

1 **Preferential Targeting of Conserved Gag Regions after Vaccination with**
2 **a Heterologous DNA prime - Modified Vaccinia Ankara (MVA) boost**
3 **HIV-1 vaccine regimen**

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39 **Abstract: 250 words**

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41 **Abstract**

42 Prime-boost vaccination strategies against HIV-1 often include multiple
43 variants for a given immunogen for better coverage of the extensive
44 viral diversity. To study the immunologic effects of this approach, we
45 characterized breadth, phenotype, function and specificity of Gag-
46 specific T cells induced by a DNA-prime Modified Vaccinia Ankara
47 (MVA)-boost vaccination strategy, which uses mismatched Gag
48 immunogens in the TamoVac 01 phase IIa trial. Healthy Tanzanian
49 volunteers received three injections of the DNA-SMI vaccine encoding
50 for a subtype B and AB-recombinant Gag_{p37} and two vaccinations with
51 MVA-CMDR encoding subtype A Gag_{p55}. Gag-specific T-cell responses
52 were studied in 42 vaccinees using fresh peripheral blood mononuclear
53 cells. After the first MVA-CMDR boost, vaccine-induced IFN- γ ⁺ Gag-
54 specific T cell responses were dominated by CD4⁺ T cells (compared to
55 CD8⁺ T cells, $p < 0.001$) that co-expressed IL-2 (66.4%) and/or
56 TNF α (63.7%). A median of 3 antigenic regions were targeted with a
57 higher median response magnitude to Gag_{p24} regions - more
58 conserved between prime and boost - as compared to regions within
59 Gag_{p15} (not primed) and Gag_{p17} (less conserved, both $p < 0.0001$). Four
60 regions within Gag_{p24} were each targeted by 45% to 74% of vaccinees
61 upon restimulation with DNA-SMI-Gag matched peptides. The response
62 rate to individual antigenic regions correlated with the sequence
63 homology between the MVA and DNA Gag encoded immunogens

64 (p=0.04, r²=0.47). In summary, after the first MVA-CMDR boost, the
65 sequence-mismatched DNA-prime MVA-boost vaccine strategy
66 induced a Gag-specific T cell response that was dominated by
67 polyfunctional CD4⁺ T cells and that targeted multiple antigenic
68 regions within the conserved Gag_{p24} Protein.

69

70 **Importance**

71 Genetic diversity is a major challenge for the design of vaccines
72 against variable viruses. While including multiple variants for a given
73 immunogen in prime-boost vaccination strategies is one approach that
74 aims to improve coverage for global virus variants, the immunologic
75 consequences of this strategy have been poorly defined so far. It is
76 unclear whether inclusion of multiple variants in prime-boost
77 vaccination strategies improves recognition of variant viruses by T cells
78 and by which mechanisms this would be achieved; either by improved
79 cross-recognition of multiple variants for a given antigenic region or
80 rather through preferential targeting of antigenic regions more
81 conserved between prime and boost. Engineering vaccines to induce
82 adaptive immune responses that preferentially target conserved
83 antigenic regions of viral vulnerability might facilitate better immune
84 control after preventive and therapeutic vaccination for HIV and for
85 other variable viruses.

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5

88 **Introduction**

89 High antigenic variability of common viruses causing either chronic
90 (e.g. Human Immunodeficiency Virus (HIV), Hepatitis C Virus (HCV)) or
91 acute (e.g. Influenza Virus, Dengue Virus) disease complicates the
92 design of efficacious vaccines. Vaccines against such variable viruses
93 should optimally induce adaptive immune responses that target all
94 variants of a vulnerable antigenic region to prevent infection or, if that
95 cannot be achieved, at least facilitate immune control of viral
96 replication to prevent disease progression. All virus proteomes, even
97 the most variable ones, contain conserved regions, where functional
98 constraints limit extensive sequence variability. Designing vaccines to
99 focus immune recognition towards such conserved regions could be a
100 viable strategy to improve vaccine efficacy against variable
101 pathogens (1, 2).

