Env in DC. Neutralization predicted HIV-1 DNA decay (57).
132 Based on these data, a 48-week randomised, double-blinded, placebo-controlled phase II study was
133 conducted in cART-treated South African adult volunteers to verify the immunogenicity and safety
134 of the B-clade Tat vaccine in a population with a different genetic background and mainly infected
135 with a C clade virus. Anti-Tat Abs were further characterised to explore cross-clade recognition and
136 their capability of cross-neutralising Tat-mediated oligomeric Env entry in DC. $CD4^+$ T-cell counts
137 were monitored for the entire trial, and the relationship between neutralization and $CD4^+$ T-cell
138 counts, as well as between anti-Tat and anti-Env Ab titers and neutralization, were also examined.
139

140 ## Methods

141 *Production and purification of the recombinant biologically active HIV-1 Tat protein for human*
142 *use.* The biologically active recombinant clade B HIV-1 Tat, selected as vaccine candidate for
143 human use, is the 86 amino acid-long protein derived from the HTLV-IIIB strain (BH-10 clone)
144 (Supplementary Figure 1). The protein was produced under Good Manufacturing Practice (GMP)
145 conditions by Diatheva-Avitech APU Srl, Fano (PU), Italy. Tat vialing, packaging and batch release
146 was performed by Injectalia Srl, Rome, Italy. Briefly, the Tat protein is obtained from a lysate of E.
147 coli cells engineered with the pET-tat plasmid, constructed for Tat expression. The pET system is
148 based on the T7 promoter-driven system originally developed by Studier and colleagues (73-75),
149 and provides vector-host combinations that enable tuning of basal expression levels to optimize
150 target gene expression (75). The GMP protein is then purified by diethylaminoethyl (DEAE)
151 chromatography followed by heparin sepharose chromatography. Following purification, the Tat
152 protein is formulated in potassium phosphate saline buffer, pH 7.4, containing 1% sucrose and 1%
153 human serum albumin (HSA). This formulation was defined in order to maintain the biological
154 activity of the protein in a liquid form, stored at -80 °C in the absence of light over 3 years.
155

156 *Study design and conduction.* The ISS T-003 (*ClinicalTrials.gov NCT01513135*) was a phase II,
157 randomised, double-blinded, placebo-controlled, clinical trial with the recombinant biologically
158 active HIV-1 B-clade Tat protein conducted at the MeCRU, University of Limpopo, Medunsa
159 Campus (now Sefako Makgatho Health Sciences University), South Africa (ISS T-003 Study
160 Protocol, Supplementary Material). The study was designed to evaluate Tat protein immunogenicity
161 and safety in HIV-1-infected, cART-treated, anti-Tat Ab-negative adult South Africans, and to
162 explore $CD4^{+}$ T-cell numbers and anti-Tat cross-clade neutralizing activity after immunization. The
163 study duration was 48 weeks including an 8-week treatment phase and a 40-week follow-up phase.
164 The allowed window for patients' screening was 35 days long.
165 Patients were recruited at the public Health Facilities located in the MeCRU catchment area
166 (Tshwane District). Patients received cART at the Health Facilities throughout the trial. Procedures
167 for patients' recruitment, access to medical records, referral to the Health Facilities for intervening
168 medical conditions were implemented under the coordination of the South African National
169 Department of Health and the Department of Health of the Gauteng Province, South Africa. A
170 community involvement program was implemented at MeCRU with the support of the South Africa
171 AIDS Vaccine Initiative, a lead program of the South Africa Medical Research Council. MeCRU
172 and local community advisory board and groups implemented community education strategies on
173 HIV/AIDS awareness, participation in clinical trials, recruitment and retention strategies. A
174 Contract Research Organization monitored study conduct, data quality and performed safety data
175 analyses, which were periodically evaluated by the Local Medical Monitor and Data Safety
176 Monitoring Board. The Local Medical Monitor was a blinded sponsor's representative expert in
177 HIV/AIDS clinical management. He reviewed safety data, assisted the Investigator in assessing
178 adverse events (AEs) severity and causality, and forwarded quarterly reports to the Data Safety
179 Monitoring Board. Data Safety Update Reports were submitted to the Competent Authorities as
180 required.
181

182 *Endpoints.* The primary endpoint of the study (immunogenicity) was measured by the induction,
183 magnitude and persistence of anti-Tat IgM, IgG and IgA in sera. The secondary endpoint (safety)
184 was assessed by collecting all AEs during the trial, which included vital signs and any clinically
185 significant change in haematological, biochemical and coagulation parameters. All the recorded
186 AEs were classified according to Medical Dictionary for Regulatory Activities (MedDRA)
187 preferred terms and system organ class, and on the basis of drug relationship and grade of severity.
188
189 *Study participants.* Two hundred adult cART-treated patients were recruited and randomised 1:1 to
190 receive Tat vaccine or placebo. Main criteria for enrolment were the following: age 18-45 years
191 (inclusive), current cART-treatment and chronically suppressed HIV-1 infection as indicated by a
192 HIV-1 plasma viremia <400 copies/mL and a CD4+ T-cell count ≥200 cells/µL at screening, and
193 documented at least once during the 12-month period prior to screening irrespective of the pre-
194 cART CD4+ nadir, B-clade anti-Tat Ab-negative, willingness and ability to provide informed
195 consent, and no acute illness at study start. Female participants of childbearing potential were
196 required to have a negative pregnancy test at screening and immediately before each vaccination
197 and to use an acceptable method of contraception for at least 3 weeks prior to the first vaccination
198 and for all duration of the trial.
199
200 *Study procedures.* All participants were randomized to receive the Tat vaccine (30 µg dose) or
201 placebo (vaccine formulation buffer), administered intradermally 3 times at 4-week intervals (ISS
202 T-003 Study Protocol, Supplementary Material). Randomisation was performed in block sizes of
203 four. Participants were allocated to a randomisation number consisting of a 3-digit sequential
204 number pre-fixed by a 1-digit unique site identifier. Upon screening completion and immediately
205 prior to vaccine administration, volunteers were randomly assigned to the next available treatment
206 number according to the randomisation schedule, which was generated by the Contract Research
207 Organization using the SAS® procedure PROC PLAN with a randomisation ratio of 1:1.

208 Participants and clinical and laboratory staff, project management personnel and anyone involved in
209 data management or analysis and the sponsor were blinded to treatment assignment. Each
210 investigational product (Tat vaccine/placebo) vial was packaged in one kit-box constituted of three
211 vials with the same label for vaccine or placebo, according to the "Guide to Good Manufacturing
212 Practice for Medicines in South Africa, Version 4.01 March 2009". Kits were provided to the
213 clinical site in a blinded fashion by the sponsor.
214 The evaluations performed at each of the 12 study visits varied according to the schedule provided
215 in the supplementary material (ISS T-003 Study Protocol). General laboratory assessments,
216 including $CD4^+$ T-cell number and HIV plasma viral load were performed by a centralized
217 laboratory (South African National Health Laboratory Service at the Dr. George Mukhari, Ga-
218 Rankuwa, Pretoria). $CD4^+$ T-cell counts were performed according to standard national laboratory
219 measurements. HIV-1 viral load was determined with the Abbott Real Time HIV-1 assay (lower
220 limit of detection 40 RNA copies/mL). Blood samples were collected and transferred according to
221 protocol-specific procedures, and tested within 3 hours from sample withdrawal. Anti-Tat binding
222 and neutralizing Abs were assessed on cryopreserved specimens shipped by a certified courier to
223 the designated Core Laboratory (*Core Laboratory of Immunology and Virology, San Gallicano
224 Institute, Istituti Fisioterapici Ospitalieri, Rome, Italy*) according to Standard Operating Procedures.
225
226 *Measurement of serum Abs against Tat proteins.* The Tat proteins used for anti-Tat Ab
227 determination and for anti-Tat Ab cross-clade analysis were, respectively, from HIV-1 B clade
228 (GenBank accession n.: AAA44199.1); C clade (GenBank accession n.: AAL06113.1); A clade
229 (GenBank accession n.: AAP33775.1); D clade (GenBank accession n.: AAP33758.1) (amino acid
230 sequences are shown in Supplementary Figure 1) and were purchased from Diatheva. All proteins
231 were biologically active as determined by the rescue assay with HLM-1 cell line carrying a Tat-
232 defective HIV provirus (19, 20), and/or by Tat uptake by monocyte-derived DC (MDDC) evaluated

233 by intracellular staining for Tat in flow cytometry (35), a potency test that is used to release the Tat
234 vaccine clinical lots.
235 Serum IgM, IgA and IgG against B-, A-, C-, and D-clade Tat were assessed by enzyme-linked
236 immunosorbent assay (ELISA), as previously described (76). Briefly, 96-well microplates (Nunc-
237 Immuno Plate MaxiSorp Surface; Nunc) were coated with Tat (100 ng/well) in 200 µL of 0.05
238 mol/L carbonate-buffer (pH 9.6), and incubated overnight at 4°C. Wells were washed 5 times with
239 phosphate-buffered solution (PBS), pH 7.4, containing 0.05% Tween-20, by an automatic plate
240 washer (Asys Hitech flexi wash). Wells were then saturated with PBS containing 1% bovine serum
241 albumin (BSA) and 0.05% Tween-20 (Sigma) (blocking buffer) for 90 min at 37 °C and then
242 washed again as above. One hundred µL of patient serum samples [diluted at 1:100 (for anti-Tat
243 IgG) or at 1:25 (for anti-Tat IgM or IgA detection) in blocking buffer] were added to the wells and
244 incubated at 37 °C for 90 min. To correct for unspecific binding, each sample was assessed in
245 duplicate against Tat and singly against the buffer in which Tat had been re-suspended. After
246 washing, wells were saturated again with blocking buffer for 15 min at 37 °C, washed again and
247 then a goat anti-human IgG, IgM, or IgA horseradish peroxidase-conjugated secondary Ab (100
248 µL/well) (PIERCE-Thermo Scientific) was added to each well, and incubated for an additional 90
249 min at 37 °C. Antigen-bound Abs were revealed by the addition of ABTS [2,2'-azino-bis(3-
250 ethylbenzothiazoline-6-sulphonic acid)] solution (Roche Diagnostics) for 60 min at 37 °C.
251 Absorbance was measured at 405 nm using a microplate reader (BIO-TEK Instruments EL800).
252 The assay was considered valid only when both the positive and negative controls were within
253 ±10% of variation of the absorbance values recorded in previous 50 assays. For the cut-off
254 calculation, both the optical density (OD) readings at 405 nm of the wells coated with Tat and the
255 delta (Δ) value were utilized. The Δ value was obtained by subtracting the OD reading of the well
256 coated with the buffer alone from the average of the OD values of the two wells coated with the Tat
257 protein. Serum samples were considered positive when both the sample OD at 405 nm and Δ values
258 were ≥0.350 and ≥0.150, respectively. The 0.350 and 0.150 OD values had been previously

259 calculated as 3 standard deviations (99% confidence interval) above the mean of each of the
260 absolute and Δ OD values obtained with sera from 89 Italian HIV-negative blood donors and 34
261 South African HIV-negative individuals. If the sample scored positive, the titer value was 100 for
262 IgG, 25 for IgM and IgA. However, if the OD reading of the sample exceeded both the absolute and
263 Δ OD values by 50%, serial two-fold dilutions of the sample were performed to determine the
264 endpoint titers. Endpoint titers were determined as the reciprocal of the last sample dilution that still
265 had ≥0.350 and ≥0.150 OD values for absolute and Δ parameters. For Tat cross-clade analysis, OD
266 values obtained with the different proteins in the same ELISA test, on the same sample, at the same
267 dilution, were compared.
268
269 *Measurement of serum Abs against the Env protein.* The same ELISA protocol and criteria for cut-
270 off determination were applied for measurement of anti-Env Abs and their titer definition (70). The
271 ΔV2-Env (Novartis Vaccine & Diagnostics) from the HIV-1 C-clade TV1 strain was used. Only
272 IgG Abs were tested, starting from a 1:100 dilution.
273
274 *Anti-Tat neutralizing Ab responses.* Anti-Tat neutralizing activity in sera was assessed by Tat-
275 mediated Env entry in DC as described (35). Briefly, MDDC from blood of healthy donors were
276 cultured and induced to maturation as described (35, 36). Purity of MDDC was always ≥99%. Sera
277 were diluted 1:30 in PBS and incubated for 60 min at 37 °C with B- or C-clade trimeric Env (0.4
278 μM in monomer) (Novartis) previously mixed for 10 min at 25 °C with B- or C-clade Tat (0.4 μM)
279 or degassed PBS (control). Samples were then added to MDDC ($2x10^5$ cells/mL) to a 1:5 final
280 dilution and incubated for 10 min at 37 °C. Cells were then washed with cold medium and treated
281 for 10 min at 37 °C with ethylene diamine tetra-acetic acid (EDTA) (Life Technologies) to remove
282 any externally bound protein. After fixation and permeabilization, DC were stained with rabbit anti-
283 gp120 polyclonal Abs (Chem Progress) or purified rabbit-IgG control Abs (Sigma-Aldrich),
284 followed by fluorescein isothiocyanate (FITC)-conjugated anti-rabbit Ig (Pierce). Fluorescence was

285 measured by flow cytometry and results expressed as the percentage of Env-positive cells as
286 compared to isotype-stained samples. Sera were defined as “neutralizing” when capable of
287 inhibiting Env entry into DC in the presence of Tat by at least 50% as compared to baseline sera
288 values (ND_{50}).
289
290 *Sample size calculation.* Sample size for this study was powered for immunogenicity evaluation.
291 The immunogenicity was assumed to be 80% for vaccinees and 60% for placebos, with alpha set at
292 0.05 (2-tailed). According to this assumption, a sample size of 91 per group had 80% power of
293 showing statistical significance ($p<0.05$). The assumed response rate for vaccinees was based on the
294 results of studies conducted at the time of protocol preparation. The response rate in placebos was
295 set in the absence of reference-controlled studies and was therefore very conservative. The actual
296 power of the study, given the percentage of spontaneous seroconversion, is 99%.
297
298 *Statistics*. Two populations were considered for statistical analyses: the immunogenicity population
299 (199 subjects), representing all randomised individuals who received at least 2 immunizations, and
300 the safety population (200 subjects), representing all randomised subjects who received at least one
301 immunization. Subjects with at least one positive anti-Tat Ab response at any given time point
302 during the study were defined as “responders”. Ninety-five % confidence intervals were estimated
303 for the primary endpoints; comparison between treatment groups was performed using the Chi-
304 Square test. Kaplan-Meier method was used to assess the cumulative probability of anti-Tat Ab
305 persistence, by treatment groups, and compared by the Log-Rank test. Anti-Tat Ab titers and the
306 percentage of DC internalizing Env were compared between vaccinees and placebos by the
307 Student’s t-test after log10 transformation to normalize the data distribution. Wilcoxon signed-rank
308 test was used to assess the intensity of cross-clade anti-Tat Abs (measured as OD units) after
309 immunization. Longitudinal analysis for repeated measures was applied for analysis of $CD4^+$ T-cell
310 number, after controlling normality assumption of variable distribution (Saphiro-Wilk test). The

311 relationship between Tat-mediated Env entry in DC and anti-Tat or anti-Env Ab-binding titers or
312 $CD4^+$ T-cell number was assessed by the longitudinal regression model using the generalized
313 estimating equations method. Wilcoxon signed-rank test was used to assess changes from baseline
314 of $CD4^+$ T-cell number in subjects not compliant to cART, while Wilcoxon-Mann-Whitney test was
315 performed in order to evaluate differences between non-CART-compliant vaccinees and placebos at
316 each visit. Statistical analyses were carried out at two-sided with a 0.05 significance level, using
317 SAS® (Version 9.2, SAS Institute Inc., Cary, NC, USA).

318

319 *Ethics.* The ISS T-003 was authorized by the South Africa Medicines Control Council and the
320 Sefako Makgatho University Research Ethics Committee (approval number: MREC/P/221/2010).
321 The study was conducted in accordance with the current Declaration of Helsinki and International
322 Conference on Harmonization Good Clinical Practice guidelines.
323 Patients gave written approval to perform the study-specific procedures, including access to source
324 medical data (demography, diagnosis, cART compliance).

325

326 Results

327 *Patients accrual and demographic data.* Seven-hundred-seventy cART-treated patients were
328 assessed for eligibility (Figure 1). Two hundred participants were enrolled between February 27,
329 2012 and 13 June, 2013. Study was completed in June 2014. Participants were randomised 1:1 to
330 one of the two treatment groups. Ninety-seven percent of enrolled participants completed the study.
331 All individuals were analyzed for safety. One subject who received only one immunization was
332 excluded from the immunogenicity population (Figure 1). Thirteen volunteers were excluded from
333 the "Per Protocol" analysis for major protocol non-compliance (Figure 1). Baseline demographic
334 and clinical characteristics of participants are shown in Table 1. Twenty-six percent of participants
335 were male and 74% female; all were black, except one volunteer. At the study entry the mean age
336 was 36 years for both vaccinees and placebos. In vaccinees, the mean $CD4^+$ T-cell count was 510

337 cells/µL, 95% of them had undetectable HIV RNA, the mean years from HIV diagnosis was 5.0,
338 while the mean time on cART was 3.5 years, with 97% on non-nucleoside reverse-transcriptase
339 inhibitors (NNRTI) or nucleoside reverse transcriptase inhibitors (NRTI)-based and 3% on PI-based
340 regimens. In placebos, the mean $CD4^{+}$ T-cell count was 563 cells/µL, HIV RNA was undetectable
341 in 96% of them, the mean years from HIV diagnosis was 4.9 years, while the mean time on cART
342 was 3.3, with 98% on NNRTI or NRTI-based and 2% on PI-based regimens.

