

1 **Therapeutic immunisation plus cytokine and hormone therapy improves CD4 T-cell counts, restores**
2 **anti-HIV-1 responses and reduces immune activation in treated chronic HIV-1 infection**

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16 Key words: HIV-1, DNA vaccine, immune-based therapy

17

18 **Abstract**

19 **Background:** This randomised, open label, phase I, immunotherapeutic study investigated the effects of
20 interleukin (IL)-2, granulocyte-macrophage colony-stimulating factor (GM-CSF), recombinant human
21 growth hormone (rhGH), and therapeutic immunisation (a Clade B DNA vaccine) on combination
22 antiretroviral therapy (cART)-treated HIV-1-infected individuals, with the objective to reverse residual T-
23 cell dysfunction.

24 **Methods:** Twelve HIV-1⁺ patients on suppressive cART with baseline CD4 T-cell counts >400 cells/mm³
25 blood were randomised into one of three groups: 1) vaccine, IL-2, GM-CSF and rhGH (n=3); 2) vaccine
26 alone (n=4); or 3) IL-2, GM-CSF and rhGH (n=5). Samples were collected at weeks 0, 1, 2, 4, 6, 8, 12, 16,
27 24 and 48. Interferon (IFN)- γ , IL-2, IL-4 and perforin ELISpot assays performed at each time point
28 quantified functional responses to Gag p17/p24, Nef, Rev, and Tat peptides; and detailed T-cell
29 immunophenotyping was undertaken by flow cytometry. Proviral DNA was also measured.

30 **Results:** Median baseline CD4 T-cell count was 757 cells/mm³ (interquartile range [IQR] 567-886
31 cells/mm³), median age 48 years (IQR 42-51 years), and plasma HIV-1-RNA <50 copies/ml for all subjects.
32 Patients who received vaccine plus IL-2, GM-CSF and rhGH (group 1) showed the most marked changes.
33 Assessing mean changes from baseline to week 48 revealed significantly elevated numbers of CD4 T cells
34 (p=0.0083) and improved CD4/CD8 T-cell ratios (p=0.0033). This was accompanied by a significant
35 reduction in expression of CD38 on CD4 T cells (p=0.0194), significantly increased IFN- γ and IL-2
36 production in response to Gag (p=0.0122) and elevated IFN- γ production in response to Tat (p=0.041) at
37 week 48 compared to baseline. Subjects in all treatment groups showed significantly reduced PD-1
38 expression at week 48 compared to baseline, with some reductions in proviral DNA.

39 **Conclusions:** Multifarious immunotherapeutic approaches in the context of fully suppressive cART further
40 reduce immune activation, and improve both CD4 T-lymphocyte counts and HIV-1-specific T-cell
41 responses. (NCT01130376)

42

1. Introduction

Combination antiretroviral therapy (cART) improves morbidity and mortality in infected patients [1]; however, it cannot eradicate HIV-1 and does not enable the immune system to control HIV-1 replication [2, 3]. Viral reservoirs persist and viral rebound is seen following treatment interruption [4-7]. Immunotherapy in chronic, treated HIV-1 infection has the potential to reduce viral reservoirs by reversing T-cell dysfunction and modifying immune responses to mirror those found in long-term nonprogressors or HIV controllers [3, 8, 9]. Ideal immunotherapeutic approaches would induce and maintain HIV-1-specific responses, increase polyfunctionality, enhance thymic function, reduce levels of immune activation and reverse T-cell anergy/exhaustion. The benefits of immunotherapy on clinical outcomes may include: more sustained viral control; suppression of residual viraemia; prevention of reservoir re-seeding; eradication of the reservoir [10]; and reduced risk of both HIV-1-related and inflammation-related morbidity and mortality.