102 HIV is a good example of a highly variable virus. It causes persistent
103 infection and rapidly escapes the HIV envelope (Env)-specific antibody
104 response. Extensive sequence variability and glycosylation of the Env
105 protein complicate Env based vaccine design (3, 4). In contrast,
106 sequence variability within the group-specific antigen (Gag) is more
107 limited due to functional constraints, but still differs between the capsid
108 protein p24, the matrix protein p17 and proteins of the p15-encoding
109 region (nucleocapsid and virion assembly proteins). An extensive body
110 of evidence supports the concept that vaccine-induction of Gag-
6

111 specific T-cell responses could be beneficial; Gag-specific T-cell
112 responses are linked to viral control during chronic HIV infection (5-10).
113 The breadth of Gag-recognition by CD8⁺ T-cells is associated with
114 better viral control and slower disease progression (9, 10). Furthermore,
115 the protective mechanism mediated by HLA class I alleles has been
116 linked to CD8⁺ T-cell recognition of defined Gag regions (10, 11).
117 Similarly, Gag-specific CD4⁺ T-cell responses appear to contribute to
118 viral control, sharing similar features with the CD8⁺ T-cell response (12-
119 14). In rhesus monkeys, vaccine induced Gag-specific T-cell responses
120 correlate with post-challenge immune control and prolonged survival
121 after SIV challenge (15). Together, these data support the concept that
122 induction of strong and broad Gag-specific T-cell responses targeting
123 common sequence variants could potentially improve HIV vaccine
124 efficacy against diverse HIV variants.

125 The multi-subtype TaMoVac DNA/MVA regimen used in the TaMoVac
126 01 phase IIa trial induced strong Gag-specific T-cell responses (16, 17).
127 This offered the opportunity to address the hypothesis that delivery of
128 non-identical, but related immunogens preferentially induces T cell
129 responses to antigenic regions more conserved between the
130 immunogens. We found that the sequence mismatched TaMoVac01
131 DNA-MVA vaccination induced broad recognition of conserved
132 antigenic regions within Gag_{p24}.

133

134 **Results**

135 **Relative conservation of Gag p24 in a mixed subtype epidemic**

136 In order to define the degree of conservation within Mbeyan HIV Gag
137 sequences, we analyzed previously published sequences and
138 determined the distribution of subtypes and recombinant forms (RF).
139 Pure subtype C sequences were found most frequently (57.1%)
140 followed by unique subtype A-containing Recombinant Forms (RFs)
141 (20.9%), pure subtype A Gag sequences (19.8%) and CD RFs (2.2%,
142 Figure 1). In order to estimate the variability at each Gag amino acid
143 position, the Shannon entropy score, was then calculated (Fig. 1b).
144 Gag p24 showed the highest conservation with 82.6% of aa positions
145 with an entropy score below 0.5 compared to the less conserved p17
146 (55.7%) and the least conserved p15 (44.8%). Similarly, the median
147 Shannon entropy score was lowest for p24 (0.14), followed by p17 (0.37)
148 and p15 (0.49).

149

150 **Preferential induction of T-cells targeting Gag_{p24} protein by sequence**
151 **mismatched DNA/MVA vaccination**

152 The DNA vaccine included two plasmids encoding for SMI-Gag_{p37} with
153 an identical p17 subtype B sequence linked to subtype B or subtype A
154 p24 sequences (Figure 2). The MVA-CMDR-Gag_{p55} boost encoded for a

155 subtype A Gag_{p55} and included the p15 region. Within the p17 region,
156 21.2% (28 of 132) of aa positions were mismatched between the DNA-
157 SMI prime and MVA-CMDR boost. The Gag_{p24} subtype A and subtype B
158 sequences included in the DNA-SMI prime differed from the MVA-
159 CMDR boost in 7.8% (18 mismatches in 231) and 11.7% (27 mismatches
160 in 231) of aa positions, respectively. The p15 region represented a 100%
161 mismatch to the DNA-SMI prime.

162

163 IFN- γ + Gag-specific T cell responses were not detected during the pre-
164 vaccination visit upon MVA-CMDR-Gag_{p55} restimulation, but were
165 present in 15% of vaccinees upon DNA-SMI-Gag_{p37} restimulation (range
166 60-165 SFC/10⁶ PBMC, data not shown). Gag-specific T cell numbers
167 already peaked after the first MVA-CMDR boost with a median of 228
168 SFC/10⁶ PBMC and significantly lower numbers of Gag-specific T cells
169 after the second boost ($p < 0.05$ for both peptide pools). Magnitude of T
170 cell responses against the control peptide pools CEF (median 83 and
171 90 SFC/10⁶ PBMC, $p = 0.58$) and CMVpp65 (median 1243 and 938
172 SFC/10⁶ PBMC, $p = 0.2$) were comparable for both visits.