343

344 *HIV-1 B-clade Tat vaccine safety and tolerability.* Tat immunization was safe and well tolerated
345 without relevant differences between vaccinees and placebos. In particular, 190 patients (96
346 vaccinees and 94 placebos) experienced at least one AE during the study, mainly of mild intensity
347 (Table 2). General disorders and administration site conditions were the most frequent AEs related
348 to study treatment both in vaccinees (73%) and placebos (58%), followed by nervous system
349 disorders (mainly headache events), which had higher incidence in placebos (38%) than vaccinees
350 (27%) (Table 3). No serious AEs (SAE) related to study treatment or suspected unexpected adverse
351 reactions were reported. The non-serious AEs related to study drug were mostly mild and local.
352 Most clinically relevant abnormal laboratory events were reported with a similar frequency in both
353 the treatment groups and were considered unrelated, since they are findings typically associated
354 with HIV-1 infection (i.e. low haemoglobin, low neutrophil and white cell counts, increased viral
355 load). Eight participants (2 placebos and 6 vaccinees) reported at least one SAE (unrelated to study
356 treatment). In particular, 1 placebo underwent hysterectomy and 1 was diagnosed with type-II
357 diabetes mellitus. Among the vaccinees, 2 participants were diagnosed with pulmonary
358 tuberculosis, 1 patient was admitted to the hospital for respiratory tract infection, bronchiectasis-
359 empyema thoracis and abdominal pain, 1 patient underwent hysterectomy, and intentional self-
360 injury was reported in 2 participants. All these SAE resolved completely, except the type II diabetes
361 mellitus.

362 Since no “important safety events” or “significant findings” emerged during the study, the Data
363 Safety Monitoring Board concluded that the Tat vaccine is safe and well tolerated.
364
365 *HIV-1 B-clade Tat vaccine induces durable anti-Tat Abs of all subclasses.* Tat immunization
366 induced anti-Tat B-clade Abs in 97% of vaccinees, whereas 20% of placebos developed
367 spontaneously anti-Tat Abs (all immunogenicity population evaluated). Anti-Tat Ab responses
368 detected in vaccinees and placebos were significantly different (Chi-Square test, $p<0.0001$, both for
369 total Abs and Ig subclasses). In particular, 81% of vaccinees developed anti-Tat B-clade IgM, 96%
370 IgG, and 76% IgA, as opposed to 10% IgM, 13% IgG, and 6% IgA of placebos, respectively
371 (Figure 2A). As shown in Table 4 and Figure 2B, 69% of vaccinees developed anti-Tat Abs of all Ig
372 subclasses, 28% developed one or two Ig subclasses, and 3% of vaccinees had no detectable anti-
373 Tat Abs. In contrast, 1% of placebos developed anti-Tat Abs of all three Ig subclasses, 19% of one
374 or two subclasses, and 80% had no detectable anti-Tat Abs (Chi-Square test, $p< 0.0001$, Figure 2B).
375 Anti-Tat B-clade Ab mean titers peaked between week 8 and week 12 for all Ig subclasses and
376 statistically significant differences for IgG titers between vaccinees and placebos were observed
377 since week 12 (weeks 12-24, $p <0.0001$; week 48 $p=0.0004$) **(**Figure 2C). Moreover, anti-Tat Ab
378 responses persisted significantly longer in vaccinees as compared to placebos (Log-Rank test,
379 $p=0.0019$) **(**Figure 3A). Further, Ab persistence was longer in vaccinees and placebos with 2 or 3
380 Ab subclasses as compared to those with 1 subclass (Figure 3B and C). The “Per Protocol” analysis
381 confirmed the results from the immunogenicity population, in particular, 88/91 (97%) and 20/96
382 (21%) of vaccinees and placebos, respectively, developed anti-Tat Abs ($p<0.0001$).
383
384 *Vaccination with the HIV-1 B-clade Tat protein elicits Abs also recognizing Tat from A, C and D*
385 *clades.* The presence of anti-Tat Abs against clades other than B (i.e. C, D, A) was evaluated in the
386 99 vaccinees. Fifty-one patients that were negative at baseline also for Abs against Tat from A, C,

387 or D clade, after immunization with the B-clade Tat protein developed anti-Tat Abs recognizing Tat
388 from one or more of these other clades (Table 5), in addition to Tat B clade.
389 At baseline (Figure 4A), 29 vaccinees, although negative for anti-Tat Abs against B-clade Tat, had
390 Abs against Tat of one or more of the other clades tested (76% C clade, 41% A clade, 14% D
391 clade). After vaccination, all of them experienced a statistically significant increase of intensity of
392 these responses (Figure 4B). In particular, changes of intensity from baseline levels were similar for
393 IgM and IgA for all clades, while for IgG changes were higher for C and D clades.
394
395 *Vaccination with the HIV-1 B-clade Tat protein induces cross-clade neutralizing anti-Tat Abs.* The
396 neutralization of B-clade Tat-mediated entry of oligomeric B-clade Env in DC was used to
397 investigate anti-Tat Ab functional activity in 24 participants. This assay permits measuring HIV
398 neutralization even in the presence of cART (57, 65), which interferes with traditional infection
399 assays (77). At baseline, entry of Env in the absence of Tat was comparable for all sera (Figure 5A,
400 left panel) and did not change after immunization (Figure 5A, right panel). As shown previously
401 (57), Tat increased entry of Env with all sera prior to immunization (baseline) (Figure 5B, left
402 panel), whereas sera from vaccinees strongly reduced Env entry (more than 60%) (week 20 and 48,
403 $p<0.0001$). This occurred to a lesser extent also with sera from anti-Tat Ab-positive placebos (about
404 37% reduction) (Figure 5B, right panel). No changes were observed in Ab-negative placebos
405 (Figure 5B**,** right panel). Differences between vaccinees and anti-Tat Ab-negative placebos were
406 statistically significant at both time points examined (week 20 and week 48 $p=0.0009$ and
407 $p=0.0003$, respectively). Further, differences in reaching 50% neutralization (ND_{50}) of Env entry
408 were observed between vaccinees (11/13, 85%) and the anti-Tat Ab-positive placebos (2/6, 33%)
409 (Fisher's Exact Test, $p=0.0460$). Neutralization of Tat-mediated Env entry in DC was also analyzed
410 for C clade Tat and Env. As shown in Figure 6, anti-Tat Abs elicited by vaccination with the B-
411 clade Tat protein induced cross-clade neutralizing Abs against B and C clade Tat/Env complex
412 entry in DC ($p<0.0001$ for both clades).

413

414 *Anti-Tat but not anti-Env Abs correlate with neutralization of Env entry in vaccinees.* To evaluate
415 the role of both anti-Tat and anti-Env humoral responses on the neutralization of Tat-mediated entry
416 of oligomeric Env in DC, anti-Env Abs were also tested (all immunogenicity population evaluated).
417 At baseline, all subjects had anti-Env Abs (geometric mean Ab-titers 72,408, range 200-409,600)
418 with titers that did not change significantly during the follow-up (*data not shown*) and correlated
419 positively with the levels of Tat-mediated Env entry in DC (r=0.42, p=0.0214) indicating lack of
420 neutralization. In contrast, after immunization, vaccinees showed a significant inverse relationship
421 between anti-Tat IgM or IgG Ab titers (p= 0.0853 and p= 0.0039, respectively) or anti-Env IgG
422 titers (p=0.0015) and the levels of Tat-mediated Env entry in DC (Table 6), indicating correlation
423 with neutralization of Env entry. Of note, anti-Env Ab titers did not correlate with neutralization of
424 Env entry in anti-Tat Ab-negative placebos, indicating that anti-Env Abs require anti-Tat Abs to
425 inhibit the Tat/Env complex formation and virus entry, as shown earlier both in vitro and in vivo
426 (65, 70, 78).

427

428 *Tat vaccination induces $CD4^+$ T cell number increases, which correlate with neutralization.*
429 Compared to placebos, $CD4^+$ T-cell counts increased significantly and progressively in vaccinees
430 (Figure 7) up to week 24 when they peaked (mean gain of 60 cells/µL p=0.0015), whereas at the
431 end of the study (week 48) the mean gain compared to baseline values was of 28 cells/µL. In
432 contrast, placebos showed a slower kinetics and lower, and not statistically significant, increases
433 (mean gain of 11 cells/µL), which peaked at 48 weeks with a mean value of 17 cells/µL as
434 compared to baseline. $CD4^+$ T-cell counts were also analyzed by treatment groups over time by
435 applying a random-effect regression model. The increase from baseline of $CD4^+$ T cells up to week
436 24 was 2.2 cells/µL (95% CI 1.1; 3.2, p<0.0001) per week in vaccinees and 0.1 cells/µL (95% CI
437 0.7; 3.4) per week in the placebo group, respectively. The difference between the coefficients of

438 regression was statistically significant (p=0.0031). The comparison between the two arms showed
439 statistically significant changes from baseline at week 20 (p=0.0466) and week 24 (p=0.0250).
440 To evaluate the effect of vaccination on the increase of $CD4^+$ T cells according to their levels at
441 study entry, baseline values were stratified by quartiles. Increases up to about 90 cells/µL were
442 detected in vaccinated subjects in Q1, Q2, and Q3, while no significant changes were observed in
443 Q4, indicating that vaccination had major effects in subjects with lower $CD4^+$ T cell number at
444 baseline (Figure 8). Placebos showed significant $CD4^+$ T-cell increases only in Q1 (up to 84
445 cells/µL). Of note, the gaining in $CD4^+$ T-cell counts in Q1 was lower (up to 58 cells/µL) in
446 placebos negative for anti-Tat Abs, who also experienced a significant $CD4^+$ T-cell decay in Q3 at
447 week 20 (Figure 8).
448 Further, $CD4^+$ T-cell increases correlated significantly with neutralization of Env entry in DC in
449 vaccinees (n= 19) (p=0.0023) as compared to placebos (n=11) (Table 7).
450
451 *Tat vaccination maintains $CD4^+$ T cells and contains viral load rebound in patients non-compliant*
452 *to therapy.* Compliance was always verified at each study visit. However, despite counseling for
453 adherence to therapy, medical records showed poor compliance (i.e. missing doses up to prolonged
454 interruptions) in 24 volunteers, particularly between week 20 and 48 after the first immunization.
455 Of them, 18 were vaccinees and 6 were placebos (1 anti-Tat Ab-positive and 5 anti-Tat Ab-
456 negative). None of the vaccinees non-compliant to cART therapy experienced a decay of $CD4^+$ T
457 cells which, instead, increased above study entry levels (median increase of 50 cells/µL at week 16,
458 p= 0.0814, 57 cells/µL at week 20, p= 0.0987 and 30 cells/µL at week 48, as compared to baseline).
459 In contrast, the anti-Tat Ab-negative placebos had $CD4^+$ T-cell decreases below study entry levels
460 (median of -33 cells/µL at week 12, p=0.0625 and -60 cells/µL at week 48 versus baseline levels).
461 Comparison between vaccinees and anti-Tat Ab negative placebos showed significant differences at
462 week 8 and week 12 (p=0.0859 and p=0.0336, respectively) (Fig. 9).

463 With regard to viral load, plasma viremia remained undetectable at week 48 in 12/18 (67%)
464 vaccinees, and in 3/5 (60%) of anti-Tat Ab-negative placebos. In addition, in patients with
465 detectable viral load at week 48, the geometric mean levels were lower in vaccinees (1,090
466 copies/mL), as compared to anti-Tat Ab-negative placebos (3,179 copies/mL) (Fig. 10).

467

468 Discussion

469 The development of therapeutic vaccination strategies for treating people already infected with
470 HIV-1 has been recently accelerated, with an increasing number of vaccine candidates being tested
471 in clinical trials, either in drug-naïve patients or in association with cART. In drug-naïve patients,
472 therapeutic vaccines are expected to contain infection (i.e., low to undetectable plasma viral load
473 and $CD4^+$ T cell preservation), preventing progression to disease as well as virus transmission,
474 while in cART-treated patients therapeutic vaccination is expected to intensify the efficacy of
475 cART, thus supporting a more effective immune restoration and virological control, particularly in
476 poor immunological responders or cART non-compliant patients, preventing progression to AIDS-
477 related as well as non AIDS-related diseases and virus transmission.
478 No therapeutic vaccines are currently market approved. However, the rapidly expanding HIV/AIDS
479 therapeutic vaccine field portraits a variety of approaches, which differ sensibly in many aspects,
480 the most relevant being the antigen chosen (unlike preventative vaccines, regulatory and accessory
481 genes are frequently targeted; in some cases almost the entire HIV genome is targeted), and the
482 delivery systems, which range from simple subcutaneous, intradermal, or intramuscular vaccine
483 administration to reinfusion of autologous DCs loaded ex vivo with the selected antigen(s) (9, 79-
484 89). In most vaccine trials conducted in treated patients cART therapy was interrupted to assess the
485 potency of the immunological control of infection provided by vaccination, while they were not
486 aimed at evaluating the immunological recovery, with the exclusion of $CD4^+$ T cell counts, which,
487 however, did not appear to go beyond the restoration provided by cART alone (9, 79).

488 Our approach has focused on Tat, a key HIV virulence factor, which is released extracellularly in a
489 biologically active form also under cART, and promotes virus reactivation, replication and
490 spreading while inducing immune activation and disabling the host immune defense (reviewed in
491 25). Thus, induction of effective anti-Tat Abs may represent a pathogenesis-driven therapeutic
492 intervention to block disease progression as indicated by the effects of long-lasting, high titers anti-
493 Tat Abs in natural infection (9, 79) or after vaccination with Tat, which induced $CD4^+$ T cell
494 recovery, immune restoration, as well as reduction of immunoactivation and of proviral DNA in
495 Italian subjects (ISS T-002 trial) (57).
496 The results of the present study indicate that B-clade Tat immunization is safe and well tolerated
497 also in South African individuals infected with a different virus subtype. Further, vaccination
498 induced anti-Tat Abs in almost all vaccinees. Abs were durable, at high titers and of multiple
499 subclasses. Remarkably, B-clade Tat vaccination induced cross-clade (A, C, D) Tat-binding Abs,
500 which were capable of neutralizing Tat-mediated entry in DC of oligomeric HIV Env from B and C
501 clade, suggesting that the B-clade Tat protein used in our vaccine program may be used for a cross-
502 clade HIV vaccine approach.
503 A natural humoral anti-Tat Ab response developed in a small number of placebos, a finding
504 expected from previous studies conducted in Italy and South Africa (53, 54, 69, 70). Indeed, as
505 compared to the other HIV proteins which elicit Abs virtually in all infected patients, production
506 of anti-Tat Abs is seen only in about 20% of the HIV-infected subjects present in all cohorts we
507 have investigated. Surprisingly, although Tat is released extracellularly, only a small percentage of
508 individuals recognizes and mounts an Ab response against this protein. One reason could be its
509 molecular mimicry for extracellular matrix proteins such as fibronectin (FN) and vitronectin (VN)
510 (90-92). As for Tat, FN and VN possess a similar basic region and RGD sequence binding to the
511 $\alpha5\beta1$, $\alpha V\beta3$ and $\alpha V\beta5$ integrins (93, 94).
512 In vaccinees, neutralization correlated positively with anti-Tat IgM and IgG Ab titers, whereas Env
513 entry was not neutralized by anti-Env Abs in the absence of anti-Tat Abs (57, 65). Indeed, anti-Env