Design of this clinical trial, immunomodulatory agents used, and timing of their administration were developed from previous studies [3, 9]. Immunisation in conjunction with cART may lead to a reversal of anergy by selective induction and activation of specific memory responses against viral proteins [3, 11]. Although the efficacy of interleukin (IL)-2 administration in HIV-1⁺ subjects has been challenged, it has been shown to increase CD4 T-cell counts [12]. In animal models relevant responses were preserved and maintained when IL-2 was administered during the antigen-specific T-cell contraction phase of the immune response [13, 14]. In contrast, IL-2 given pre-immunisation in HIV-1⁺ cART-treated patients failed to increase specific T-cell proliferation [15, 16], although IL-2 administered following tetanus immunisation in HIV-1-infected persons was found to better sustain tetanus-specific responses than IL-2 given together with vaccine or before immunisation [17]. Here we describe administration of IL-2 following immunisation as part of an immunotherapeutic programme also including granulocyte-macrophage colony-stimulating factor (GM-CSF) and recombinant human growth hormone (rhGH). In the context of HIV-1 infection, GM-CSF has been reported to increase CD4 T-cell counts and reduce plasma HIV-1 RNA [18]. GM-CSF activates antigen presenting cells, and stimulates macrophage differentiation and proliferation [19, 20]. IL-2 and GM-CSF have shown efficacy as adjuvants for DNA- or peptide-based vaccines [21]. In 1996, the United States FDA approved rhGH to treat HIV-1 associated wasting (although it is not approved for use in the European Union or Switzerland), with demonstrable improvements in a number of factors including quality of life [22].

Immunological benefits of rhGH include: reversal of thymic involution [23-26]; increase in total and naive CD4 T-cell counts [23, 24, 26, 27]; restoration of T-cell responses against HIV-1 [27-29]; and reduction in expression of activation (CD38, HLA-DR) and apoptosis (CD95) markers [24].

In this study, immunisation was used to prime specific cellular responses within treated-HIV-1-infected individuals followed by the administration of a regime of cytokines/rhGH along with further vaccine boosts designed to augment and sustain memory responses to HIV-1 [3], which in turn were postulated to confer clinical benefit.

2. Materials and methods

2.1. Study design

This randomised, open label, phase I, immunotherapeutic study (NCT01130376) was conducted in compliance with GCP guidelines and approved by appropriate review boards and ethics committees. All study participants provided written informed consent prior to study entry and were considered eligible if they were chronically infected with HIV-1, on stable cART, with plasma viral load <50 copies/ml, and CD4 T-cell count >400 cells/mm³. It was intended to recruit and screen 50 patients, of which 30 were to be randomised into the study (with subjects randomised equally into each arm). The sample size was determined accounting for an estimated 10% drop out by week 48, and we calculated an 80% power to show significant changes ($p=0.05$) if we recruited 10 patients into each arm. Patients were randomised into one of three arms with treatment schedules as shown in Fig. 1. The primary endpoint of the study was analysis of safety and toxicity data in relation to grade 3 or above laboratory or clinical serious adverse events (SAE), which could be attributed to the treatments given. Lymphocyte subsets and plasma viral load were determined at each time point as previously described [30].

2.2. Vaccine

GTU®-MultiHIV DNA Clade B vaccine (Han-2 isolate) is comprised of both regulatory and structural HIV-1 proteins (Rev, Nef, Tat, p17, p24, and stretch of eleven cytotoxic T-lymphocyte [CTL] epitope clusters from Pol and Env) representing B clade, cloned into a vector termed the Gene Transport Unit (GTU®). GTU® also codes for a nuclear anchoring protein (bovine papilloma virus E2) [31, 32]. The vaccine has been found to be safe and well-tolerated in phase I and II clinical trials [11, 31].

100 **2.3. HIV-1 peptides**

101 HIV-1 peptides were sourced from NIBSC (Potters Bar, Herts, UK): Gag p17 (ARP703.1-.13, ARP789.1-
102 .4); Gag p24 (ARP788.1-.22, EVA7046.18, EVA7080.51-.52, EVA7080.69); Rev (EVA780.1-.11,
103 EVA7085.1-.3); Tat (EVA779.1-.8, EVA7084.1-.3); and Nef (ARP7074.1-.20, EVA7081.2, EVA7081.44,
104 EVA7086.6). These were 9mers, 15mers overlapping by 5, and 20mers overlapping by 10, and used at a
105 final concentration of 5µg/ml. A second set of peptides run in parallel were from FIT Biotech (Tartu,
106 Estonia; kindly provided by Rein Sikut): Gag p17/p24, Rev, Tat, Nef, and CTL epitope-stretch peptides
107 (15mers with an 11 amino acid overlap) for the MultiHIV-B consensus antigen [33], which were used at a
108 final concentration of 2µg/ml.