173 Because vaccine-induced IFN- γ + Gag-specific T cell responses peaked
174 after the first MVA-CMDR boost, we focus on this time point in the
175 subsequent analyses. ICS data from 41 vaccine recipients were eligible
176 for further analyses. IFN- γ + Gag-specific CD4 and CD8⁺ T cell responses

177 were detected in 30 and 14 of 41 subjects, respectively after
178 restimulation with the MVA-CMDR-Gag_{p55} peptide pool (Fisher`s Exact
179 test; p=0.0008). Likewise, significantly higher frequencies of CD4⁺ as
180 compared to CD8⁺ IFN- γ ⁺ Gag-specific T cells were detected after re-
181 stimulation with either the MVA-CMDR-Gag_{p55} (median; 0.04% versus
182 0.01%; p<0.0001, figure 3A) or the DNA-SMI-Gag_{p37} (median; 0.06% v
183 0.01% p=0.0002) peptide pool after the first MVA-CMDR boost. IFN- γ ⁺
184 Gag-specific CD4 T cells frequently co-expressed IL-2 (66.4%, mean of
185 both Gag peptide pools) or TNF- α (63.7%) with 49.9% co-expressing IL-2
186 and TNF- α and high concordance between the two tested Gag
187 peptide pools (Figure 3B). The CCR5-ligand Mip-1 β and degranulation
188 marker CD107 were co-expressed by only 20% (CMDR-Gag_{p55}: 13%)
189 and 11% (CMDR-Gag_{p55}: 7%) of IFN- γ ⁺ Gag-specific CD4⁺ T cells,
190 respectively. By contrast high frequencies of IFN- γ ⁺ CD4⁺ T cells co-
191 expressed Mip-1 β (59%) and/or CD107 (53%) after re-stimulation with
192 CMV_{pp65} peptides, including a significant proportion (16%) of
193 polyfunctional cells expressing all assessed functional markers, which is
194 concordant with previous reports (18). Almost 75% of IFN- γ ⁺ Gag-
195 specific CD8 T cells co-expressed Mip-1 β and almost 50% were CD107⁺
196 indicating degranulation of cytotoxic granula.

197

198 Breadth and specificity of Gag-specific T cell recognition were
199 determined in 42 vaccine recipients using the IFN- γ -ELISPOT on freshly
10

200 isolated PBMCs, after re-stimulation with linear consecutive peptide
201 pools matching for MVA-CMDR-Gag_{p55} (n=9 pools) and DNA-SMI-
202 Gag_{p37} (n=7 pools). Median breadth of Gag recognition was 3 peptide
203 pools (range 0 - 8, Fig. 3c). The majority of the 42 vaccine recipients
204 responded to at least one DNA-SMI-Gag_{p37} (n=36, 85.7%) or MVA-
205 CMDR-Gag_{p55} peptide pool (n=33, 78.6%). In order to compare
206 magnitude of T cell responses against the different Gag regions and
207 account for sequence lengths of p17, p24, and p15, we calculated
208 average SFC values as per 15_{mer} peptide for each Gag region.
209 Antigenic regions within p24 were recognized at a higher median
210 magnitude (14.55 SFC/10⁶ PBMC/15mer_peptide) compared to those
211 in the more variable p17 and p15 regions (both 6.52 SFC/10⁶
212 PBMC/15mer_peptide, p<0.0001, Fig. 4a). The magnitude of response
213 to p17 (median of 6.52 SFC/10⁶ PBMC/15mer_peptide for MVA-CMDR
214 and 6.0 SFC/10⁶ PBMC/15mer_peptide for DNA-SMI) was similar to the
215 response to p15 (p=0.95).

216 Ten of the 42 vaccine recipients (23.8%) mounted SMI Gag_{p17} specific T-
217 cell responses and 36 volunteers (85.7%) responded to SMI-Gag_{p24}.
218 Eight vaccine recipients (19.0%) responded to CMDR-Gag_{p17}, 32
219 (76.2%) to CMDR-Gag_{p24}, and 8 (19.0%) to CMDR-Gag_{p15}. Recognition
220 of individual antigenic regions within Gag_{p24} was comparable between
221 DNA and MVA encoded variants (Fig. 4b). Pools 5 (HxB aa 225-290)
222 and 6 (aa 275-347) were immunodominant with 57.1% (CMDR-Gag

223 peptides: 47.6%) and 73.8% (CMDR-Gag peptides: 57.1%) responders
224 after restimulation with the SMI Gag peptides, respectively. Pool 5 was
225 targeted with a median magnitude of 38.65 SFC/10⁶ PBMCs/15mer (SMI
226 Gag peptides) and 35.19 SFC/10⁶ PBMCs/15mer (CMDR-Gag
227 peptides), respectively. Pool 6 was targeted with a median magnitude
228 27.74 SFC/10⁶ PBMCs/15mer (SMI-Gag peptides) and 29.79 SFC/10⁶
229 PBMCs/15mer (CMDR-Gag peptides).