514 Abs measured prior to immunization both in vaccinees and placebos had a positive correlation with
515 increased levels of Tat-mediated Env entry in DC. This reproduces what has been seen earlier with
516 sera from Italian vaccinees (ISS T-002 trial) or in monkey studies (65). In particular, by forming a
517 complex with Env, Tat increases virus entry in DC and blocks neutralization by anti-Env Abs,
518 which is restored and further increased only in the presence of anti-Tat Abs (65).
519 Tat vaccination was associated with significant increases of $CD4^+$ T cells above baseline levels,
520 whereas placebos showed a slower kinetics and lower, and not statistically significant, increases, as
521 expected in individuals on cART for a mean of about 3 years. Increases of $CD4^+$ T cells in
522 vaccinees correlated significantly with neutralization. Of note, $CD4^+$ T cells increased particularly
523 in vaccinees with lower $CD4^+$ T cell counts at baseline. This is of particular relevance since poor
524 immunological response to therapy is frequent either in patients starting cART late, even if
525 virologically suppressed (4-8), or in patients with persistent immune activation (95-99) or low
526 compliant (100-103). A poor $CD4^+$ T cell recovery (<500 T cells/µl) is associated with disease
527 progression, co-morbidities**,** hospitalization and death (104-107). These patients are those that most
528 require ART intensification.
529 While in the ISS T-002 trial conducted in Italy patients were highly compliant to therapy,
530 compliance was lower in the ISS T-003 study, a finding particularly frequent in Southern Africa
531 where scarce adherence to cART therapy represents a relevant clinical problem since it is associated
532 with disease progression, virus drug resistance and transmission (108-111). Of interest, none of the
533 vaccinees non-compliant to cART therapy experienced a decay of $CD4^+$ T cells, and in most of
534 them plasma viremia remained undetectable at week 48 while, in those with detectable viremia,
535 viral load levels were low. In contrast, the anti-Tat Ab-negative placebos had $CD4^+$ T-cell decreases
536 below entry levels, and in most of them plasma viremia rebounded to geometric mean levels higher
537 than those recorded in non-compliant vaccinees. Although these results are only descriptive, since
538 the groups are too small to draw any firm conclusion, they indicate the need of ad hoc studies to
539 address whether cART intensification by Tat therapeutic immunization may mitigate the effects of

540 low adherence to therapy. To this end, structured therapy interruption studies after cART
541 intensification by the Tat vaccine are being planned.
542 The results of the ISS T-003 trial are highly consistent with those of the ISS T-002 (53, 57),
543 although the two trials were conducted in individuals with different genetic background, infected
544 with HIV from different subtypes (B versus C clade), and on cART for different periods of time
545 (i.e., mean of 6 years in the ISS T-002 trial versus about 3 years in the ISS T-003 trial). Indeed,
546 safety and immunogenicity results were remarkably similar, sometimes identical, as were the $CD4^+$
547 T-cell increments, particularly in subjects with lower levels at baseline (53, 57), suggesting that
548 poor immunological responders to therapy could greatly benefit from Tat immunotherapy.
549 The results from the Italian trial (ISS T-002) clearly indicate that proviral DNA reduction (as
550 opposed to $CD4^+$ T cell increase) is a late event, particularly under NNRTI-based drug regimens
551 requiring 108 weeks for detecting a significant proviral reduction (57). Indeed, no significant
552 reductions of proviral DNA are seen at week 48 in both (ISS T-002 and ISS T-003) trials (*data not*
553 *shown*). Furthermore, it appears that time on effective cART is also relevant, in that proviral DNA
554 decay plateau after about 4-5 years of successful therapy (112, 113). Thus, unlike the Italian trial in
555 which vaccinees had been on therapy on average for 6 years, subjects enrolled in the South African
556 study had been on cART for around 3 years. Altogether these data indicate that longer periods of
557 time are required to see an effect on proviral DNA in NNRTI-treated South African subjects, which
558 represent 97% of the trial population.
559 Therefore, similarly to the ISS T-002 trial, a roll-over observational study (ISS T-003 EF-UP) has
560 been initiated for the South African trial to ensure the extended follow-up of the volunteers, in order
561 to evaluate the persistence of vaccine-induced immune responses as well as the immunological and
562 virological effects of Tat immunization. In particular, proviral DNA will be monitored to verify
563 whether Tat vaccination is capable of reducing it, as observed for the ISS T-002 trial after 3 years
564 from vaccination (57).
565

Conclusions

These data indicate that immunization with B clade Tat induced functionally effective cross-clade anti-Tat Abs and $CD4^{+}$ T-cell increases and reinforce the notion that B clade Tat is a suitable candidate for therapeutic immunization against different HIV clades in different geographical areas, thus supporting the future conduct of phase III studies in South Africa.

Abbreviations

Ab: antibody; ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid; AE: adverse event; cART: combined antiretroviral therapy; BSA: bovine serum albumin; CCR5: C-C chemokine receptor 5; DC: dendritic cell; DEAE diethylaminoethyl; EDTA: ethylene diamine tetra-acetic acid; ELISA: enzyme-linked immunosorbent assays; Env: Envelope; FITC: fluorescein isothiocyanate; GMP: Good Manufacturing Practice; HCT: HIV counselling and testing campaign; HIV: human immunodeficiency virus; HLA-DR: human leukocyte antigen-D related; Ig: immunoglobulin; HSA: human serum albumin; min: minutes; MDDC: monocyte-derived dendritic cell; MedDRA: medical dictionary for regulatory activities; ND50: 50% neutralization; NK: natural killer; NNRTI: non-nucleoside reverse-transcriptase inhibitors; NRTI: nucleoside reverse transcriptase inhibitors; OD: optical density; PBS: phosphate-buffered solution; PI: protease inhibitor; SAE: serious AEs; Tat: Transactivator of Transcription; WHO: World Health Organization.

Competing interests

Mauro Magnani owns interest in Vaxxit S.r.l.; the remaining authors declare that they have no competing interests.

Funding

This work was funded by the General Directorate for Development Cooperation of the Italian Ministry of Foreign Affairs through the “Program to support the Ministry of Health of South Africa

592 in the implementation of a national program of global response to HIV & AIDS" - Project N. AID
593 8421".
594

595 Author contributions

596 B.E. and E.G. conceived and designed the clinical study and program, supervised the experimental
597 work, data analysis and interpretation, and manuscript preparation.
598 M.N. contributed to Study Protocol definition and acted as the Principal Investigator for clinical
599 trial conduct.
600 F.E. and A.C. contributed to Study Protocol definition, supervised immunological and virological
601 laboratory investigations, and contributed to trial data analysis and manuscript preparation
602 A.T. coordinated immunological and virological laboratory investigations.
603 V.F., S.M., M.R.P.C., BC A.A. and G.P. performed immunological and virological laboratory
604 investigations.
605 S.B. and O.P. contributed to Study Protocol definition, performed the statistical analyses and
606 contributed to manuscript preparation.
607 C.S and O.L. contributed to Study Protocol definition, supervised clinical study management and
608 contributed to manuscript preparation.
609 S.Bu. contributed to laboratory trial Standard Operating Procedures definition and quality
610 assurance.
611 L.T. and D.J. contributed to clinical trial preparation and management, and trial producers
612 implementation at the site catchment area.
613 An.C. and M.M. contributed to second line testing design and implementation.
614 E.L. coordinated clinical site capacitation and community involvement strategies.
615 J.V.N. coordinated the implementation of trial-related activities at the public health facilities in the
616 Tshwane District.

B.A. and Y.P. coordinated and supervised, respectively, the activities of the Department of Health in relation to trial preparation and conduct.

P.M. contributed to Study Protocol definition, acted as Head of the clinical program and contributed data and manuscript critical review.

Acknowledgments

The authors thank all the volunteers who participated to the clinical trial. Moreover, they thank the MeCRU (Gauteng, South Africa) Team for study conduct: Matsontso Peter Mathebula (Sub-Investigator); Ayoola Komolafe (Sub-Investigator), Nazira Carrim-Ganey (Sub-Investigator), Sylvia Pieter (Sub-Investigator), Granny Pila (Sub-Investigator), Nontando Moeketsi (Project Manager), Innocentia Matjila (Study Coordinator); Ronald Moate (Community Liaison Officer), Aaron Manyabeane Phaahla (Laboratory Manager), Famola Suprise Ngobeni (Laboratory Manager). Further, the authors thank Triclinium (Clinical Trial Project Management, LTD, Sandown, South Africa) for invaluable support in trial preparation and conduct, and data management and analysis; the National Health Laboratory System (Johannesburg, South Africa) for trial testing and laboratory data management; the Foundation for Professional Development (FPD, Pretoria, South Africa) for health care professionals training and development; the Health Information System Project (HISP, East London, South Africa) for support to the public Health Facilities toward medical records management.

The authors thank Glenda Gray and Michelle Mulder (South African Medical Research Council, Cape Town, South Africa) for invaluable support.

The authors thank Tshilizi Tuwani (Masikhulisane Community Educator) for support to MeCRU; Patricia Southwood (SAAVI Material Developer) for coordinating material development; Merlin Osborne (SAAVI Administrator) for assisting with financial control and management.

641 The authors also thank G.B. Cozzone (previous affiliation: Consultant to Istituto Superiore di Sanità
642 (ISS), Rome, Italy; current affiliation: 3i Consulting s.r.l. Rome, Italy) for his advice in the conduct
643 of the “Program to support the Ministry of Health of South Africa in the implementation of a
644 national program of global response to HIV & AIDS” and for the preparation of all contractual
645 agreements among the parties; the MRC Legal Unit and Thomas Smit (SAAVI) for the preparation
646 of the contractual agreements with the CRUs; S. Marcotullio [National AIDS Center (NAC),
647 Istituto Superiore di Sanità (ISS), Rome, Italy] for support to communication to HIV-1
648 communities; A. Scoglio, M. Campagna, M.J. Ruiz Alvarez, C. Ariola (NAC, ISS, and Pathology
649 and Microbiology, San Gallicano Hospital, "Istituti Fisioterapici Ospitalieri", Rome, Italy) and C.
650 Orlandi (Department of Biomolecular Science, University of Urbino, Urbino, Italy) for laboratory
651 support; F. Cammisa, S. Ceccarelli and G. Fornari Luswergh (NAC, ISS) for support to study
652 management and editorial assistance, respectively; S. De Menna, A. Biondi, S. Tobelli and F. Fedeli
653 (NAC, ISS) for administrative support.
654

655 ## References

656 1. The Gap report UNAIDS 2014. UNAIDS Web Site. July 2014, updated September 2014.
657 http://www.unaids.org/sites/default/files/media_asset/UNAIDS_Gap_report_en.pdf.
658 Accessed 7 Apr 2016.
659 2. Guideline on when to start antiretroviral therapy and on pre-exposure prophylaxis for HIV.
660 WHO Web Site. September 2015.
661 http://apps.who.int/iris/bitstream/10665/186275/1/9789241509565_eng.pdf. Accessed 7 Apr
662 2016.
663 3. Gupta RK, Jordan MR, Sultan BJ, Hill A, Davis DH, Gregson J, Sawyer AW, Hamers RL,
664 Ndembi N, Pillay D, Bertagnolio S: **Global trends in antiretroviral resistance in**
665 **treatment-naive individuals with HIV after rollout of antiretroviral treatment in**
666 **resource-limited settings: a global collaborative study and meta-regression analysis**.
667 *Lancet* 2012, **380**:1250-8.
668 4. Hocqueloux L, Avettand-Fènoël V, Jacquot S, Prazuck T, Legac E, Mélard A, Niang M,
669 Mille C, Le Moal G, Viard JP, Rouzioux C; AC32 (Coordinated Action on HIV Reservoirs)
670 of the Agence Nationale de Recherches sur le Sida et les Hépatites Virales (ANRS): **Long-**
671 **term antiretroviral therapy initiated during primary HIV-1 infection is key to achieving**
672 **both low HIV reservoirs and normal T cell counts.** *J Antimicrob Chemother* 2013, **68**,
673 1169–78.
674 5. Grinsztejn B, Hosseinipour MC, Ribaudo HJ, Swindells S, Eron J, Chen YQ, Wang L, Ou
675 SS, Anderson M, McCauley M, Gamble T, Kumarasamy N, Hakim JG, Kumwenda J, Pilotto
676 JH, Godbole SV, Chariyalertsak S, de Melo MG, Mayer KH, Eshleman SH, Piwowar-
677 Manning E, Makhema J, Mills LA, Panchia R, Sanne I, Gallant J, Hoffman I, Taha TE,
678 Nielsen-Saines K, Celentano D, Essex M, Havlir D, Cohen MS; HPTN 052-ACTG Study
679 Team: **Effects of early versus delayed initiation of antiretroviral treatment on clinical**

680 **outcomes of HIV-1 infection: results from the phase 3 HPTN 052 randomised**
681 **controlled trial.** *Lancet Infect Dis* 2014, **14**,281–290.
682 6. Gazzola L, Tincati C, Bellistrì GM, Monforte AD, Marchetti G: **The absence of CD4+ T**
683 **cell count recovery despite receipt of virologically suppressive highly active**
684 **antiretroviral therapy: clinical risk, immunological gaps, and therapeutic options.** *Clin*
685 *Infect Dis* 2009, **48**,328–337.
686 7. Cenderello G, De Maria A: **Discordant responses to cART in HIV-1 patients in the era of**
687 **high potency antiretroviral drugs: clinical evaluation, classification, management**
688 **prospects**. *Expert Rev Anti Infect Ther* 2016, **14**:29-40.
689 8. Massanella M, Negredo E, Clotet B, Blanco J: **Immunodiscordant responses to HAART--**
690 **mechanisms and consequences.** *Expert Rev Clin Immuno* 2013, **9**:1135-49.
691 9. Ensoli B, Cafaro A, Monini P, Marcotullio S, Ensoli F: **Challenges in HIV Vaccine**
692 **Research for Treatment and Prevention.** *Front Immunol* 2014, **5**:417.
693 10. Jeang KT, Xiao H, Rich EA: **Multifaceted activities of the HIV-1 transactivator of**
694 **transcription, Tat.** *J Biol Chem* 1999, **274**: 28837-40.
695 11. Li JC, Yim HC, Lau AS: **Role of HIV-1 Tat in AIDS pathogenesis: its effects on cytokine**
696 **dysregulation and contributions to the pathogenesis of opportunistic infection.** *AIDS*
697 2010, **24**, 1609–23.
698 12. Fisher AG, Feinberg MB, Josephs SF, Harper ME, Marselle LM, Reyes G, Gonda MA,
699 Aldovini A, Debouk C, Gallo RC. & Wong-Staal F: **The trans-activator gene of HTLV-III**
700 **is essential for virus replication.** *Nature* 1986, **320**:367-71.
701 13. Dayton AI, Sodroski JG, Rosen CA, Goh WC Haseltine WA: **The trans-activator gene of**
702 **the human T cell lymphotropic virus type III is required for replication.** *Cell* 1986, **44**:
703 941-7.

704 14. Hauber J, Perkins A, Heimer EP Cullen BR: **Trans-activation of human immunodeficiency virus gene expression is mediated by nuclear events.** *Proc Natl Acad Sci USA* 1987, **84**: 6364-68.

707 15. Berkhout B, Silverman RH, Jeang KT: **Tat trans-activates the human immunodeficiency virus through a nascent RNA target.** *Cell* 1989, **59**:273-282.

709 16. Weinberger LS, Burnett JC, Toettcher JE, Arkin AP, Schaffer DV: **Stochastic gene expression in a lentiviral positive-feedback loop: HIV-1 Tat fluctuations drive phenotypic diversity.** *Cell* 2005, **122**:169-82.

712 17. Wu Y, Marsh JW: **Selective transcription and modulation of resting T cell activity by preintegrated HIV DNA.** *Science* 2001, **293**:1503-6.

714 18. Mediouni S, Darque A, Baillat G, Ravaux I, Dhiver C, Tissot-Dupont H, Mokhtari M, Moreau H, Tamalet C, Brunet C, Paul P, Dignat-George F, Stein A, Brouqui P, Spector SA, Campbell GR, Loret EP: **Antiretroviral therapy does not block the secretion of the human immunodeficiency virus Tat protein.** *Infect Disord Drug Targets* 2012, **12:**81-86.

718 19. Ensoli B, Buonaguro L, Barillari G, Fiorelli V, Gendelman R, Morgan RA, Wingfield P, Gallo RC: **Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation.** *J Virol* 1993, **67:**277–87.

722 20. Chang HC, Samaniego F, Nair BC, Buonaguro L, Ensoli B: **HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region.** *AIDS* 1997, **11**:1421-31.

725 21. Rayne F, Debaisieux S, Yezid H, Lin YL, Mettling C, Konate K, Chazal N, Arold ST, Pugnière M, Sanchez F, Bonhoure A, Briant L, Loret E, Roy C, Beaumelle B: **Phosphatidylinositol-(4,5)-bisphosphate enables efficient secretion of HIV-1 Tat by infected T-cells.** *EMBO J* 2010, **29:**1348-62.