109 **2.4. HIV-1-specific T-cell responses**

110 IFN-γ, IL-2, IL-4, and perforin production in response to HIV-1 peptide pools was investigated using
111 enzyme-linked immune spot (ELISpot) assays at each time point. Briefly, 2.5×10^5 peripheral blood
112 mononuclear cells (PBMC) were added per well in duplicate and stimulated with peptides (described in
113 section 2.3) or positive/negative controls, and processed as previously described [34]. IFN-γ, IL-2 and IL-4
114 responses were positive if >50 spot forming cells (SFC) were present per million PBMC after background
115 (negative control) was subtracted and if this number was at least twice the background. Perforin assay cut-off
116 was >100 SFC (due to higher background).

117 **2.5. T-cell phenotyping**

118 Flow cytometry was carried out using a six-colour BD LSR II Flow Cytometer (Becton Dickinson, Oxford,
119 UK). Fresh PBMC were labelled with murine anti-human monoclonal antibodies (mAbs) according to
120 manufacturer's instructions, including: PerCP Cy5.5 anti-CD3; APC-H7 anti-CD8; FITC anti-CD57, anti-
121 programmed death (PD)-1 (CD279), anti-CD27; PE anti-CD38, anti-CD25, anti-CD31; APC anti-CD95,
122 anti-CD45RA, anti-CD28, anti-CTLA-4; PECy7 anti-HLA-DR, anti-PD-L1 (CD274), anti-CD45RO, anti-
123 CD69 (BD Biosciences, Oxford, UK); PE anti-CCR7 (CD197), PE anti-TIM-3 (R&D Systems, Abingdon,
124 UK); and APC anti-PTK-7 (Miltenyi Biotech Ltd, Bisley, UK). Following acquisition, forward and side
125 scatter properties were used to determine the lymphocyte population and 50,000 gated lymphocyte events
126 were collected for each sample. Appropriate isotype-matched control mAbs were run in parallel for each
127 sample, and data analysis was performed using FACSDiva™ Software version 6.1.2 (BD Biosciences, San
128 Jose, CA).

129 **2.6. DNA extraction and proviral DNA**

130 DNA was isolated from cryopreserved baseline (week 0) and week 48 PBMC samples using the Nucleon
131 BACC2 Genomic DNA Extraction Kit (Gen-Probe Life Sciences Ltd, Manchester, UK) as per
132 manufacturer's instructions. Proviral DNA was quantified using the COBAS® Taqman® HIV-1 Test,
133 version 2.0 (Roche Molecular Systems, Inc, Branchburg, NJ) and copies/μg cellular DNA calculated.

134 **2.7. Sequencing**

135 Patient HIV-1 subtypes were determined at FIT Biotech Ltd (Tartu, Estonia). Two proviral DNA regions
136 were sequenced and genotyped from cryopreserved PBMC (5×10^6), as described previously in detail [35].

137 **2.8. Statistical analysis**

138 All summary statistics of quantitative data are presented as median with interquartile range (IQR).
139 Qualitative data are presented as numbers and percentages. Since longitudinally collected data were available
140 at study visit time points, linear mixed model was used for between treatment arm comparisons to calculate
141 the difference in averages which represent the time weighted difference in immunological (functional and
142 phenotypic) results from baseline to each study time points. A random intercept model using MIXED
143 procedure in SAS was generated by fitting all clinical and immunological results as dependent variables by
144 study time points, stratified by the three randomised treatment arms. Mean changes from baseline to each of
145 the study time points in all clinical and immunological parameters are given as point estimates, presented
146 with a 95% confidence interval (CI). For the graphs presented herein, where the 95% CI does not cross the
147 line of origin, a statistically significant difference can be inferred (above, a statistically significant increase
148 from baseline, and below, a statistically significant decrease from baseline). Where the 95% CI from the
149 groups do not overlap for an individual time point, there is a statistically significant difference between the
150 means of the groups. The small numbers in each treatment arm were taken into consideration and descriptive
151 statistics were also carried out. All statistical analyses were performed using SAS version 9.3 (Institute Inc,
152 Cary, NC) statistical software; all p-values presented are two-tailed and a statistically significant change
153 from baseline to each of the study time points are indicated with asterisks (*) in figures.