230 Next we mapped individual MVA-CMDR-Gag_{p55} peptide responses
231 (Figure 4c) in 39 vaccine recipients using cryopreserved PBMC. Results
232 from 3 vaccine recipients were excluded because of invalid ELISpot
233 results. Positive peptide-specific ELISpot responses were detected in 23
234 of 39 (60%) vaccine recipients. The 23 responders almost exclusively
235 recognized peptides located within the p24 region. Four individual
236 peptide responses were detected in p17 and two within p15. When
237 counting responses to consecutive peptides as a single antigenic
238 region, these 23 responders recognized a mean of 4 antigenic regions -
239 ranging from one (26% of subjects (6/23)) to 12 antigenic regions
240 recognized (4.3% (1/23)). The peptide YVDRFYKTLRAEQAT (pool 6, HxB
241 position aa 296-310) was the most frequently recognized with 39.13% (9
242 of 23) responders, followed by the peptide YKRWILGLNKIVRMY (pool 5,
243 aa 262-277, 30.43% responders). Four peptides within p24 were
244 recognized with an equal frequency of 26.09%.; GATPQDLNMMLNIVGG
245 (pool 4, aa 178-193), IAGTTSTLQEQIGWMT (pool 5, aa 236-251),
12

246 ILGLNKIVRMYPVSI(9) (pool 5, aa 267-282) and WMTETLLVQNaNPDCK
247 (pool 6, aa 316-331). As shown in Figure 4d, the six most frequently
248 recognized antigenic regions had only 0 or 1 aa difference between
249 the SMI-DNA-prime encoded Gag_{p37} sequences (DNA B or BA) and the
250 MVA-CMDR boost encoded Gag_{p55} sequences. These data indicate
251 that hotspots of the vaccine-induced Gag recognition by T cells were
252 exclusively located within p24, whereas recognition of antigenic regions
253 within p17 and p15 appeared attenuated.

254

255 We next compared the frequency of responders to a given antigenic
256 region with the region-specific amino acid mismatches between the
257 DNA- and the MVA-encoded Gag sequences using linear regression
258 analysis (Figure 5). Plotting the frequency of responders against the
259 sequence heterogeneity between the encoded aa sequence of the
260 DNA-SMI-Gag_{p37} prime and the MVA-CMDR-Gag_{p55} boost for each
261 linear peptide pool, we found a linear correlation between region-
262 specific aa mismatches and the frequency of responders to a given
263 antigenic region ($r^2=0.69$, $p=0.04$ (DNA-SMI-Gag_{p37}), Figure 5a) and
264 $r^2=0.45$, $p=0.07$ (MVA-CMDR-Gag_{p55}), suggesting that higher levels of
265 sequence conservation between DNA-SMI-Gag_{p37} prime and MVA-
266 CMDR-Gag_{p55} boost contribute to higher frequencies of recognition
267 within more conserved Gag regions.

268 The link between the level of sequence conservation within a given
269 antigenic region and its recognition could further be substantiated by
270 linear regression analysis. The number of aa mismatches between
271 priming DNA Gag_{p24} encoded subtype B sequence and boosting Gag
272 aa sequences (MVA) correlated with the frequency of responders
273 (Figure 5b, $r^2=0.01$ and $p=0.0332$) and the magnitude of response
274 (Figure 5c, $p=0.04$, $r^2=0.04$) to a given Gag_{p24} peptide. These data
275 support the concept that the sequence heterologous prime-boost
276 vaccination strategy applied during TaMoVac 01 contributed to
277 preferential recognition of the more conserved antigenic regions within
278 Gag.

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280

281

282 **Discussion**

283 Genetic diversity poses a major challenge to the design of efficacious
284 vaccines against HIV-1 and other variable viruses. Several vaccination
285 strategies have been tested to address this extensive diversity of HIV-1
286 (2). The TaMoVac 01 study incorporated multiple variants of the same
287 immunogen in the vaccine formulation (16, 17, 19). The immunologic
288 consequences of such vaccination strategies incorporating multiple
289 variants and in particular the impact on the pattern of antigen
290 recognition - have only been poorly defined so far. Inclusion of three
291 immunogen variants elicited strong Gag-specific T-cell responses in the
292 TaMoVac 01 and HIVIS studies (16, 17) providing the opportunity to
293 study parameters that potentially influence the immunodominance
294 pattern of vaccine-induced Gag recognition by T cells. The analysis of
295 91 Gag sequences confirmed previous reports that the capsid antigen
296 p24 shows the highest degree of conservation followed by the less
297 conserved p17 and p15 (20).