729 22. Ensoli B, Barillari G, Salahuddin SZ, Gallo RC, Wong-Staal F: **Tat protein of HIV-1**
730 **stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients.**
731 *Nature* 1990, **345**:84-6.
732 23. Ensoli B, Gendelman R, Markham P, Fiorelli V, Colombini S, Raffeld M, Cafaro A, Chang
733 HK, Brady JN, Gallo RC: **Synergy between basic fibroblast growth factor and HIV-1 Tat**
734 **protein in induction of Kaposi's sarcoma.** *Nature* 1994, **371:**674-80.
735 24. Li CJ, Ueda Y, Shi B, Borodyansky L, Huang L, Li YZ, Pardee AB: **Tat protein induces**
736 **self-perpetuating permissivity for productive HIV-1 infection.** *Proc Natl Acad Sci USA*
737 1997, **94:**8116-8120.
738 25. Huang L, Bosch I, Hofmann W, Sodroski J, Pardee AB: **Tat protein induces human**
739 **immunodeficiency virus type 1 (HIV-1) coreceptors and promotes infection with both**
740 **macrophage-tropic and T- lymphotropic HIV-1 strains.** *J Virol* 1998, **72**: 8952-60.
741 26. Albini A, Ferrini S, Benelli R, Sforzini S, Giunciuglio D, Aluigi MG, Proudfoot AE, Alouani
742 S, Wells TN, Mariani G, Rabin RL, Farber JM, Noonan DM: **HIV-1 Tat protein mimicry of**
743 **chemokines.** *Proc Natl Acad Sci USA* 1998, **95**: 13153-8.
744 27. Johnson TP, Patel K, Johnson KR, Maric D, Calabresi PA, Hasbun R, Nath A: **Induction of**
745 **IL-17 and nonclassical T-cell activation by HIV-Tat protein.** *Proc Natl Acad Sci USA*
746 2013, **110:**13588-93.
747 28. Ott M, Emiliani S, Van Lint C Herbein G, Lovett J, Chirmule N, McCloskey T, Pahwa
748 S, Verdin E: **Immune hyperactivation of HIV-1-infected T cells mediated by Tat and the**
749 **CD28 pathway.** *Science* 1997, **275:**1481-1485.
750 29. Viscidi RP, Mayur K, Lederman HM & Frankel AD: **Inhibition of antigen-induced**
751 **lymphocyte proliferation by Tat protein from HIV-1.** *Science* 1989, **246**, 1606–8.
752 30. Westendorp MO, Li-Weber M, Frank RW, Krammer PH: **Human immunodeficiency virus**
753 **type 1 Tat upregulates interleukin-2 secretion in activated T cells.** *J Virol* 1994, **68**:
754 4177-85.

755 31. Zauli G, Gibellini D, Celeghini C, Mischiati C, Bassini A, La Placa M, Capitani S:
756 **Pleiotropic effects of immobilized versus soluble recombinant HIV-1 Tat protein on**
757 **CD3-mediated activation, induction of apoptosis, and HIV-1 long terminal repeat**
758 **transactivation in purified CD4+ T lymphocytes.** *J Immunol* 1996, **157**:2216-24.
759 32. Buonaguro L, Barillari G, Chang HK, Bohan CA, Kao V, Morgan R, Gallo RC, Ensoli B:
760 **Effects of the human immunodeficiency virus type 1 Tat protein on the expression of**
761 **inflammatory cytokines.** *J Virol* 1992, **66**: 7159-67.
762 33. Scala G, Ruocco MR, Ambrosino C, Mallardo M, Giordano V, Baldassarre F, Dragonetti E,
763 Quinto I, Venuta S: **The expression of the interleukin 6 gene is induced by the human**
764 **immunodeficiency virus 1 TAT protein**. *J Exp Med* 1994, **179**: 961-71.
765 34. Matsui M, Warburton RJ, Cogswell PC, Baldwin AS, Jr., Frelinger JA: **Effects of HIV-1**
766 **Tat on expression of HLA class I molecules.** *J Acquir Immune Defic Syndr Hum Retrovirol*
767 1996, **11**: 233-40.
768 35. Fanales-Belasio E, Moretti S, Nappi F, Barillari G, Micheletti F, Cafaro A, Ensoli B: **Native**
769 **HIV-1 Tat protein targets monocyte-derived dendritic cells and enhances their**
770 **maturation, function, and antigen-specific T cell responses.** *J Immunol* 2002, **168:**197-
771 206.
772 36. Fanales-Belasio E, Moretti S, Fiorelli V, Tripiciano A, Pavone Cossut M.R, Scoglio A,
773 Collacchi B, Nappi F, Macchia I, Bellino S, Francavilla V, Caputo A, Barillari G, Magnani
774 M, Laguardia ME, Cafaro A, Titti F, Monini P, Ensoli F, Ensoli B: **HIV-1 Tat addresses**
775 **dendritic cells to induce a predominant Th1-type adaptive immune response that**
776 **appears prevalent in the asymptomatic stage of infection.** *J Immunol* 2009, **182**:2888-97.
777 37. Planès R, BenMohamed L, Leghmari K, Delobel P, Izopet J & Bahraoui E: **HIV-1 Tat**
778 **Protein Induces PD-L1 (B7-H1) Expression on Dendritic Cells through Tumor Necrosis**
779 **Factor Alpha- and Toll-Like Receptor 4-Mediated Mechanisms.** *J Virol* 2014, **88**, 6672–
780 89.

781 38. Planès R & Bahraoui E: **HIV-1 Tat Protein Induces the Production of IDO in Human
782 Monocyte Derived-Dendritic Cells through a Direct Mechanism: Effect on T Cells
783 Proliferation.** *PLoS One* 2013, **8**(9), e74551.

784 39. Li JC, Lee DC, Cheung BK, Lau AS: **Mechanisms for HIV Tat upregulation of IL-10 and
785 other cytokine expression: kinase signaling and PKR-mediated immune response.** *FEBS
786 Lett* 2005, **579**: 3055-62.

787 40. Poggi A & Zocchi: **HIV-1 Tat triggers TGF-beta production and NK cell apoptosis that
788 is prevented by pertussis toxin B.** *Clin & Develop Immunol* 2006, **13**, 369–72.

789 41. Gupta S, Boppana R, Mishra GC, Saha B, Mitra D: **HIV-1 Tat suppresses gp120-specific T
790 cell response in IL-10-dependent manner.** *J Immunol* 2008, **180**: 79-88.

791 42. Cheng SM, Li JC, Lin SS, Lee DC, Liu L, Chen Z & Lau AS: **HIV-1 transactivator protein
792 induction of suppressor of cytokine signaling-2 contributes to dysregulation of
793 IFN{gamma} signaling.** *Blood* 2009, **113**, 5192–201.

794 43. Faller EM, Sugden SM, McVey MJ, Kakal JA, MacPherson PA: **Soluble HIV Tat protein
795 removes the IL-7 receptor alpha-chain from the surface of resting CD8 T cells and
796 targets it for degradation**. *J Immunol* 2010, **185**: 2854-66.

797 44. Gavioli R, Gallerani E, Fortini C, Fabris M, Bottoni A, Canella A, Bonaccorsi A, Marastoni
798 M, Micheletti F, Cafaro A, Rimessi P, Caputo A, Ensoli B: **HIV-1 Tat protein modulates
799 the generation of cytotoxic T cell epitopes by modifying proteasome composition and
800 enzymatic activity.** *J Immunol* 2004, **173:**3838-3843.

801 45. Sforza F, Nicoli F, Gallerani E, Finessi V, Reali E, Cafaro A, Caputo A, Ensoli B, Gavioli R:
802 **HIV-1 Tat affects the programming and functionality of human CD8^{+} T cells by
803 modulating the expression of T-box transcription factors.** *AIDS* 2014, **28:**1729-1738.

804 46. Nicoli F, Finessi V, Sicurella M, Rizzotto L, Gallerani E, Destro F, Cafaro A, Marconi P,
805 Caputo A, Ensoli B, Gavioli R: **The HIV-1 Tat protein induces the activation of CD8^{+} T**

806 **cells and affects in vivo the magnitude and kinetics of antiviral responses.** *PLoS One*
807 2013, **8:**e77746.
808 47. Chiozzini C, Collacchi B, Nappi F, Bauer T, Arenaccio C, Tripiciano A, Longo O, Ensoli F,
809 Cafaro A, Ensoli B, Federico M: **Surface-bound Tat inhibits antigen-specific CD8$^+$ T-cell**
810 **activation in an integrin-dependent manner.** *AIDS* 2014, **28:**2189-200.
811 *48.* Debaisieux, S, Lachambre S, Gross A, Mettling C, Besteiro S, Yezid H, Henaff D, Chopard
812 C, Mesnard JM, Beaumelle B: **HIV-1 Tat inhibits phagocytosis by preventing the**
813 **recruitment of Cdc42 to the phagocytic cup.** *Nat Commun.* 2015, **6**:6211.
814 49. Li CJ, Friedman DJ, Wang C, Metelev V, Pardee AB: **Induction of apoptosis in uninfected**
815 **lymphocytes by HIV-1 Tat protein.** *Science* 1995, **268**: 429-31.
816 50. Campbell GR, Pasquier E, Watkins J, Bourgarel-Rey V, Peyrot V, Esquieu D, Barbier P, de
817 Mareuil J, Braguer D, Kaleebu P, Yirrell DL, Loret EP: **The glutamine-rich region of the**
818 **HIV-1 Tat protein is involved in T-cell apoptosis.** *J Biol Chem* 2004, **279**: 48197-204.
819 51. Campbell GR, Watkins JD, Esquieu D, Pasquier E, Loret EP, Spector SA: **The C terminus**
820 **of HIV-1 Tat modulates the extent of CD178-mediated apoptosis of T cells**. *J Biol Chem*
821 2005, **280**: 38376-82.
822 52. Kim N, Kukkonen S, Gupta S, Aldovini A: **Association of Tat with promoters of PTEN**
823 **and PP2A subunits is key to transcriptional activation of apoptotic pathways in HIV-**
824 **infected CD4+ T cells.** *PLoS Pathog* 2010, **6**: e1001103.
825 53. Ensoli B, Bellino S, Tripiciano A, Longo O, Francavilla V, Marcotullio S, Cafaro A, Picconi
826 O, Paniccia G, Scoglio A, Arancio A, Ariola C, Ruiz Alvarez MJ,Campagna M, Scaramuzzi
827 D, Iori C, Esposito R, Mussini C, Ghinelli F, Sighinolfi L, Palamara G, Latini A, Angarano
828 G, Ladisa N, Soscia F, Mercurio VS, Lazzarin A, Tambussi G, Visintini R, Mazzotta F, Di
829 Pietro M, Galli M, Rusconi S, Carosi G, Torti C, Di Perri G, Bonora S, Ensoli F, Garaci E:
830 **Therapeutic immunization with HIV-1 Tat reduces immune activation and loss of**

831 **regulatory T-cells and improves immune function in subjects on HAART.** *PLoS One*
832 2010, **5**:e13540.
833 54. Ensoli B, Fiorelli V, Ensoli F, Lazzarin A, Visintini R, Narciso P, Di Carlo A, Monini
834 P, Magnani M, Garaci E: **The therapeutic phase I trial of the recombinant native HIV-1**
835 **Tat protein.** *AIDS* 2008, **22**:2207-2209.
836 55. Ensoli B, Fiorelli V, Ensoli F, Lazzarin A, Visintini R, Narciso P, Di Carlo A, Tripiciano A,
837 Longo O, Bellino S, Francavilla V, Paniccia G, Arancio A, Scoglio A, Collacchi B, Ruiz
838 Alvarez MJ, Tambussi G, Tassan Din C, Palamara G, Latini A, Antinori A, D'Offizi G,
839 Giulianelli M, Giulianelli M, Carta M, Monini P, Magnani M, Garaci E. **The preventive**
840 **phase I trial with the HIV-1 Tat-based vaccine.** *Vaccine* 2009, **28:**371-378.
841 56. Longo O, Tripiciano A, Fiorelli V, Bellino S, Scoglio A, Collacchi B, Ruiz Alvarez MJ,
842 Francavilla V, Arancio A, Paniccia G, Lazzarin A, Tambussi G, Tassan Din C, Visintini R,
843 Narciso P, Antinori A, D'Offizi G, Giulianelli M, Carta M, Di Carlo A, Palamara G, Giuliani
844 M, Laguardia ME, Monini P, Magnani M, Ensoli F, Ensoli B: **Phase I therapeutic trial of**
845 **the HIV-1 Tat protein and long term follow-up.** *Vaccine* 2009, **27:**3306-3312.
846 57. Ensoli F, Cafaro A, Casabianca A, Tripiciano A, Bellino S, Longo O, Francavilla V, Picconi
847 O, Sgadari C, Moretti S, Pavone Cossut MR, Arancio A, Orlandi C, Sernicola L, Maggiorella
848 MT, Paniccia G, Mussini C, Lazzarin A, Sighinolfi L, Palamara G, Gori A, Angarano G, Di
849 Pietro M, Galli M, Mercurio VS, Castelli F, Di Perri G, Monini P, Magnani M, Garaci E,
850 Ensoli B: **HIV-1 Tat immunization restores immune homeostasis and attacks the**
851 **HAART-resistant blood HIV DNA: results of a randomized phase II exploratory**
852 **clinical trial.** *Retrovirology* 2015, 12:33.
853 58. Cafaro A, Tripiciano A, Sgadari C, Bellino S, Picconi O, Longo O, Francavilla V, Buttò S,
854 Titti F, Monini P, Ensoli F, Ensoli B: **Development of a novel AIDS vaccine: the HIV-1**
855 **Tat protein vaccine.** *Expert Opin on Biol Ther* 2015, **15 Suppl 1**:S13-29.

856 59. Marchetti G, Gori A, Casabianca A, Magnani M, Franzetti F, Clerici M, Perno CF, Monforte
857 Ad, Galli M, Meroni L: **Comparative analysis of T-cell turnover and homeostatic**
858 **parameters in HIV-infected patients with discordant immune-virological responses to**
859 **HAART.** *AIDS* 2006*,* **20**, 1727–36.
860 60. Migueles SA, Weeks KA, Nou E, Berkley Am, Rood JE, Osborne CM, Hallahan CW,
861 Cogliano-Shutta NA, Metcalf JA, McLaughlin M, Kwan R, Mican JM, Davey RT Jr,
862 Connors M: **Defective human immunodeficiency virus-specific CD8+ T-cell**
863 **polyfunctionality, proliferation, and cytotoxicity are not restored by antiretroviral**
864 **therapy.** *J Virol* 2009, **83:**11876-89.
865 61. Guihot A, Bourgarit A, Carcelain G, Autran B: **Immune reconstitution after a decade of**
866 **combined antiretroviral therapies for human immunodeficiency virus.** *Trends Immunol*
867 2011, **32**:131-7.
868 62. Costiniuk CT, Angel JB: **Human immunodeficiency virus and the gastrointestinal**
869 **immune system: does highly active antiretroviral therapy restore gut immunity?**
870 *Mucosal Immunol* 2012, **5**:596-604.
871 63. Tincati C, Merlini E, Braidotti P, Ancona G, Savi F, Tosi D, Borghi E, Callegari ML,
872 Mangiavillano B, Barassi A, Bulfamante G, Monforte AD, Romagnoli S, Chomont N,
873 Marchetti G: **Impaired gut junctional complexes feature late-treated individuals with**
874 **suboptimal CD4+ T-cell recovery upon virologically-suppressive cART**. *AIDS* 2016,
875 [Epub ahead of print January 8, 2016]
876 64. Chertova E, Chertov O, Coren LV, Roser JD, Trubey CM, Bess JW Jr, Sowder RC 2nd,
877 Barsov E, Hood BL, Fisher RJ, Nagashima K, Conrads TP, Veenstra TD, Lifson JD, Ott DE:
878 **Proteomic and biochemical analysis of purified human immunodeficiency virus type 1**
879 **produced from infected monocyte-derived macrophages.** *J Virol* 2006, **80:**9039-9052.
880 65. Monini P, Cafaro A, Srivastava IK, Moretti S, Sharma VA, Andreini C, Chiozzini
881 C, Ferrantelli F, Cossut MR, Tripiciano A, Nappi F, Longo O, Bellino S, Picconi O, Fanales-

882 Belasio E, Borsetti A, Toschi E, Schiavoni I, Bacigalupo I, Kan E, Sernicola L, Maggiorella
883 MT, Montin K, Porcu M, Leone P, Leone P, Collacchi B, Palladino C, Ridolfi B, Falchi
884 M, Macchia I, Ulmer JB, Buttò S, Sgadari C, Magnani M, Federico MP, Titti F, Banci
885 L, Dallocchio F, Rappuoli R, Ensoli F, Barnett SW, Garaci E, Ensoli B: **HIV-1 Tat
886 Promotes Integrin-Mediated HIV Transmission to Dendritic Cells by Binding Env
887 Spikes and Competes Neutralization by Anti-HIV Antibodies.** *PLoS One* 2012,
888 **7:**e48781.
889 66. Reiss P, de Wolf F, Kuiken CL, de Ronde A, Dekker J, Boucher CA, Debouck C, Lange JM,
890 Goudsmit J: **Contribution of antibody response to recombinant HIV-1 gene-encoded
891 products nef, rev, tat, and protease in predicting development of AIDS in HIV-1-
892 infected individuals.** *J Acquir Immune Defic Syndr* 1991, **4:**165-72.
893 67. Re MC, Furlini G, Vignoli M, Ramazzotti E, Roderigo G, De Rosa V, Zauli G, Lolli S,
894 Capitani S, La Placa M: **Effect of antibody to HIV-1 Tat protein on viral replication in
895 vitro and progression of HIV-1 disease in vivo.** *J Acquir Immune Defic Syndr Hum
896 Retrovirol* 1995, **10:**408-16.
897 68. Richardson MW, Mirchandani J, Duong J, Grimaldo S, Kocieda V, Hendel H, Khalili
898 K, Zagury JF, Rappaport J: **Antibodies to Tat and Vpr in the GRIV cohort: differential
899 association with maintenance of long-term non-progression status in HIV-1 infection.**
900 *Biomed Pharmacother* 2003, **57**:4–14.
901 69. Rezza G, Fiorelli V, Dorrucci M, Ciccozzi M, Tripiciano A, Scoglio A, Collacchi B, Ruiz-
902 Alvarez M, Giannetto C, Caputo A, Tomasoni L, Castelli F, Sciandra M, Sinicco A, Ensoli
903 F, Buttò S, Ensoli B: The **presence of anti-Tat antibodies is predictive of long-term
904 nonprogression to AIDS or severe immunodeficiency: findings in a cohort of HIV-1
905 seroconverters.** *J Infect Dis* 2005, **191**:1321-1324.
906 70. Bellino S, Tripiciano A, Picconi O, Francavilla V, Longo O, Sgadari C, Paniccia G, Arancio
907 A, Angarano A, Ladisa N, Lazzarin A, Tambussi G, Nozza S, Torti C, Focà E, Palamara G,

908 Latini A, Sighinolfi L, Mazzotta M, Di Pietro M, Di Perri G, Bonora S, Mercurio VS,
909 Mussini C, Gori A, Galli M, Monini P, Cafaro A, Ensoli F, Ensoli B: **The presence of anti-**
910 **Tat antibodies in HIV-infected individuals is associated with containment of CD4$^+$ T-**
911 **cell decay and viral load, and with delay of disease progression: results of a 3-year**
912 **cohort study.** *Retrovirology* 2014, **11:**49.