154 3. Results

155 3.1. Baseline patient characteristics

156 Of ninety-three patient referrals, twelve patients met the eligibility criteria for enrolment (baseline patient
157 characteristics and randomisation group allocation as per Table 1) and were randomised into one of three
158 arms of the trial, with the treatment schedule as per Fig. 1. There were no statistically significant differences
159 determined, using the Kruskal-Wallis test, in the clinical characteristics presented in Table 1 between the
160 three arms of the study at baseline, in terms of: age ($p=0.621$); baseline CD4 T-cell count ($p=0.724$); nadir
161 CD4 T-cell count (0.800); time since HIV-1 diagnosis ($p=0.914$); duration of cART ($p=0.536$); and
162 CD4/CD8 T-cell ratio (0.070).

163 3.2. Lymphocyte subsets and HIV-1 RNA

164 Administration of IL-2 and GM-CSF for five days resulted in mean change in CD4 counts of 1307 cells/mm³
165 and 1511 cells/mm³ in groups 1 and 3 ($p < 0.0001$ for both), at week 2 compared to baseline. This was
166 significantly higher than for group 2 (in which patients received vaccine alone; Fig. 2A). At week 6, CD4
167 counts remained elevated compared to baseline in both groups that received cytokines and rhGH, and the
168 mean increase was 413 cells/mm³ and 239 cells/mm³ in groups 1 and 3 ($p=0.0001$ and $p=0.0039$,
169 respectively), whereas for group 2 there was a marginal but statistically significant decrease in the mean CD4
170 count at week 6 compared to baseline of -191 cells/mm³ ($p=0.0369$). At the final study time point, week 48,
171 only patients from group 1 (who received immunisation plus cytokines and rhGH) demonstrated a sustained
172 increase in mean CD4 count compared to baseline of 281 cells/mm³ ($p=0.0083$; Fig. 2A). Mean changes
173 from baseline, 95% CI and p values for parameters presented in sections 3.2 and 3.3 are summarised in
174 Supplementary Table 1.

175

176 Throughout the course of 48 weeks, CD8 counts followed a similar pattern to CD4 counts. Patients in groups
177 1 and 3 demonstrated statistically significant increases in their CD8 counts at week 2, following
178 administration of IL-2 and GM-CSF, compared to baseline and compared to group 2. Mean increases from
179 baseline at week 2 were 634 cells/mm³ and 999 cells/mm³ ($p < 0.0001$ for both; Fig. 2B). The increase was
180 more prominent for group 3 for which CD8 counts remained elevated at weeks 4 and 6, with mean increases
181 from baseline of 248 cells/mm³ and 220 cells/mm³ ($p=0.0168$ and $p=0.0330$, respectively). Patients in group

182 2 experienced a marginal but significant reduction in the numbers of CD8 T cells at weeks 6 and 12 with a
183 mean decreases of -283 cells/mm³ and -239 cells/mm³ (p=0.0151 and p=0.0387, respectively). By week 48,
184 there was no difference from baseline in CD8 counts for any of the groups (Fig. 2B).

185

186 CD4/CD8 ratios were elevated at week 2 for individuals in groups 1 and 3 with mean increases from baseline
187 of 0.43 and 0.34 (p <0.0001 for both). This increased CD4/CD8 ratio was maintained for all other time
188 points for group 1 patients with mean increase from baseline to week 48 of 0.27 (p=0.0033; Fig. 2C).

189

190 Plasma viral load was <50 copies/ml for all individuals at screen visits, baseline and week 48.

191 **3.3. T-cell function and phenotype**

192 Patients in group 1 demonstrated noticeable increase in IFN- γ production in response to stimulation with Gag
193 p17/p24 peptides at week 4, although this did not reach statistical significance (mean increase 223 SFC/10⁶
194 PBMC; p=0.071; Fig. 3A). This increase was maintained throughout the remaining study period and reached
195 significance at week 48 (mean increase 312 SFC/10⁶ PBMC; p=0.0122; Fig. 3A). Similarly, IL-2 production
196 in response to Gag p17/p24 stimulation was significantly elevated at week 48 compared to baseline for these
197 subjects (mean increase 79 SFC/10⁶ PBMC; p=0.0017; Fig. 3B). Subjects randomised to this group also
198 showed an elevated IFN- γ response to Tat peptides at week 48 (data not shown).