298

299 In TaMoVac 01 vaccinees, antigenic regions within conserved Gag_{p24}
300 regions were preferentially targeted after the first MVA-CMDR boost. A
301 comparative analysis of HIV-1 rAd-5 T-cell based vaccine clinical trials
302 Merck16, HVTN 054 and HVTN 502/Step, which included closely related
303 Gag immunogen sequences and did not include DNA priming, found

304 considerable variation in the recognition of Gag regions (21). HVTN 054
305 showed an accumulation of hotspots within Gag_{p24}, whereas hotspots
306 of T cell recognition were more evenly distributed within Gag for
307 Merck16 and HVTN 502/Step. In comparison, the sequence
308 mismatched DNA/MVA TaMoVac vaccine “focused” T-cell responses
309 even more on highly conserved Gag_{p24} regions. The strength of
310 induced T cell responses against individual antigenic regions in Gag_{p24}
311 correlated with the degree of conservation between the SMI-DNA
312 encoded subtype B and MVA-CMDR encoded subtype A sequence
313 variants. While no such correlation was detected for the DNA encoded
314 subtype A variant, the six most frequently targeted peptides showed
315 only none or one aa mismatch when taking both DNA encoded
316 Gag_{p24} variants into account. Hence, immune recognition of peptides
317 with high degree of variability between MVA-CMDR and DNA-Gag_{p37.1}
318 (subtype A, Figure 2) were probably primed by the other DNA- Gag_{p37}
319 variant (subtype B).

320 What is the mechanism underlying this preferential targeting of more
321 conserved antigenic regions within Gag_{p24} in TaMoVac01 vaccinees?
322 One possibility is that induction of T-cell responses towards more
323 variable epitopes - more common in Gag_{p17} and Gag_{p15} - was
324 negatively affected by increasing numbers of mismatched amino acid
325 positions within a given epitope. Immunodominant Gag-specific CD8⁺ T-
326 cell populations targeting the epitope variants TL9M7 (TPQDLNMML)

327 and TL9T7 (TPQDLNNTML) during natural HIV subtype A and subtype C
328 infections are completely different in their clonotypic composition(22).
329 It is therefore plausible that certain T-cell clonotypes induced by one
330 immunogen sequence cannot be boosted by certain other sequence
331 variants or that boosting is suboptimal; however, if T-cell clonotypes
332 partially overlap in their recognition of the two epitope variants, these
333 cross-reactive clonotypes would be boosted stronger, resulting in
334 improved variant cross-recognition and "focusing" towards such cross-
335 reactive clonotypes. Our data show that recognition of epitopes with
336 more than 2 mismatches is completely abrogated (Figure 5 C, D). A
337 high number of epitope variant mismatches is therefore likely to abolish
338 any efficient boosting. Of note, based on our analyses of 91 Mbeyan
339 sequences, the tested CMDR-Gag sequence variants for the peptides
340 P1 to P6 always closely matched the most frequent Mbeyan variants
341 with ≤ 2 amino acid substitutions. In summary a variety of mechanisms
342 probably contributed to preferential T cell recognition of more
343 conserved Gag regions in the TaMoVac01 study using mismatched
344 immunogen sequences.

345

346 All immunodominant regions recognized by the TaMoVac 01
347 vaccinees have also been identified in previous studies. P1
348 (YVDRFYKTLRAEQAT) is an immunodominant target for CD4⁺ and CD8⁺
349 T-cells during early and chronic infection (14, 23, 24) and was also a
17

350 hotspot in the HVTN502/Step and HVTN 054 trials (21). P2
351 (YKRWIILGLNKIVRMY) is frequently targeted by CD4⁺ T cells during early
352 and chronic HIV infection (14) and was a hotspot in the Step trial
353 (21)(39). P3 (GATPQDLNMMLNIVGG) contains the highly
354 immunodominant B42/B81 restricted TL9 epitope (22, 24-26). P4 to P6
355 also contained previously described epitopes (14, 23). Inherent
356 immunogenicity of these peptide sequences P1-P6 could also
357 contribute to their preferential recognition as these were also
358 frequently recognized by T cells in natural infection or in other trials.

359

360 Most of individual peptide-specific T cell responses after the first MVA-
361 CMDR boost were most likely mediated by CD4⁺ compared to CD8⁺ T
362 cells, even though we were not able to phenotype individual vaccine-
363 induced peptide-specific T-cell responses from cryopreserved PBMC.
364 After the second MVA-CMDR boost, 73% (32 of 44) of vaccinees had
365 higher frequencies of Gag-specific IFN- γ ⁺ CD8⁺ T-cell as compared to
366 after the first MVA boost, suggesting that optimal induction of Gag-
367 specific CD8 T cell responses probably need a second MVA boost.