913 71. Sáez-Cirión A, Lacabaratz C, Lambotte O, Versmisse P, Urrutia A, Boufassa F, Barré-
914 Sinoussi F, Delfraissy JF, Sinet M, Pancino G, Venet A; Agence Nationale de Recherches
915 sur le Sida EP36 HIV Controllers Study Group: **HIV controllers exhibit potent CD8 T cell**
916 **capacity to suppress HIV infection ex vivo and peculiar cytotoxic T lymphocyte**
917 **activation phenotype**. *Proc Natl Acad Sci USA* 2007, **104**:6776–81.

918 72. Hua S, Lécuroux C, Sáez-Cirión A, Pancino G, Girault I, Versmisse P, Boufassa F, Taulera
919 O, Sinet M, Lambotte O, Venet A: **Potential Role for HIV-Specific CD38−/HLA-DR+**
920 **CD8+ T Cells in Viral Suppression and Cytotoxicity in HIV Controllers**. *PLoS One*
921 2014, **9**:e101920.

922 73. Studier FW, Moffatt BA: **Use of bacteriophage T7 RNA polymerase to direct selective**
923 **high-level expression of cloned genes**. *J Mol Biol* 1986, **189**:113-130.

924 74. Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW: **Use of T7 RNA polymerase to**
925 **direct expression of cloned genes**. *Methods Enzymol* 1990, **185**:60-89.

926 75. Rosenberg AH, Lade BN, Chui DS, Lin SW, Dunn JJ, Studier FW: **Vectors for selective**
927 **expression of cloned DNAs by T7 RNA Polimerase**. *Gene* 1987, **56**:125-135.

928 76. Buttò S, Fiorelli V, Tripiciano A, Ruiz-Alvarez MJ, Scoglio A, Ensoli F, Ciccozzi M,
929 Collacchi B, Sabbatucci M, Cafaro A, Guzmán CA, Borsetti A, Caputo A, Vardas E, Colvin
930 M, Lukwiya M, Rezza G, Ensoli B; Tat Multicentric Study Group: **Sequence conservation**
931 **and antibody cross-recognition of clade B human immunodeficiency virus (HIV) type 1**
932 **Tat protein in HIV-1-infected Italians, Ugandans, and South Africans**. *J Infects Dis*
933 2003, **188:**1171-1180.

934 77. Medina-Ramírez M, Sánchez-Merino V, Sánchez-Palomino S, Merino-Mansilla A, Ferreira
935 CB, Pérez I, González N, Alvarez A, Alcocer-González JM, García F, Gatell JM, Alcamí J,
936 Yuste E: **Broadly cross-neutralizing antibodies in HIV-1 patients with undetectable**
937 **viremia.** *J Virol* 2011, **85:**5804-5813.
938 78. Demberg T, Florese RH, Heath MJ, Larsen K, Kalisz I, Kalyanaraman VS, Lee EM, Pal R,
939 Venzon D, Grant R, Patterson LJ, Korioth-Schmitz B, Buzby A, Dombagoda D, Montefiori
940 DC, Letvin NL, Cafaro A, Ensoli B, Robert-Guroff M: **A replication-competent**
941 **adenovirus-human immunodeficiency virus (Ad-HIV) tat and Ad-HIV env priming/Tat**
942 **and envelope protein boosting regimen elicits enhanced protective efficacy against**
943 **simian/human immunodeficiency virus SHIV89.6P challenge in rhesus macaques**. *J*
944 *Virol* 2007, **81**:3414-3427.
945 79. García F, León A, Gatell JM, Plana M, Gallart T: **Therapeutic vaccines against HIV**
946 **infection**. *Hum Vaccin Immunother* 2012, **8**:569-581
947 80. Turner JL, Kostman JR, Aquino A, Wright D, Szabo S, Bidwell R, Goodgame J, Daigle A,
948 Kelley E, Jensen F, Duffy C, Carlo D, Moss RB: **The effects of an HIV-1 immunogen**
949 **(Remune) on viral load, CD4 cell counts and HIV-specific immunity in a double-blind,**
950 **randomized, adjuvant-controlled subset study in HIV infected subjects regardless of**
951 **concomitant antiviral drugs**. *HIV Med* 2001, **2**:68-77.
952 81. Chantratita W, Sukeepaisarncharoen W, Chandeying V, Kulpradist S, Israngkura Na
953 Ayudhtaya B, Rugpao S, Sirawaraporn W, Boonshuyar C, Churdboonchart V: **Delayed**
954 **progression to AIDS in volunteers treated with long-term HIV-1 Immunogen**
955 **(REMUNE®) therapy in Thailand**. *HIV Med* 2004, **5**:317-325.
956 82. Hardy G, Imami N, Nelson MR, Sullivan AK, Moss R, Aasa-Chapman MM, Gazzard B,
957 Gotch FM: **A phase I, randomized study of combined IL-2 and therapeutic**
958 **immunisation with antiretroviral therapy.** *J Immune Based Ther Vaccines* 2007, **5**:6.

959 83. Herasimtschuk A, Downey J, Nelson M, Moyle G, Mandalia S, Sikut R, Adojaan M,
960 Stanescu I, Gotch F, Imami N: **Therapeutic immunisation plus cytokine and hormone**
961 **therapy improves CD4 T-cell counts, restores anti-HIV-1 responses and reduces**
962 **immune activation in treated chronic HIV-1 infection**. *Vaccine* 2014, **32**:7005-7013.
963 84. Lori F: **DermaVir: a plasmid DNA-based nanomedicine therapeutic vaccine for the**
964 **treatment of HIV/AIDS**. *Expert Rev Vaccines* 2011, **10**:1371-1384.
965 85. Vardas E, Stanescu I, Leinonen M, Ellefsen K, Pantaleo G, Valtavaara M, Ustav M, Reijonen
966 K: **Indicators of therapeutic effect in FIT-06, a Phase II trial of a DNA vaccine,**
967 **GTU(®)-Multi-HIVB, in untreated HIV-1 infected subjects**. *Vaccine* 2012, **30**:4046-54.
968 86. Lind A, Sommerfelt M, Holmberg JO, Baksaas I, Sørensen B, Kvale D: **Intradermal**
969 **vaccination of HIV-infected patients with short HIV Gag p24-like peptides induces**
970 **$CD4^+$ and $CD8^+$ T cell responses lasting more than seven years**. *Scand J Infect Dis* 2012,
971 **44**:566-572.
972 87. Goldstein G, Damiano E, Donikyan M, Pasha M, Beckwith E, Chicca J: **HIV-1 Tat B-cell**
973 **epitope vaccination was ineffectual in preventing viral rebound after ART cessation:**
974 **HIV rebound with current ART appears to be due to infection with new endogenous**
975 **founder virus and not to resurgence of pre-existing Tat-dependent viremia**. *Hum Vaccin*
976 *Immunother* 2012, **8:**1425-30.
977 88. García F, Climent N, Guardo AC, Gil C, León A, Autran B, Lifson JD, Martínez-Picado J,
978 Dalmau J, Clotet B, Gatell JM, Plana M, Gallart T: **A Dendritic Cell-Based Vaccine Elicits**
979 **T Cell Responses Associated with Control of HIV-1 Replication**. *Sci Transl Med* 2013,
980 **5**:166ra2.
981 89. Achenbach CJ, Assoumou L, Deeks SG, Wilkin TJ, Berzins B, Casazza JP, Lambert-Niclot
982 S, Koup RA, Costagliola D, Calvez V, Katlama C, Autran B, Murphy RL: **Effect of**
983 **therapeutic intensification followed by HIV DNA prime and rAd5 boost vaccination on**

984 **HIV-specific immunity and HIV reservoir (EraMune 02): a multicentre randomised**
985 **clinical trial.** *Lancet HIV* 2015, **2**:e82-91.
986 90. Barillari G, Gendelman R, Gallo RC, Ensoli B: **The Tat protein of human**
987 **immunodeficiency virus type 1, a growth factor for AIDS Kaposi sarcoma and**
988 **cytokine-activated vascular cells, induces adhesion of the same cell types by using**
989 **integrin receptors recognizing the RGD amino acid sequence**. *Proc Natl Acad Sci U S A*
990 1993, **90**:7941-7945.
991 91. Barillari G, Ensoli B: **Angiogenic effects of extracellular human immunodeficiency virus**
992 **type 1 Tat protein and its role in the pathogenesis of AIDS-associated Kaposi's**
993 **sarcoma**. *Clin Microbiol Rev* 2002, **15**:310-326.
994 92. Toschi E, Bacigalupo I, Strippoli R, Chiozzini C, Cereseto A, Falchi M, Nappi F, Sgadari C,
995 Barillari G, Mainiero F, Ensoli B: **HIV-1 Tat regulates endothelial cell cycle progression**
996 **via activation of the Ras/ERK MAPK signaling pathway**. *Mol Biol Cell* 2006, **17:**1985-
997 1994.
998 93. Stupack DG, Cheresh DA: **Integrins and angiogenesis**. *Curr Top Dev Biol* 2004, **64**:207-38.
999 94. Ruoshlati E: **Integrin Signaling and Matrix Assembly**. *Tumour Biol* 1996, **17**:117-124.
1000 95. Hunt PW, Martin JN, Sinclair E, Bredt B, Hagos E, Lampiris H, Deeks SG: **T cell activation**
1001 **is associated with lower CD4+ T cell gains in human immunodeficiency virus-infected**
1002 **patients with sustained viral suppression during antiretroviral therapy**. *J Infect Dis*
1003 2003, **187**:1534-1543.
1004 96. French MA, King MS, Tschampa JM, da Silva BA, Landay AL: **Serum immune activation**
1005 **markers are persistently increased in patients with HIV infection after 6 years of**
1006 **antiretroviral therapy despite suppression of viral replication and reconstitution of**
1007 **CD4+ T cells**. *J Infect Dis* 2009, **200**:1212-1215.
1008 97. Neuhaus J, Jacobs DR Jr, Baker JV, Calmy A, Duprez D, La Rosa A, Kuller LH, Pett SL,
1009 Ristola M, Ross MJ, Shlipak MG, Tracy R, Neaton JD: **Markers of inflammation,**

1010 **coagulation, and renal function are elevated in adults with HIV infection**. *J Infect Dis*
1011 2010, **201**:1788-95.

1012 98. Burdo TH, Lo J, Abbara S, Wei J, DeLelys ME, Preffer F, Rosenberg ES, Williams KC,
1013 Grinspoon S: **Soluble CD163 made by monocyte/macrophages is a novel marker of HIV**
1014 **activity in early and chronic infection prior to and after anti-retroviral therapy**. *J Infect*
1015 *Dis* 2011, **204**:154-163.

1016 99. Lederman MM, Calabrese L, Funderburg NT, Clagett B, Medvik K, Bonilla H, Gripshover
1017 B, Salata RA, Taege A, Lisgaris M, McComsey GA, Kirchner E, Baum J, Shive C, Asaad R,
1018 Kalayjian RC, Sieg SF, Rodriguez B: **Immunologic failure despite suppressive**
1019 **antiretroviral therapy is related to activation and turnover of memory CD4 cells**. *J*
1020 *Infect Dis* 2011, **204**:1217-26.

1021 100.Wood E, Hogg RS, Yip B, Harrigan PR, O'Shaughnessy MV, Montaner JS: **Effect of**
1022 **Medication Adherence on Survival of HIV-Infected Adults Who Start Highly Active**
1023 **Antiretroviral Therapy When the CD4+ Cell Count Is 0.200 to 0.350** $\times$ **10**^{9} **cells/L.** *Ann*
1024 *Intern Med* 2003, **139**:810–6.

1025 101.Wood E, Hogg RS, Yip B, Harrigan PR, O'Shaughnessy MV, Montaner JS: **The impact of**
1026 **adherence on CD4 cell count responses among HIV-infected patients.** *J Acquir Immune*
1027 *Defic Syndr* 2004, **35**:261-8.

1028 102.Lucas GM: **Antiretroviral adherence, drug resistance, viral fitness and HIV disease**
1029 **progression: a tangled web is woven**. *J Antimicrob Chemoth* 2005, **55**:413–6.

1030 103.Pasternak AO, de Bruin M, Bakker M, Berkhout B, Prins JM: **High Current CD4+ T Cell**
1031 **Count Predicts Suboptimal Adherence to Antiretroviral Therapy.** *PLoS One* 2015,
1032 **10**:e0140791.

1033 104.Marin B, Thiébaut R, Bucher HC, Rondeau V, Costagliola D, Dorrucci M, Hamouda O,
1034 Prins M, Walker S, Porter K, Sabin C, Chêne G: **Non-AIDS-defining deaths and**

1035 **immunodeficiency in the era of combination antiretroviral therapy.** *AIDS* 2009,
1036 **23:**1743–53.
1037 105.Phillips AN, Neaton J & Lundgren JD: The **role of HIV in serious diseases other than**
1038 **AIDS.** *AIDS* 2008, **22:** 2409–18.
1039 106.Schouten J, Wit FW, Stolte IG, Kootstra NA, van der Valk M, Geerlings SE, Prins M, Reiss
1040 P; AGEhIV Cohort Study Group: **Cross-sectional Comparison of the Prevalence of Age-**
1041 **Associated Comorbidities and Their Risk Factors Between HIV-Infected and**
1042 **Uninfected Individuals: The AGEhIV Cohort Study.** *Clin Infect Diseases* 2014, **59**, 1787–
1043 97.
1044 107.Mirani G, Williams PL, Chernoff M, Abzug MJ, Levin MJ, Seage GR 3rd, Oleske JM,
1045 Purswani MU, Hazra R, Traite S, Zimmer B, Van Dyke RB; IMPAACT P1074 Study Team:
1046 **Changing Trends in Complications and Mortality Rates Among US Youth and Young**
1047 **Adults With HIV Infection in the Era of Combination Antiretroviral Therapy.** *Clin*
1048 *Infect Diseases* 2015, **61**:1850-61.
1049 108. Barth RE, van der Loeff MF, Schuurman R, Hoepelman AI, Wensing AM: **Virological**
1050 **follow-up of adult patients in antiretroviral treatment programmes in sub-Saharan**
1051 **Africa: a systematic review.** *Lancet Infect Dis* 2010, **10**:155-66.
1052 109. Rosen S, Fox MP: **Retention in HIV care between testing and treatment in sub-Saharan**
1053 **Africa: a systematic review**. *PLoS Med* 2011, **8**:e1001056.
1054 110. Kozal MJ, Hullsiek KH, Macarthur RD, Berg-Wolf Mv, Peng G, Xiang Y, Baxter JD, Uy J,
1055 Telzak EE, Novak RM; Terry Beirn Community Programs for Clinical Research on AIDS
1056 (CPCRA): **The Incidence of HIV Drug Resistance and Its Impact on Progression of HIV**
1057 **Disease Among Antiretroviral-Naïve Participants Started on Three Different**
1058 **Antiretroviral Therapy Strategies.** *HIV Clin Trials* 2007, **8**,357-70.
1059 111. Ketseoglou I, Lukhwareni A, Steegen K, Carmona S, Stevens WS, Papathanasopoulos MA:
1060 **Viral tropism and antiretroviral drug resistance in HIV-1 subtype C-infected patients**

1061 **failing highly active antiretroviral therapy in Johannesburg, South Africa.** *AIDS Res*
1062 *Hum Retroviruses* 2014, **30**:289-93.
1063 112. Besson GJ, Lalama CM, Bosch RJ, Gandhi RT, Bedison MA, Aga E, Riddler SA, McMahon
1064 DK, Hong F, Mellors JW: **HIV-1 DNA decay dynamics in blood during more than a**
1065 **decade of suppressive antiretroviral therapy.** *Clin Infect Dis* 2014, **59**, 1312–21.
1066 113. Buzón MJ, Martin-Gayo E, Pereyra F, Ouyang Z, Sun H, Li JZ, Piovoso M, Shaw A,
1067 Dalmau J, Zangger N, Martinez-Picado J, Zurakowski R, Yu XG, Telenti A, Walker BD,
1068 Rosenberg ES, Lichterfeld M: **Long-term Antiretroviral Treatment Initiated in Primary**
1069 **HIV-1 Infection Affects the Size, Composition and Decay Kinetics of the Reservoir of**
1070 **HIV-1 Infected CD4 T Cells.** *J Virol* 2014, **88**:10056-65.
1071