199

200 As expected, the percentage of T cells expressing the activation marker, CD38, was significantly increased at
201 week 2 in all study groups and was more pronounced for individuals who received IL-2 and GM-CSF (Fig.
202 3C and D). By week 4, CD38 expression on the CD4 T cells had normalised for patients in all study groups
203 and by week 6, there was a significant reduction in the percentage expression of CD38 on CD4 T cells for
204 individuals who received cytokine and rhGH that was maintained throughout the remainder of the study,
205 where the mean decrease from baseline was -8.3% (p=0.0194) and -6.1% (p=0.0254; Fig. 3C). At week 24,
206 individuals in groups 1 and 3 showed a significant reduction in the percentage expression of CD38 on their
207 CD8 T cells, and this was only maintained for individuals in group 3, with mean decrease from baseline to
208 week 48 of -8.8% (p=0.0139), although there was a trend towards a decrease in this marker for individuals in
209 group 1 with a mean decline from baseline of -8.4% (p=0.0651; Fig. 3D). The percentage of CD4 and CD8 T
210 cells expressing PD-1 was significantly reduced at week 48 for all treatment groups (Fig. 3E and F). CD4 T-

cell mean fluorescence intensity (MFI) of CD95 was significantly reduced by week 48, despite significant increases at week 2, and mean decreases from baseline were -1562 ($p=0.0077$) and -1675 ($p=0.0003$) for groups 1 and 3 respectively (Fig. 3G). CD95 expression in terms of MFI followed the same pattern on the CD8 T cells, with a significant increase at week 2 compared to baseline for individuals that received IL-2 and GM-CSF and a significant reduction by week 48 for patients in group 3, with trend towards reduction for those in group 1 (Fig. 3H).

The most marked changes in T-cell function and phenotype at week 48 (compared to baseline) were seen for individuals in group 1. Representative plots looking at changes on an individual level are shown in Fig. 4. Subject G739 demonstrated reduced CD38 expression on CD4 and CD8 T cells at week 48, which was paralleled by increased IFN- γ response to Gag (Fig. 4A). For patient B784, CD8 T-cell activation was below the baseline value at week 48 while expression of CD38 on CD4 T cells had returned to the baseline level for this individual. At this time point, the subject demonstrated increased IFN- γ and IL-2 production in response to Gag stimulation (Fig. 4B). Finally, as a comparison, subject C319 (in group 3) showed small fluctuations in these parameters throughout the course of the study, and despite slightly elevated functional responses at week 48, activation levels were unaltered compared to baseline (Fig. 4C).

3.4. Proviral DNA

In each treatment group, distinct patients demonstrated a decrease in proviral DNA at week 48 compared to baseline, while others revealed an increase (Table 2). Statistical analyses comparing changes in proviral DNA levels from baseline to week 48 between the treatment groups demonstrated no significant differences. Patients G739 (group 1) and F810 (group 3) had the greatest reduction in proviral DNA at week 48 (with a reduction of 1052 and 1527 copies/ μ g cellular DNA, respectively; Table 2).

3.5. Toxicology, adverse event monitoring and endpoints

No differences were observed in the plasma chemistry data in any of the groups, throughout the course of the study confirming that the interventions were well-tolerated. The adverse events documented were mostly mild and were usually limited to erythema at the injection site. Subject C241 (randomised to group 3) was admitted with headache following one dose of rhGH. Due to the patient's SAE, it was agreed that this subject should receive no further rhGH and continued on observational basis only. Data were re-analysed

239 excluding all data captured post day 14 for this patient, which did not alter outcome of the findings (results
240 not presented). Overall, the treatments were determined to be safe and well-tolerated.

241 **4. Discussion**

242 This study demonstrates that a novel combination immunotherapy with an HIV-1 vaccine leads to enhanced
243 anti-HIV-1 responses and reversal of unfavourable immunophenotypes. The approach was well-tolerated,
244 and subjects who received immunisation, IL-2, GM-CSF and rhGH (group 1) showed the most marked
245 changes at final study time point compared to baseline. CD4 T-cell counts were significantly increased at
246 week 2 and remained elevated at the majority of time points, compared to baseline, after week 2. We propose
247 that this is an effect of the combination of cytokines and subsequent hormone administration, in addition to
248 vaccine, that is driving these immunological changes. Furthermore, CD4/CD8 T-cell ratios improved, and
249 these changes were accompanied by enhanced functional responses to Gag and a reversal of unfavourable
250 immunophenotypes. Sullivan *et al.*, reported HIV-1-specific lymphoproliferative responses (particularly to
251 p24) at week 24 when IL-2 was administered during weeks 0, 4 and 8, and the response was relatively
252 unchanged even at week 12 [36]. Here we report a delayed Gag-specific response (at week 48, when the last
253 dose of vaccine was given at week 12). Administration of IL-2 and GM-CSF, in cART-treated patients with
254 incomplete/failed immune reconstitution despite virological suppression, was found to result in HIV-1-
255 specific proliferative responses and IL-2 production [37, 38]. We further documented the beneficial effects
256 of rhGH on T-cell function in treated HIV-1 infection [28, 29], and concurred with findings describing a
257 reduction in T-cell activation and apoptosis markers following rhGH therapy in such subjects [24, 29].
258 Changes observed herein were most striking for subjects who received all the interventions, suggesting a
259 combinatorial outcome of these agents with distinct biological effects.