368 One limitation of this study is that cryopreservation negatively affected
369 our ability to detect vaccine-induced T cell responses. We did not
370 observe this phenomenon during previous studies for natural HIV- or

371 MTB-specific T-cell responses(22, 27). However, this phenomenon is not
372 unknown (28).

373

374 In conclusion, our results show that after one MVA-CMDR boost the
375 sequence mismatched TaMoVac 01 DNA prime/MVA boost vaccine
376 regimen induced Gag specific T-cell responses that were dominated
377 by CD4⁺ T cells co-expressing IL-2 and TNF- α and targeted multiple
378 conserved antigenic regions within Gag_{p24}.

379

380 **Methods**

381 **Study Design and samples**

382 The TaMoVac 01 phase 2a trial was described in detail, previously (16).
383 Briefly, this randomized controlled trial was performed at Muhimbili
384 University of Health and Allied Science (MUHAS) and the National
385 Institute for Medical Research -Mbeya Medical Research Center (NIMR-
386 MMRC) in Mbeya, Tanzania with a total of 120 healthy, HIV-negative
387 individuals, aged 18-40 years. Immunosuppressive medications were
388 exclusion criteria. The TaMoVac 01 trial participants received two or
389 five intradermal (ID) injections of DNA/Placebo at weeks 0, 4 and 12
390 and were boosted with two MVA-CMDR/Placebo intramuscular (IM)
391 injections at weeks 30 and 46. The DNA/Placebo was administered
392 intradermally in the skin over both deltoids by using a Biojector 2000®
393 needleless device (Bioject Medical Technologies, Inc., Tualatin, OR,
394 USA), the MVA injections were administered into the left deltoid muscle.

395 The DNA-SMI vaccine (Vecura, Huddinge, Stockholm Sweden) was
396 composed of 7 plasmids encoding the HIV-1 genes Env (subtype A, B
397 and C respectively), Rev clade B, Gag (subtype A and A/B) and
398 reverse transcriptase subtype B (for details see (29)) and was
399 administered at either 600µg or 1000µg total. The recombinant
400 modified vaccinia virus Ankara (MVA) expressing HIV-1 gp150 clade E
401 as well as gag and pol clade A (MVA-CMDR) was manufactured by

402 Walter Reed Army Institute of Research (WRAIR) (30) and administered
403 at 10^8 pfu.

404

405 The study was reviewed and approved by the National Ethics
406 Committee, the Institutional Review Board (IRB) at the NIMR-MMRC and
407 the MUHAS IRB in compliance with national guidelines and institutional
408 policies (Clinical Trials Registration: ATM2010050002122368), and
409 informed consent was obtained in accordance with the Declaration of
410 Helsinki.

411 In the present study, samples collected at two weeks after the first
412 MVA-CMDR vaccination - when vaccine-induced T cell responses
413 peaked - from 42 vaccine recipients at the National Institute for
414 Medical Research – Mbeya Medical Research Center and who did not
415 become HIV infected during the trial were used for analyses.

416

417 **HIV genetic sequence analyses**

418 HIV-1 Gag sequences included in the phylogenetic analyses were from
419 91 HIV-positive subjects from the Mbeya region and have the
420 Genebank accession numbers FJ853501 to FJ85359 (22). The subtypes
421 and recombinant forms were determined using the jpHMM-HIV tool
422 (http://jphmm.gobics.de/submission_hiv).

423

424 Peptide antigen and peptide pool design

425 **DNA-SMI-Gag_{p37} and MVA-CMDR-Gag_{p55} peptide sets consisted of 15-**
426 **to 18-mer peptides overlapping by 11 amino acids had a purity of**
427 **>80% (JPT peptide technologies, Berlin). Individual peptide variants of**
428 **identical length for a given Gag region were used to allow for direct**
429 **comparison of T-cell responses targeting MVA or DNA-encoded**
430 **peptide variants and to prevent artifacts with linked to intrapeptide**
431 **epitope location (24, 31). Peptide maxipools including all peptides for**
432 **the DNA-SMI-Gag_{p37} and MVA-CMDR-Gag_{p55} immunogens were used**
433 **to study phenotypic and functional characteristics of vaccine-induced**
434 **Gag-specific T cell responses; Linear peptide pools were used to test T-**
435 **cell responses against distinct antigenic regions (Figure 2); nine pools**
436 **for MVA-CMDR-Gag_{p55} and 7 pools for DNA-SMI-Gag_{p37}.**

437

438 Intracellular cytokine staining (ICS)

439 A 8-colour ICS assay was performed on fresh PBMC were stimulated in
440 the presence of Brefeldin A (5(g/ml Sigma-Aldrich) for 6 hours with
441 either MVA-CMDR-Gag_{p55} or DNA-SMI-Gag_{p37} maxipools
442 (1(g/ml/peptide), nothing (negative control) or the control antigens
443 (CMV_{pp65} peptide pool (0.5(g/ml/peptide) and Staphylococcus