1072 **Table 1. Baseline characteristics of study participants**

	n	**Vaccinees**	n	**Placebo**
Gender				
Male	32	32.0%	20	20.0%
Female	68	68.0%	80	80.0%
Race				
Black	100	100.0%	99	99.0%
Caucasian	0	0.0%	0	0.0%
Mixed	0	0.0%	1	1.0%
Age				
Mean ± s.d.[a]	100	36.1 ± 5.6	100	36.0 ± 6.2
Range		21.1-45.8		19.6-45.4
$CD4^+$ (cells/μl)				
Mean ± s.d.	99	510 ± 229	100	563 ± 195
Range		137-1530		242-1252
$CD4^+$ (%)				
Mean ± s.d.	99	28± 8	100	29 ± 7
Range		7-49		17-42
HIV RNA (copies/mL)				
<40 (assay cut-off)	94	95.0%	96	96.0%
≥ 40	5	5.0%	4	4.0%
Years from HIV diagnosis				
Mean ± s.d.	100	5.0 ± 3.0	100	4.9 ± 3.3
Range		1.0-14.0		1.0-19.0
Years from cART initiation				
Mean ± s.d.	100	3.5 ± 2.0	100	3.3 ± 2.1
Range		0.7-8.2		0.6-8.9
cART regimen				
NNRTI or NRTI-based	97	97.0%	98	98.0%
PI-based	3	3.0%	2	2.0%
Previous Tuberculosis	29	29.0%	34	34.0%

1073 *n* indicates the number of individuals; [a]Standard deviation

1074 **Table 2. Total adverse events observed in study participants reported by relationship to study drug and intensity**

	Treatment group								
	Tat vaccine			Placebo			Total		
	n	(m)	%	n	(m)	%	n	(m)	%
Number of subjects in safety population	100			100			200		
Number of subjects with at least one adverse event	96	(883)	96.0	94	(581)	94.0	190	(1464)	95.0
Relationship with study medication									
Certain	72	(541)	72.0	59	(250)	59.0	131	(791)	65.5
Probable	14	(35)	14.0	23	(38)	23.0	37	(73)	18.5
Possible	25	(45)	25.0	28	(56)	28.0	53	(101)	26.5
Unlikely	41	(80)	41.0	37	(63)	37.0	78	(143)	39.0
Not related	76	(182)	76.0	74	(174)	74.0	150	(356)	75.0
Not assessable	0	(0)		0	(0)		0	(0)	
Not known	0	(0)		0	(0)		0	(0)	
Intensity									
Mild	94	(774)	94.0	91	(522)	91.0	185	(1296)	92.5
Moderate	42	(90)	42.0	28	(48)	28.0	70	(138)	35.0
Severe	15	(17)	15.0	9	(10)	9.0	24	(27)	12.0
Not applicable	1	(1)	1.0	0	(0)		1	(1)	0.5
Not known	1	(1)	1.0	1	(1)	1.0	2	(2)	1.0
Serious adverse events	6	(8)	6.0	2	(2)	2.0	8	(10)	4.0
Related	0	(0)	0.0	0	(0)	0.0	0	(0)	0.0
Not related	6	(8)	6.0	2	(2)	2.0	8	(10)	4.0

n=number of subjects, (m)=number of mentions, %=all percentages are expressed as the percentage of the number of subjects in the safety population in each treatment group.

1075

1076 **Table 3. Incidence of related adverse events by system organ class and relationship to study treatment**

	Treatment group												Total		
	Tat vaccine						Placebo								
MedDRA system organ class	Related[1]			Not related[2]			Related[1]			Not related[2]					
	n	(m)	%	n	(m)	%	n	(m)	%	n	(m)	%	n	(m)	%
Number of subjects	100			100			100			100			200		
Number of subjects with at least one adverse event	77	(621)	77.0	85	(262)	85.0	72	(344)	72.0	81	(237)	81.0	190	(1464)	95.0
General disorders and administration site conditions	73	(520)	73.0	8	(10)	8.0	58	(199)	58.0	11	(12)	11.0	133	(741)	66.5
Infections and infestations	1	(1)	1.0	50	(77)	50.0	2	(2)	2.0	56	(88)	56.0	107	(168)	53.5
Nervous system disorders	27	(40)	27.0	19	(24)	19.0	38	(67)	38.0	10	(13)	10.0	81	(144)	40.5
Musculoskeletal and connective tissue disorders	16	(25)	16.0	14	(16)	14.0	17	(31)	17.0	15	(28)	15.0	54	(100)	27.0
Gastrointestinal disorders	9	(12)	9.0	20	(25)	20.0	14	(18)	14.0	13	(19)	13.0	49	(74)	24.5
Skin and subcutaneous tissue disorders	10	(12)	10.0	15	(15)	15.0	10	(18)	10.0	13	(16)	13.0	47	(61)	23.5
Reproductive system and breast disorders	0	(0)		22	(24)	22.0	0	(0)		22	(27)	22.0	44	(51)	22.0
Investigations	2	(3)	2.0	19	(28)	19.0	2	(2)	2.0	8	(10)	8.0	30	(43)	15.0
Blood and lymphatic system disorders	7	(7)	7.0	7	(9)	7.0	5	(6)	5.0	3	(3)	3.0	21	(25)	10.5
Injury, poisoning and procedural complications	0	(0)		10	(12)	10.0	0	(0)		5	(7)	5.0	15	(19)	7.5
Vascular disorders	1	(1)	1.0	6	(6)	6.0	0	(0)		3	(3)	3.0	10	(10)	5.0
Respiratory, thoracic and mediastinal disorders	0	(0)		2	(2)	2.0	0	(0)		4	(5)	4.0	6	(7)	3.0
Eye disorders	0	(0)		3	(3)	3.0	0	(0)		2	(2)	2.0	5	(5)	2.5
Metabolism and nutrition disorders	0	(0)		3	(3)	3.0	0	(0)		1	(1)	1.0	4	(4)	2.0
Renal and urinary disorders	0	(0)		2	(2)	2.0	0	(0)		1	(1)	1.0	3	(3)	1.5
Surgical and medical procedures	0	(0)		2	(2)	2.0	0	(0)		1	(1)	1.0	3	(3)	1.5
Psychiatric disorders	0	(0)		2	(4)	2.0	0	(0)		0	(0)		2	(4)	1.0
Cardiac disorders	0	(0)		0	(0)		1	(1)	1.0	0	(0)		1	(1)	0.5
Immune system disorders	0	(0)		0	(0)		0	(0)		1	(1)	1.0	1	(1)	0.5

n= number of subjects, (m)= number of mentions, %= all percentages are expressed as the percentage of subjects in the safety population in each treatment group. Adverse event data were coded using the MedDRA dictionary version 15.0.

[1] Related refers to events whose relationship to the study treatment was regarded as certain, probable or possible.

[2] Not related refers to events whose relationship to the study treatment was regarded as unrelated or unlikely related.

Table 4. Anti-Tat Ab response by Ig subclasses in vaccinees and placebos

	Vaccinees (*n* = 99)		**Placebos** (*n* = 100)	
	n	Percentage	n	Percentage
IgM+	1	1.0	5	5.0
IgG+	9	9.1	5	5.0
IgA+	0	0.0	2	2.0
IgM+IgG+	11	11.1	4	4.0
IgM+IgA+	0	0.0	0	0.0
IgG+IgA+	7	7.1	3	3.0
IgM+IgG+IgA+	68	68.7	1	1.0
Ab-negative	3	3.0	80	80.0

n indicates the number of subjects. Percentage of subjects positive for 1, 2 or 3 anti-Tat Ab subclasses at any given time point after the first immunization

Table 5. Induction of anti-Tat cross-clade Abs after immunization in vaccinees negative at baseline for any anti-Tat Abs

HIV Clades	n	%
C	5	9.8
D	7	13.7
A	4	7.8
C+D	12	23.5
C+A	1	2.0
D+A	7	13.7
C+D+A	15	29.4
Total	**51**	**100.0**

Sera from 51 vaccinees negative at baseline also for anti-Tat Abs against C, D and A clades were tested between week 12 and week 24 (99 tested) after immunization with the B-clade Tat protein. All patients mounted anti-Tat Ab responses against A, C, and/or D clade.

Table 6. Relationship between anti-Tat or anti-Env Ab titers and Tat-mediated Env entry in DC in vaccinees

Parameter	Estimate	95% CI[a]		p-value
Vaccinees				
anti-Tat IgM (log_{10} titers)	-0.15	-0.31	0.02	0.0853
anti-Tat IgG (log_{10} titers)	-0.12	-0.20	-0.04	0.0039
anti-Tat IgA (log_{10} titers)	-0.02	-0.12	0.08	0.7579
anti-Env IgG (log_{10} titers)	-0.06	-0.09	-0.02	0.0015
Placebos				
anti-Env IgG (log_{10} titers)	0.00	-0.02	0.02	0.9471

[a]Confidence interval. A longitudinal analysis for repeated measures by generalized estimating equation method was used for the analysis. Vaccinees anti-Tat Ab-positive n=19 (86 observations), Placebos anti-Tat Ab-negative n=5 (30 observations).

Table 7. Longitudinal analysis of Tat-mediated Env entry in DC versus $CD4^+$ T-cell counts

Treatment	Estimate	95% CI[a]		p-value
Vaccinees	-127	-208	-45	0.0023
Placebo	-72	-194	51	0.2515

[a]Confidence interval. A significant inverse relationship was observed between $CD4^+$ T cells and the Tat-mediated Env entry in DC in the presence of sera from vaccinees (n=19) but not from placebo (n=11) indicating a positive relationship of $CD4^+$ T-cell increases with neutralization of Env entry

in DC. A longitudinal analysis for repeated measures by generalized estimating equation method was used for the analysis.

Figure Legends

Figure 1. CONSORT flow diagram. The number of participants screened, enrolled, randomized, followed-up and analyzed is shown for vaccine and placebo groups. Two hundred participants were randomised to one of the two treatment groups and analyzed for safety (safety population). One subject who received only one immunization was excluded from the immunogenicity population (Total = 199). Thirteen volunteers were excluded from the "Per protocol" analysis: 4 received less than three immunizations, 4 did not complete three or more visits of follow-up and 5 had major protocol non-compliance (Total = 187).

Figure 2. Anti-Tat humoral immune response elicited in study participants. (**A**) Percentage of responders for anti-Tat Abs (see Methods) in vaccinees (n=99) or placebos (n=100). The absolute number of vaccines/placebos developing anti-Tat Ig subclasses are reported on the top of each histogram. Statistical significant differences were detected between vaccinees and placebos for each Ig and for total response ($p<0.0001$, Chi-Square test). (**B**) Percentage of responders for anti-Tat Abs stratified according to the presence of one or more Ab isotype in vaccinees (n=99) or placebos (n=100). The absolute number of vaccines/placebos developing one or more Ab isotype are reported on the top of each histogram. Statistical significant differences were detected between vaccinees and placebos ($p<0.0001$, Chi-Square test). (**C**) IgM, IgG and IgA Ab mean titers (with standard error) in responders (vaccinees: n=79 for IgM, n=95 for IgG and n=75 for IgA; placebos: n=9 for IgM, n=12 for IgG and n=6 for IgA). Significant differences were detected between vaccinees and placebos for anti-Tat IgG Abs from week 12 to week 48 (Student's t-test).

Figure 3. Anti-Tat Ab durability in responders. (**A**) Kaplan-Meier estimates showing the cumulative probability of anti-Tat Ab durability during follow-up in responders (see Methods) (vaccinees: n=96; placebos: n=18). Anti-Tat Abs persisted significantly longer in vaccinees as

compared to the placebo group (p=0.0019, Log-Rank test). (**B**) Kaplan-Meier estimates showing the cumulative probability of anti-Tat Ab durability during follow-up in vaccinees (left panel) or placebo (right panel) responders, according to the number of anti-Tat Ab isotypes (vaccinees: 1 subclass n=10, 2 or 3 subclasses n=86; placebo: 1 subclass n=10, 2 or 3 subclasses n=8).

Figure 4. Increase of cross-clades anti-Tat Abs elicited in vaccinees. (**A**) baseline OD values of anti-Tat IgM, IgG and IgA against clades C, D and A in vaccinees prior to immunization (n=29, 76% C clade, 41% A clade, 14% D clade). (**B**) Changes from baseline of IgM, IgG and IgA Ab responses (OD) against Tat from other clades (C, D and A) after vaccination. Testing was performed at the peak of Ab responses (between 12 and 24 weeks). Statistical analysis was performed using the Wilcoxon signed-rank test. P-values assess the increase from baseline.

Figure 5. Neutralization of Tat/Env complex entry in DC. Baseline values (left panels) and changes from baseline after immunization (right panels) of B-clade Env entry in DC in the absence (**A**) or presence (**B**) of B-clade Tat in anti-Tat Ab-positive (n=13) vaccinees, and anti-Tat Ab-positive (n=6) or anti-Tat Ab-negative (n=5) placebos at week 20 and 48 from the first immunization. Reduction of Env entry in DC by sera indicates neutralization. Student's t-test was applied to evaluate the changes from baseline within and between treatment groups.

Figure 6. Neutralization of B- and C-clade Tat/Env complex entry in DC in vaccinees. Neutralization of B- (n=13) and C- (n=10) clade Env entry in DC in the presence or absence of (B- or C-clade) Tat by sera of Ab-positive vaccinees, measured at week 20 or week 48 after immunization. Data are presented as mean values with standard errors. Student's t-test for paired data was used for the analyses.

Figure 7. Changes from baseline of $CD4^+$ T-cell number in vaccinees and placebos. Baseline values (left panel) and changes from baseline (right panel) of $CD4^+$ T-cell counts in vaccinees (n=99) and placebos (n=100). Data are presented as mean values with standard errors. Longitudinal analysis for repeated measures by the generalized estimating equations method was applied for the analysis. P-values assess the changes from baseline within and between treatment groups.

Figure 8. $CD4^+$ T-cell numbers up to week 48 in vaccinees and placebo stratified by quartiles according to baseline values. Baseline values (left panels) and changes from baseline (right panels) of $CD4^+$ T cells in (**A**) vaccinees (n=98), **(B)** placebo (n=100) and (**C**) anti-Tat Ab-negative placebo (n=80). Data are presented as mean values with standard errors. Longitudinal analysis for repeated measures was used. P-values assess the changes from baseline within each treatment group.

Figure 9. Changes from baseline of $CD4^+$ T-cell number in vaccinees and placebos non compliant to therapy. Baseline values (left panel) and changes from baseline after immunization (right panel) of $CD4^+$ T-cell counts in vaccinees (n=18) and placebos (n=5). Data are presented as box plots. Wilcoxon signed rank sum test for paired data and Wilcoxon-Mann-Whitney test were used for the analyses. P-values assess the changes from baseline within and between treatment groups.

Figure 10. Plasma viremia up to week 48 in vaccinees and placebo non compliant to therapy. Percentage of vaccinees and anti-Tat Ab-negative placebos non-compliant to cART with detectable plasma viremia (upper panel), and plasma viremia values (log_{10} copies/mL) in patients with detectable viral load at each study visit (lower panel).

Legends to Supplementary Figures

Supplementary Figure 1. B, C, A and D Tat clades sequences and GeneBank accession numbers. Based on data published in Hemelaar J et al (AIDS 2011, 2:679-689), which refer to the time period 2004-2007, a frequency of 0.98%, 27.28%, 11.54%, and 3.61% for HIV-1 subtypes B, C, A and D, respectively, was calculated for the African continent.