260

261 Initial cytokine-induced immune activation at week 2 was short-lived. Overall there was a reduction in
262 activation state for individuals who received IL-2 and GM-CSF. Furthermore, this was accompanied by
263 decreased expression of the negative regulator, PD-1. Expression of this exhaustion marker was found to be
264 reduced on both CD4 and CD8 T cells at week 48 compared to baseline for all treatment groups regardless of
265 randomisation, which may be an effect of prolonged cART. Kassu *et al.*, found the percentage of HIV-1-
266 specific CD4⁺IFN- γ ⁺PD-1⁺ T cells to be significantly lower in cART-treated patients compared to untreated

267 viraemic subjects [39], and PD-1 has been proposed as a marker to identify individuals on long-term
268 suppressive cART with incomplete immune reconstitution [40].
269
270 Vaccine was anticipated to induce recovery of virus-specific responses [11], and these responses were
271 expected to be enhanced by the administration of cytokines [41] and rhGH [3]. Delivery route of
272 immunisation may have influenced the resulting antigen-specific responses. The FIT Biotech DNA vaccine
273 was administered via ten intradermal injections, whereas another therapeutic DNA vaccine study reported
274 enhanced HIV-1-specific CD8 T-cell responses following immunisation on the skin [42]. *In vivo*
275 electroporation (EP) delivery of a DNA vaccine was found to significantly improve cellular immune
276 responses in terms of rate, magnitude, duration and breadth to a number of antigens [43], which concurred
277 with results from animal models demonstrating improved responses with EP [44]. The GTU® Multi-HIVB
278 vaccine used in untreated HIV-1⁺ individuals in South Africa induced antigen-specific responses, with
279 beneficial effects on plasma HIV-1 RNA and CD4 T-cell recovery, particularly following intramuscular
280 administration, and in individuals with certain HLA haplotypes [11].
281
282 Patients across all groups showed reductions in proviral DNA but these could not be attributed to a particular
283 study arm. Subject B784, who showed the most improved Gag-specific responses at week 48 (in terms of
284 both IFN- γ and IL-2 production), also showed a reduction in proviral DNA. Recovery of IL-2 responses in
285 this individual may be due to low-level proviral DNA at baseline and the fact that this patient started cART
286 0.3 months after diagnosis. Studies have sought to optimise the timing and duration of cART. Initiation of
287 cART in early HIV-1 infection results in lower residual viral reservoirs [2, 45], lower levels of immune
288 activation and improved polyfunctionality of HIV-1-specific responses [45]. Furthermore, earlier initiation of
289 cART and higher nadir CD4 counts have been associated with functional responses to immunisation [46]. In
290 a study investigating IL-2 administration and immunisation with therapeutic HIV-1 vaccine Remune™,
291 functional responses were not boosted in cART-treated HIV-1-infected patients who had low pre-cART CD4
292 counts [41]. This lack of immune recovery was postulated to be due to protracted immunosuppression and/or
293 clonal dysfunction [41], and differed from previous Remune™ studies which demonstrated proliferative
294 responses in patients that had higher pre-cART CD4 counts [47]. For this reason, patients enrolled in this
295 study were carefully selected, particularly in terms of CD4 counts and suppressed viraemia. Our findings

296 from subject B784 suggest that, in addition to such criteria, earlier cART in combination with
297 immunotherapy might represent a future approach.

298

299 Responses were not measured at week 3 of the study, when the effect of five days treatment with rhGH may
300 have been most apparent. Hence, the authors appreciate that the benefit of such a short course of rhGH may
301 be difficult to detect immunologically, particularly as the majority of previous rhGH studies in the context of
302 HIV-1 infection tend to give rhGH for 12 weeks or more [23-29].