22

444 enterococcus Toxin B (1(g/ml, Sigma-Aldrich). Stimulated PBMCs were
445 then stained with α -CD3 APC-Cy7 (BD Biosciences Europe,
446 Erembodegem, Belgium), α -CD4 PerCp-Cy5.5 (ebioscience, San Diego,
447 CA), α -CD8 V500 (BD Bioscience), α -TNF- α Pe-Cy7 (BD), α -IFN- γ V450
448 (BD), α -IL-2 APC (BD), α -MIP-1 β PE (BD), α -CD107 FITC (ebioscience).
449 Acquisition of samples was performed using a FACSCanto II flow
450 cytometer with acquisition-defined compensation using BD
451 CompBeads (BD). Flow Cytometry results were analysed using FlowJo
452 software, version 9.6.4 (Tree Star, Ashland, OR) and SPICE, version 5.35,
453 downloaded from <http://exon.niaid.nih.gov> (32). A minimum of 50.000
454 CD3⁺ lymphocytes were required for a sample to be included in the
455 analysis. Background reactivity, defined by using unstimulated
456 negative controls, was subtracted for analyses of antigen-specific T cell
457 responses. IFN- γ ⁺ T cell frequencies >0.025% were considered a positive
458 response.

459

460 **IFN γ ELISPOT assay**

461 The IFN- γ ELISPOT^{PLUS} kit (Mabtech, Nacka, Sweden) was used
462 according to the instructions of the manufacturer. Fresh Peripheral
463 Blood Mononuclear Cells (PBMC) were stimulated with linear peptide
464 pools matching DNA-SMI-Gag_{p37} and MVA-CMDR-Gag_{p55} (described
465 below, JPT, Berlin, Germany) (Figure 2). Frequencies of antigen-specific

466 spot-forming cells (SFC) were measured with an automated ELISPOT
467 reader (Immunospot, C.T.L., Bonn, Germany). Responses were
468 considered positive when the number of Spot Forming Cells (SFC) was
469 at least four times the medium control and >55 SFC/ 10^6 PBMCs (33).
470 Mapping of individual peptide responses was performed based on the
471 peptide pool matrix ELISPOT results and testing cryopreserved PBMC.
472 Responses of stimulated PBMCs with a SFC count higher than 2-fold the
473 unstimulated medium control and ≥ 50 SCF/ 10^6 PBMCs cells were
474 considered positive. Samples with a medium control of >60 SFC/PBMCs
475 were excluded from analyses.

476

477 **Statistical analyses**

478 Statistical analyses were performed using Prism version 6.0 (GraphPad,
479 Inc). Comparisons of two groups were performed with the Mann-
480 Whitney test. Magnitudes of CD4 and CD8 T cell responses were
481 performed with the Wilcoxon signed-rank test. Number of responders
482 with IFN- γ + CD4 and CD8 T cell responses were compared using The
483 Fisher`s Exact test. The linear relationship between the number of
484 mismatched amino acid positions within a given antigenic region and
485 the corresponding frequency of recognition and response magnitude
486 was calculated using linear regression analyses. Tests used for statistical
487 analysis are mentioned in the figure legends.

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498

499

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- 629
- 630
- 631

632 **Figure legends**

633 **Figure 1. Diversity of HIV-1 Gag protein sequences originating from the**
634 **Mbeya Region.**

635 The distribution of subtypes and unique recombinant forms of Gag
636 polyprotein sequences from 91 HIV infected subjects from the Mbeya
637 Region is shown in the pie chart (A). A Shannon Entropy Plot generated
638 from these Gag sequences is shown in (B).

639

640 **Figure 2. Immunogen sequences included in the DNA-Gag prime and**
641 **Modified Vaccinia Ankara (MVA)-Gag boost and their coverage by**
642 **peptide pools**

643 The seven DNA peptide pools covered the Gag_{p37} region consisting of
644 p17 (blue) and p24 (grey), whereas the nine MVA-Gag peptide pools
645 covered the Gag_{p55} precursor protein including the p15 region (red) in
646 addition to p17 and p24. The p15 region was only covered by MVA-
647 Gag peptide pools 8 and 9.