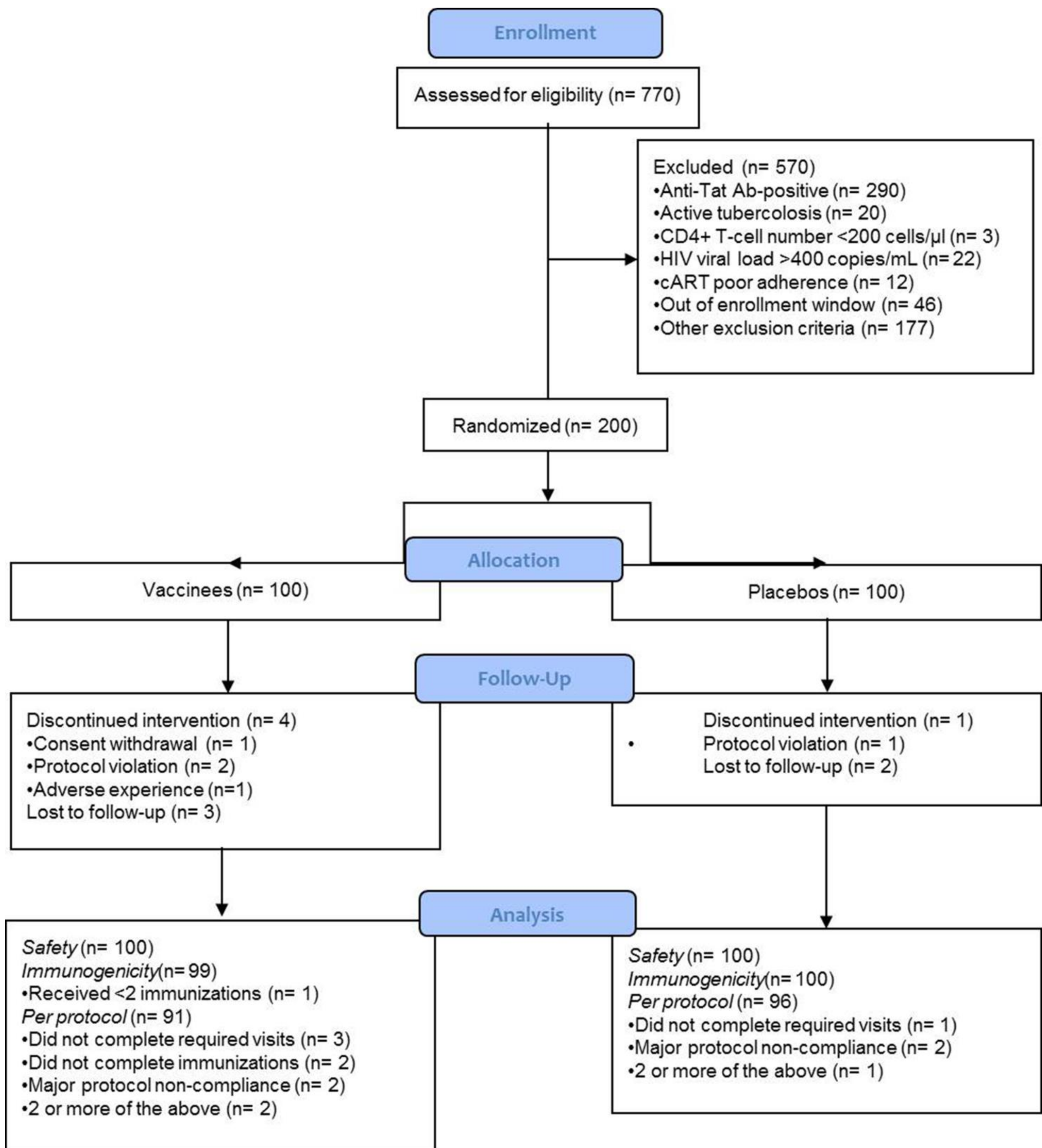
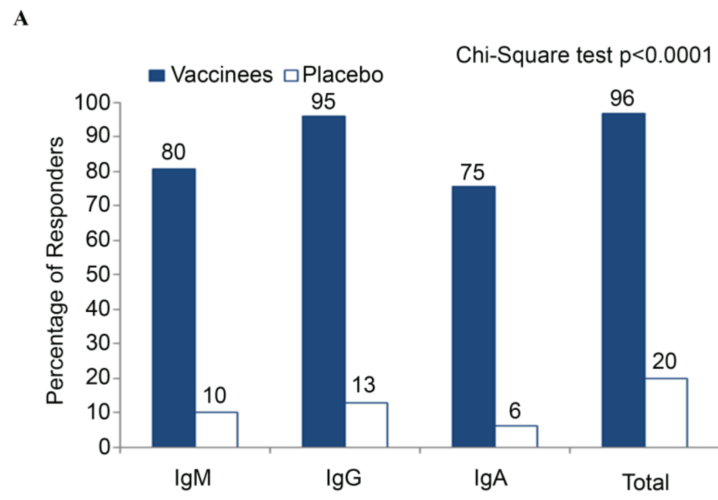
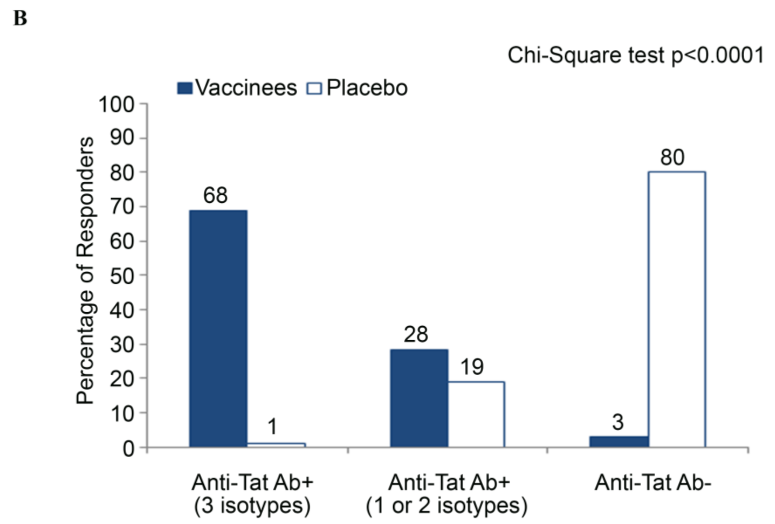


Fig 1

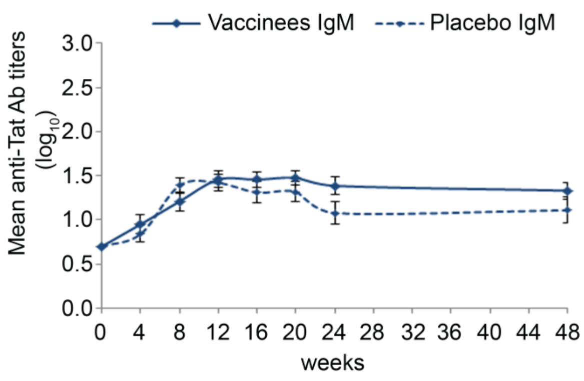
A

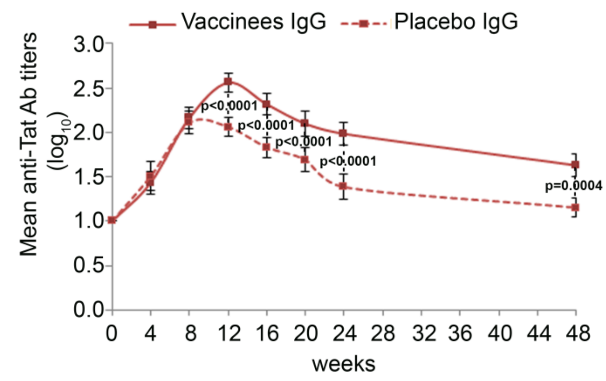


B



C





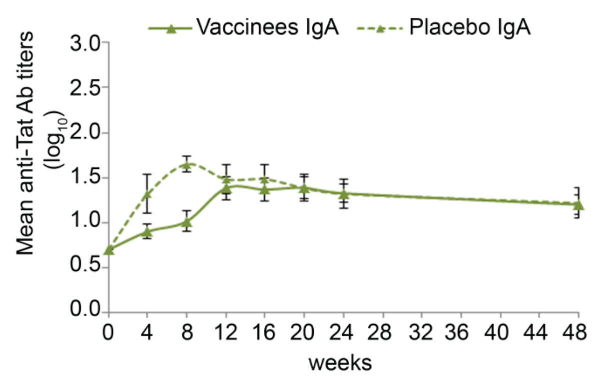
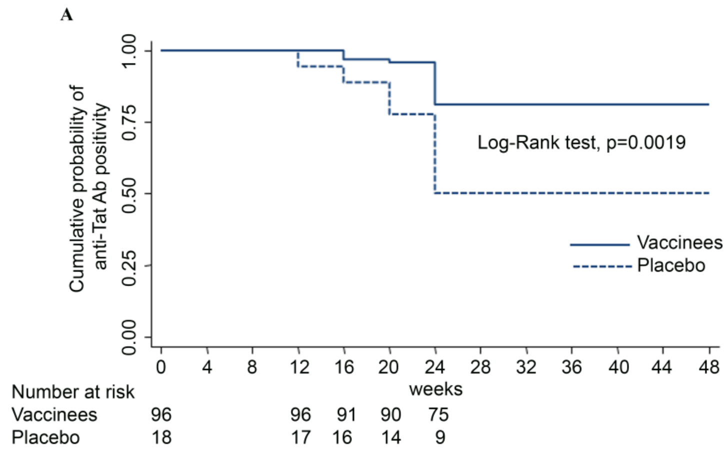