303

304 Despite ninety-three patient referrals and twenty-one screen visits, only 12 patients who met the eligibility
305 criteria were enrolled onto the trial. This discrepancy (why so few referred patients were screened) was
306 primarily due to: an inability to commit to the treatment schedule/work commitments, subjects not meeting
307 the baseline CD4 T-cell requirement, acute illness prior to baseline and previous immunotherapy. We
308 acknowledge that due to small numbers we have lost the power to show a significant difference as predicted
309 when the sample size was estimated, however, despite the low numbers, we showed significant changes in
310 CD4 T-cell counts and CD4/CD8 T-cell ratios for individuals who received therapeutic immunisation, IL-2,
311 GM-CSF, and rhGH, in addition to significant changes in T-cell function and phenotype for subjects in this
312 group at week 48 compared to baseline. The results have been presented with 95% confidence intervals, and
313 the width of these depend directly on the sample size: large studies produce narrow intervals and, therefore,
314 more precise results. When we looked at individual patient responses, importantly, all subjects randomised to
315 group 1 demonstrated increased CD4 T-cell counts and improved CD4/CD8 T-cell ratios at week 48
316 compared to baseline, however we acknowledge with a small sample size it is difficult to distinguish
317 between a real effect and random variation. Despite this limitation, our research hypothesis was tested in a
318 small number of subjects thus avoiding spending too many resources, e.g. subjects, time and financial costs,
319 on finding an association. With the described associations found, a larger confirmatory study is needed to
320 confirm our findings. Another limitation of small study size is an over estimation of the magnitude of an
321 association. Furthermore, lack of statistical significance in the work presented should also be viewed with
322 caution, as with small sample sizes, results may have just missed statistical significance. Despite the
323 limitations highlighted, linear mixed model was utilised to analyse the data, in addition to simple descriptive
324 methods, as the intended sample size was not recruited. Both methods showed similar data trends, and data

presented in this manuscript are from the mixed model (as detailed in section 2.8). The normality of the data could be assumed, as within patient changes from baseline to each of the study time points are presented, and these time points are spaced out relatively closely in time from baseline. The overall objective of this repeated measures study design was to make inferences about the expected values of the immunological (functional and phenotypic) data, and about the means of the populations from which study subjects were sampled. This was done in terms of treatment and time effects in the mixed model – in order to estimate differences between means at different study time points for the same treatment. These are inferences about the fixed effects in the model. The problems with statistical inference for fixed effects have been associated with missing or unbalanced data and complex covariance structures. The design of this study was intended to apply repeated measures statistical method.

The authors appreciate that larger studies employing these agents and/or others are needed to fully elucidate the clinical benefit of such therapies. Longer treatment with rhGH at a lower dose represents a possible addition to such a study design, as this has been associated with improved thymic function, improved T-cell functionality, and few adverse events [25, 29]. In the lymphocytic choriomeningitis virus (LCMV) mouse model, blockade of IL-10 or PD-1 during therapeutic DNA immunisation or vaccinia vector vaccination, respectively, enhanced control of LCMV, suggesting concurrent blockade of both might be beneficial [48]. Combining IL-2 administration and PD-L1 blockade in the mouse model of LCMV reduced viral load and increased virus-specific CD8 T-cell responses [49], again lending weight to the idea of synergistic approach. Longer term follow up would show whether or not these improvements are maintained. As we strive towards a unified consensus regarding combinatorial strategies [50], optimal timing and dosing regimens, given the antigen loss post-cART [3], and as the field moves toward a curative approach [10], the focus of prospective immunotherapy will not only be to induce and maintain increased T-cell functionality, improve immunophenotypes and confer clinical benefit, but also to target the latent viral reservoir. Such successful immune-based therapy may require different step-wise novel approaches and larger patient cohorts.

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358

359 **Conflict of interest statement**

360 The authors declare that the research was conducted in the absence of any commercial or financial
361 relationships that could be construed as potential conflict of interest.