Fig 2

A



B

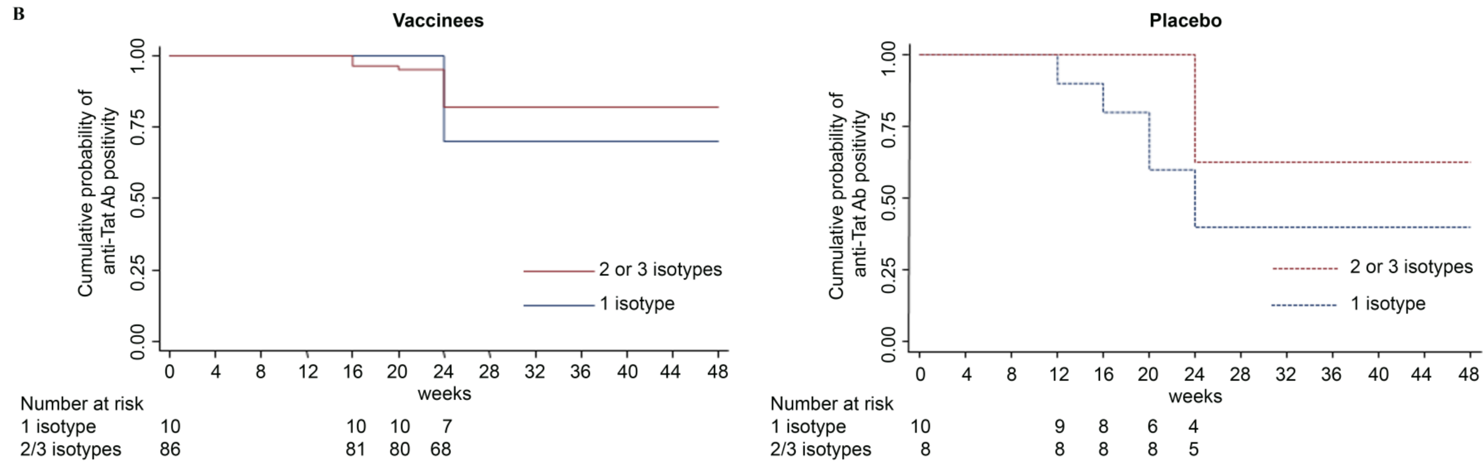


Fig 3

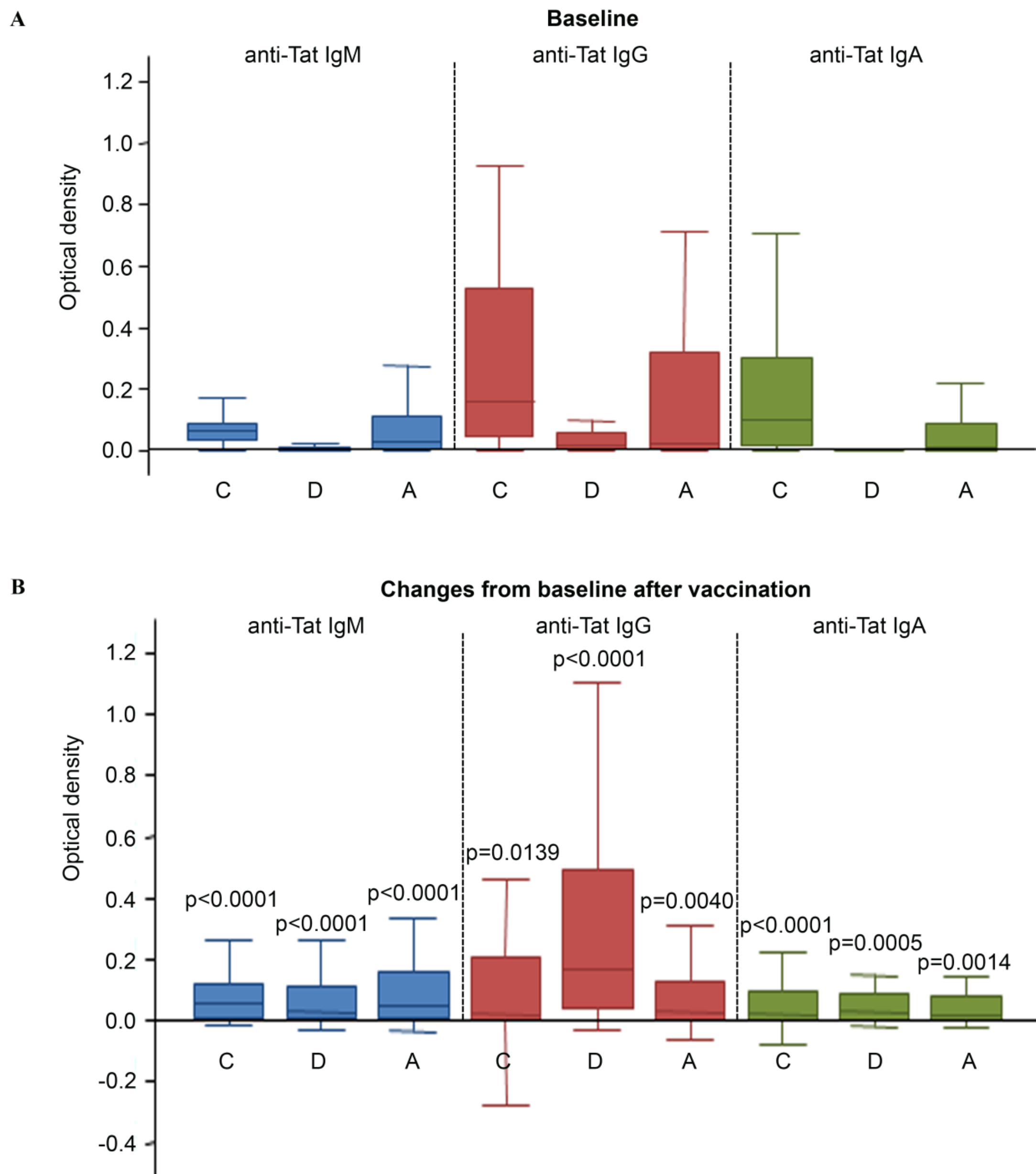


Fig 4

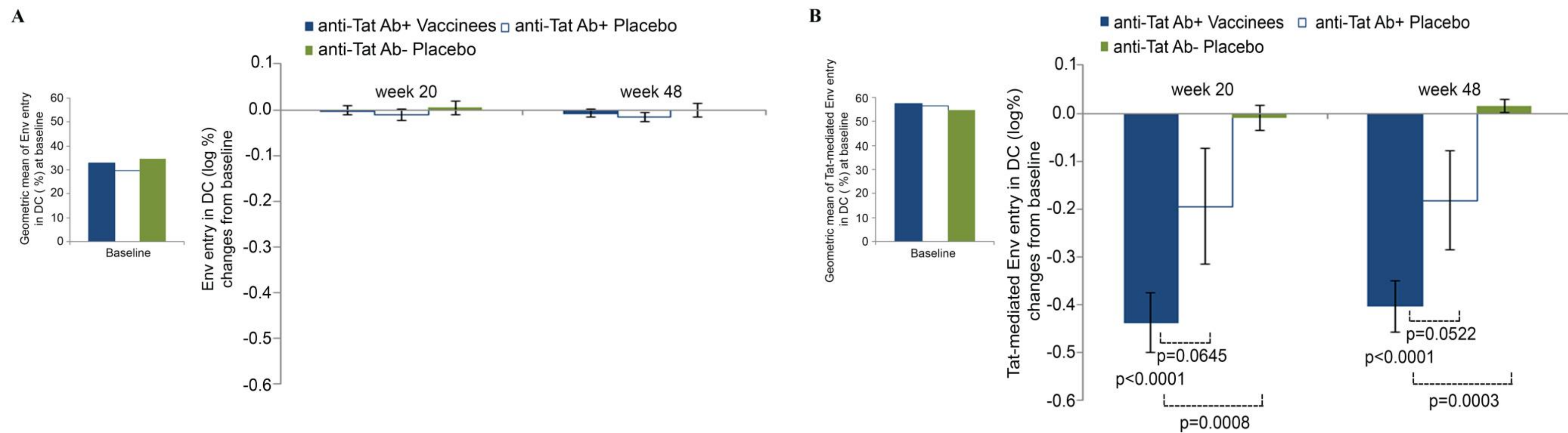


Fig 5

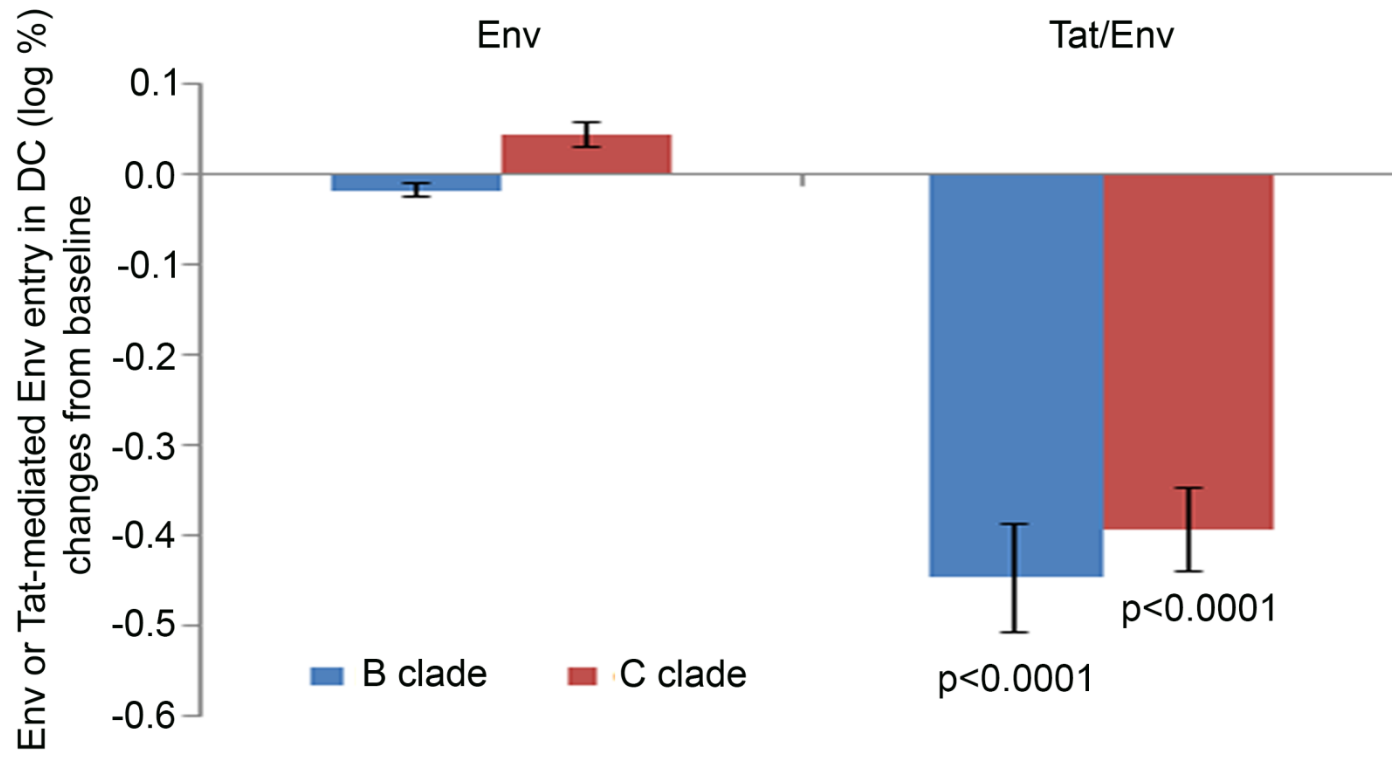


Fig 6

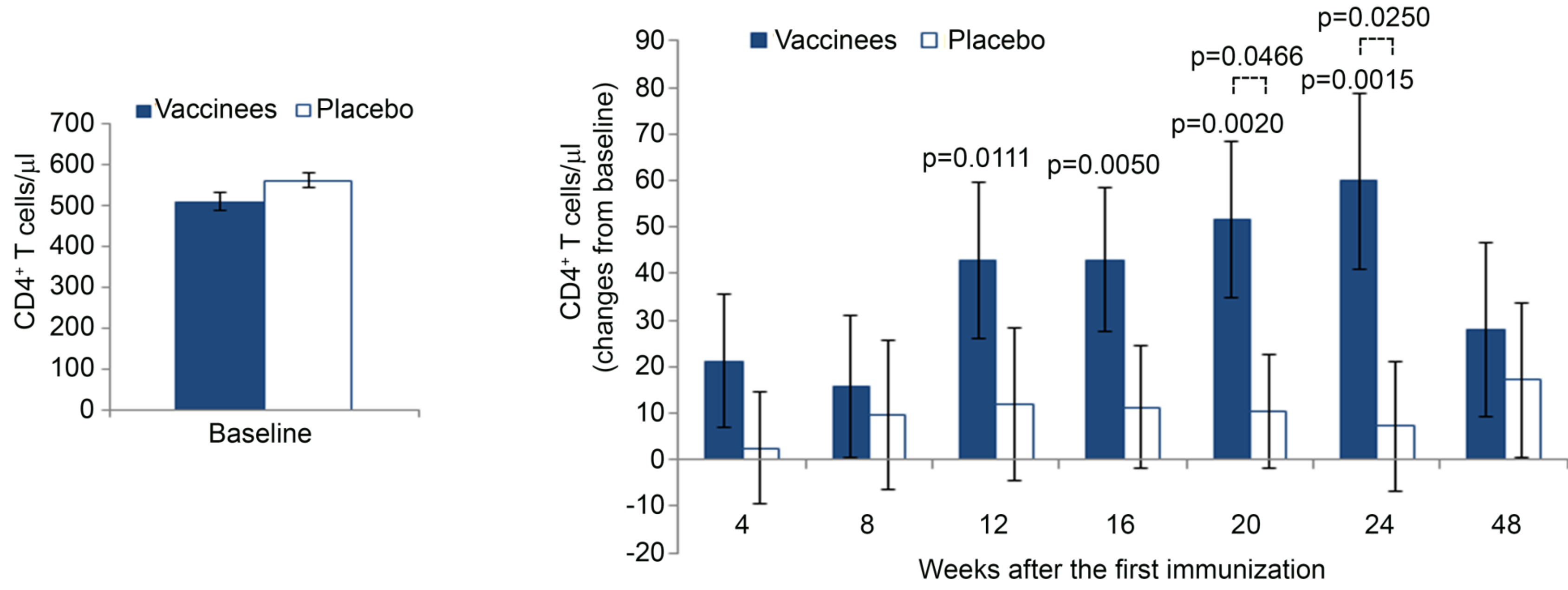
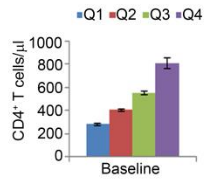
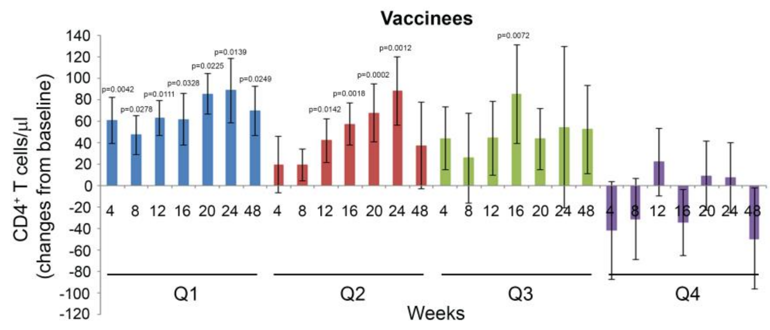


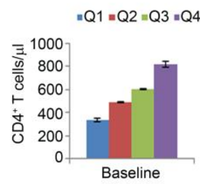
Fig 7

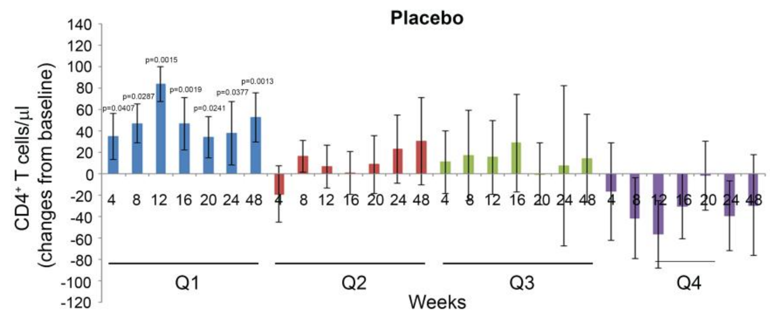
A



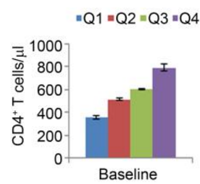


B





C



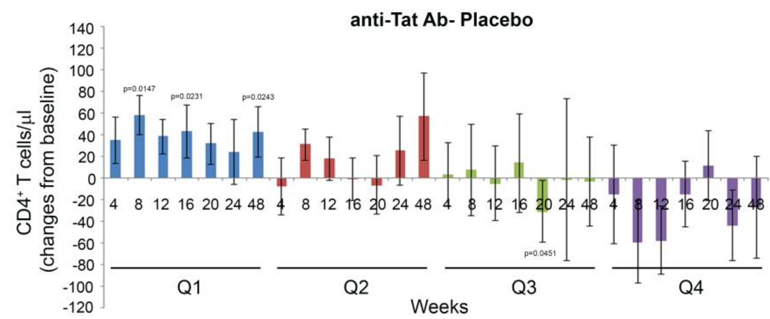


Fig 8

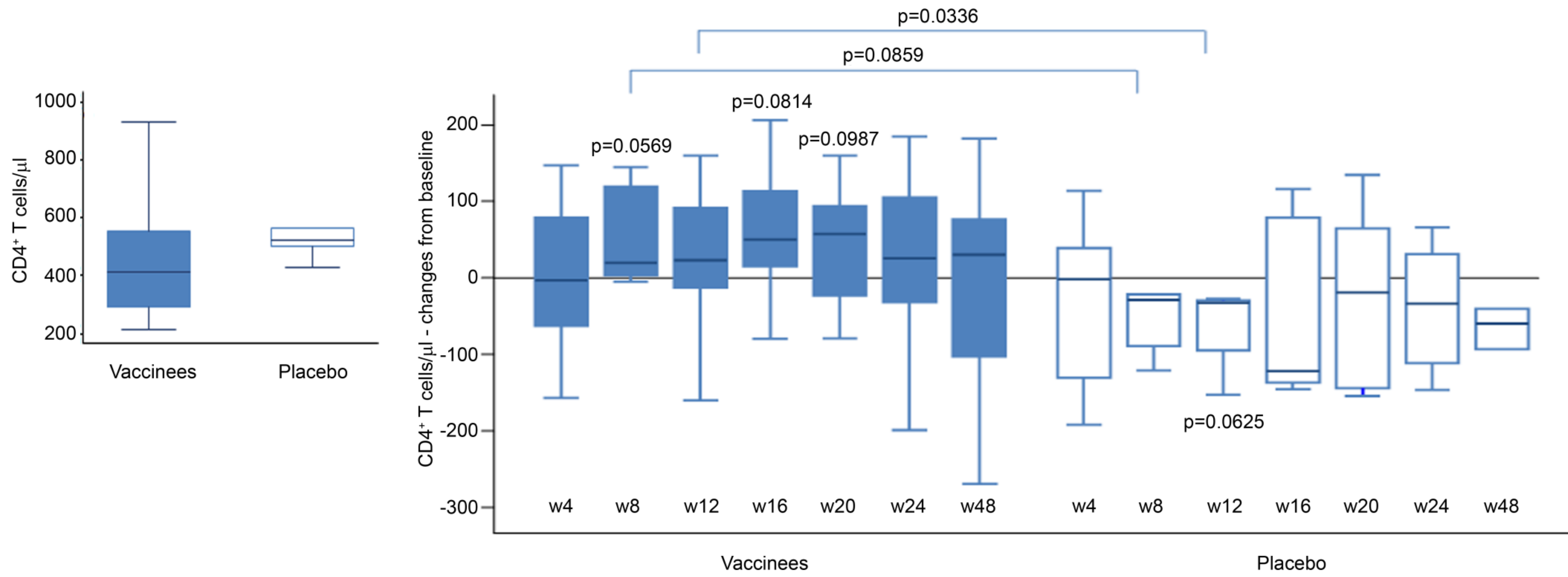
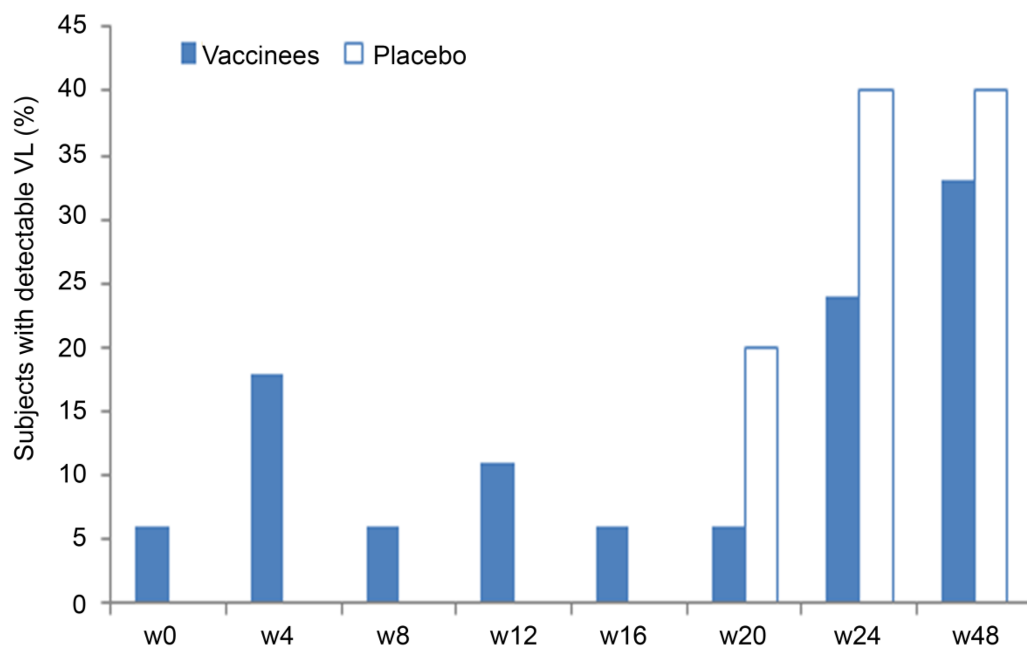


Fig 9



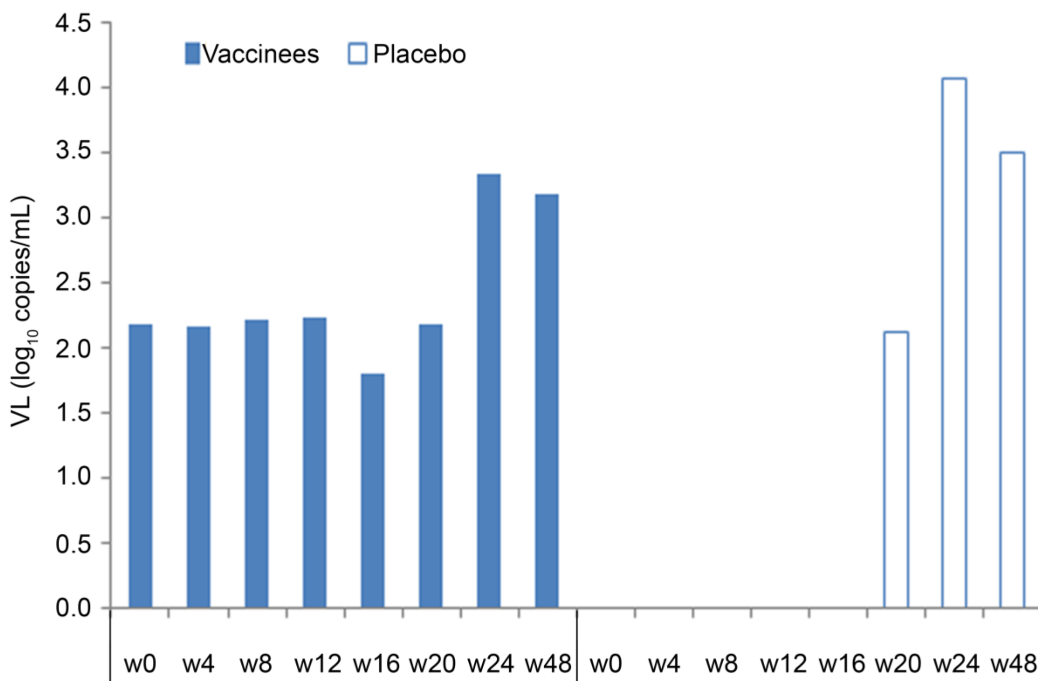


Fig 1

```
                           10        20        30        40        50        60        70        80        90        100
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
AAA44199.1 (B)     MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTGPKE---------------
AAL06113.1 (C)     ......N....N..........NT......SY..L...Q..G..............SA.PS.ED..NPI....LPRTQ.....SE.SKKKVESKTETDPFD
AY194047.1 (A)     .D....N....N......R.P.N........Y...K..L..G.........W.P...SHPDGPN..DPIPN..S..P.........---------------
AY194029.1 (D)     ......................................T..G.A.............A..DN.............S......NSE.---------------
```

1

2 Supp Fig 1

3

4