362

363 **Fig. 1.** Treatment schedule overview. Patients were eligible to participate in the study if they were over 18
364 years of age, chronically infected with HIV-1, on a stable cART regimen for ≥ 6 months, if they had a current
365 CD4 T-cell count of >400 cells/mm³, and a plasma viral load of <50 copies/ml. Subjects could not be
366 receiving or have received immunomodulatory drugs or immunisation. Twelve patients who met the
367 eligibility criteria were randomised into one of three arms of the trial: i) to receive the GTU®-MultiHIV
368 DNA Clade B vaccine (FIT Biotech Plc, Tampere, Finland) at baseline, followed by administration of IL-2
369 (Aldesleukin, Proleukin; Novartis, Camberley, UK) and GM-CSF (Sargramostim, Leukine™; Berlex,
370 Seattle, WA) for five days during week 1, and rhGH (Somatropin, Saizen™; Merck-Serono International,
371 Geneva, Switzerland) for five days during week 2, with vaccine further administered at weeks 6 and 12
372 (n=3); ii) to receive vaccine alone (n=4); or iii) to receive cytokines and rhGH alone (n=5), all at the
373 aforementioned time points. Vaccine was administered at 1mg/ml as 10 intradermal injections (five 100µl
374 injections per arm); IL-2 given twice daily, 5×10^6 Units, administered by subcutaneous injection, 8 hours
375 apart; GM-CSF, 150µg, was administered subcutaneously once daily, 4 hours from the IL-2; and rhGH self-
376 administered subcutaneously daily at 4mg/day. Blood was drawn at two screen visits, at baseline (week 0)
377 and at weeks 1, 2, 4, 6, 8, 12, 24 and 48. Throughout the period of the study, routine analysis of serum
378 glucose along with chemical gauges of urinary and endocrine function (sodium, potassium, chloride, urea,
379 creatinine), liver function (bilirubin, ALT, AST, alkaline phosphatase), thyroid function (Free T4, TSH),
380 lipids (cholesterol, triglycerides, LDL, HDL), and other metabolic indicators (total protein, albumin,
381 globulin, calcium, phosphate, amylase) was undertaken. It is notable that prior to beginning the study the
382 majority of patients had levels of serum triglycerides above normal range (<2.0 mmol/L). As patients had

383 well controlled HIV-1 infection, elevations in the levels of triglycerides could either be genetic and/or
384 cART-related.

385 **Fig. 2.** CD4 and CD8 T-cell dynamics following immunotherapy. CD4 (A) and CD8 (B) T-cell counts and
386 CD4/CD8 T-cell ratios (C) are depicted for each of the treatment groups: group 1 in blue circles, group 2 in
387 green squares, and group 3 in red triangles. Mean change from baseline to the study time point (week) is
388 plotted in each instance, with error bars displaying the 95% confidence interval. Asterisks indicate statistical
389 significance, where $p \leq 0.05$ (as detailed in materials and methods, section 2.8).

390 **Fig. 3.** Impact of immunotherapy on T-cell function and phenotype. Plots show mean change from baseline
391 to each of the study time points with the error bars representing the 95% confidence interval. PBMC were
392 stimulated with overlapping Gag p17/p24 peptides (FIT Biotech); IFN- γ (A) and IL-2 (B) production was
393 determined by calculating the number of spot forming cells (SFC) per million PBMC. Surface expression of
394 the activation marker CD38 was assessed on both CD4 (C) and CD8 (D) T cells, as was the percentage
395 expression of the exhaustion marker PD-1 on both T-cell subsets (E and F, respectively). The mean
396 fluorescence intensity (MFI) of the apoptosis marker CD95 was also evaluated on CD4 (G) and CD8 (H) T
397 cells. This was done for each study time point, and the treatment groups are as previous: group 1 in blue
398 circles, group 2 in green squares, and group 3 in red triangles. Asterisks indicate statistical significance,
399 where $p \leq 0.05$.

400 **Fig. 4.** Individual patient changes in T-cell function and phenotype. Representative plots show IFN- γ (light
401 grey bar) and IL-2 (dark grey bar) production in response to Gag p17/p24 peptide stimulation throughout the
402 course of the study, and the simultaneous alterations in CD4 and CD8 T-cell activation (in terms of CD38
403 expression; light blue circles and dashed line, and dark blue circles and solid line, respectively) for
404 individuals in group 1: G739 (A) and B784 (B). For comparison, a representative plot from a subject in
405 group 2 (C319) is also shown (C), but here CD4 activation is shown in light green squares and dashed lines,
406 and CD8 activation in dark green squares and solid lines. The right y-axis corresponds to the functional
407 response and changes in spot forming cells (SFC) compared to baseline are shown. The left y-axis
408 corresponds to changes in percentage CD38 expression at each of the study time points compared to
409 baseline.

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