648

649 **Figure 3. Phenotype, function and breadth of vaccine-induced Gag-**
650 **specific T cell responses**

651 **Representative Dot Plots for the analyses of Gag-specific CD4 and CD8**
652 **T cell functions are shown in (A). Frequency of IFN- γ + CD4⁺ and CD8⁺ T**
653 **cell after stimulation of freshly isolated PBMC with whole MVA-CMDR-**
654 **Gag_{p55} (left panel) or DNA-SMI-Gag_{p37} peptide pools (right panel) in 41**
655 **vaccinees are shown in (B). Co-expression of additional functions**
656 **(TNF α , Mip-1 β , IL2 and the degranulation marker CD107) is shown in (C)**
657 **for IFN γ +CD4 (left) and IFN γ +CD8⁺ (right) T cells. The four colour coded**
658 **arcs indicate the proportion of cells co-expressing the four additional**
659 **functions. The colour-coded pies symbolize the 16 possible functional**
660 **combinations. Intracellular cytokine staining was performed using fresh**
661 **PBMC stimulated overnight with the indicated antigens as well as the**
662 **control antigens Staphylococcus enterotoxin B and CMV_{pp65}. The**
663 **number of different antigenic regions (linear peptide pools, x-axis)**
664 **recognized by TaMoVac vaccinees (n=42) is shown in (D) and was**
665 **determined using the IFN- γ ELISpot. The frequency of subjects with a**
666 **given Gag response breadth is indicated on the y-axis. Nine and 7**
667 **linear peptide pools matching MVA-CMDR-Gag_{p55} (gray bars) or DNA-**
668 **SMI-Gag_{p37} (black bars) subdivided Gag into distinct antigenic regions,**
669 **respectively. Statistical comparison in (B) was performed using**
670 **Wilcoxon-matched pairs-signed rank-test.**

671

672 **Figure 4. Recognition of Gag antigenic regions by vaccine induced T-**
673 **cells**
30

674 A comparison of the magnitude of Gag-specific T-cell responses (Y-
675 axis) targeting antigenic regions within p17, p24 and p15 normalized as
676 per 15mer peptide is shown in **(A)**. The frequency of responders is
677 shown for different Gag regions **(B)**. Vaccine-induced T-cell responses
678 were characterized using IFN- γ ELISpot in 42 participants after
679 stimulation of fresh PBMCs with 9 and 7 peptide pools matching MVA-
680 CMDR-Gag_{p55} (grey bars) and DNA-Gag_{p37} (black bars), respectively.
681 The frequency of responders for individual MVA-CMDR-Gag_{p55}
682 matched peptides is shown in **(C)** and is based on 23 subjects with at
683 least 1 detectable response against on individual peptide during fine
684 mapping using cryopreserved instead of fresh PBMC. The key data for
685 the most frequently recognized peptides are shown in **(D)**. Cut off for a
686 positive response was 2-fold the unstimulated control. Corresponding
687 Gag regions p15, p17 and p24 are indicated in **(B)** and **(C)**.

688

689 **Figure 5. Linear regression analyses between DNA-SMI-Gag_{p37} and**
690 **MVA-CMDR-Gag_{p55} sequence mismatches and induced T-cell**
691 **responses targeting specific antigenic regions.**

692 A linear regression analyses was performed to study the association of
693 immunogen-encoded amino acid mismatches and the respective T
694 cell response rate to the DNA-SMI-Gag_{p37} pools 1 to 6 **(A)**. T cell
695 responses were detected using the IFN- γ ELISPOT and freshly isolated

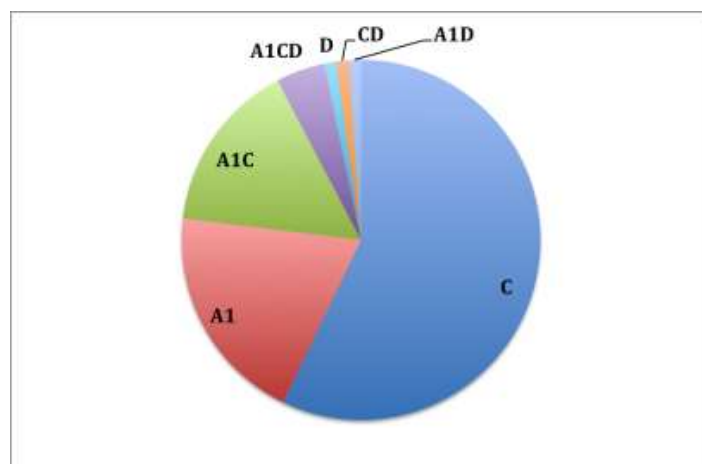
696 PBMC Pool 7 was excluded because it only contains 3 instead of 11
697 peptides. A comparison of mismatches between MVA-CMDR-Gag_{p55}
698 to the DNA-SMI-Gag_{p37B} sequence and the respective T cell response
699 rate (**B**) or the magnitude of responses (**C**) for single peptide-specific T
700 cell responses as detected using the IFN- γ ELISPOT and cryopreserved
701 PBMC.
702

703 **Figures**

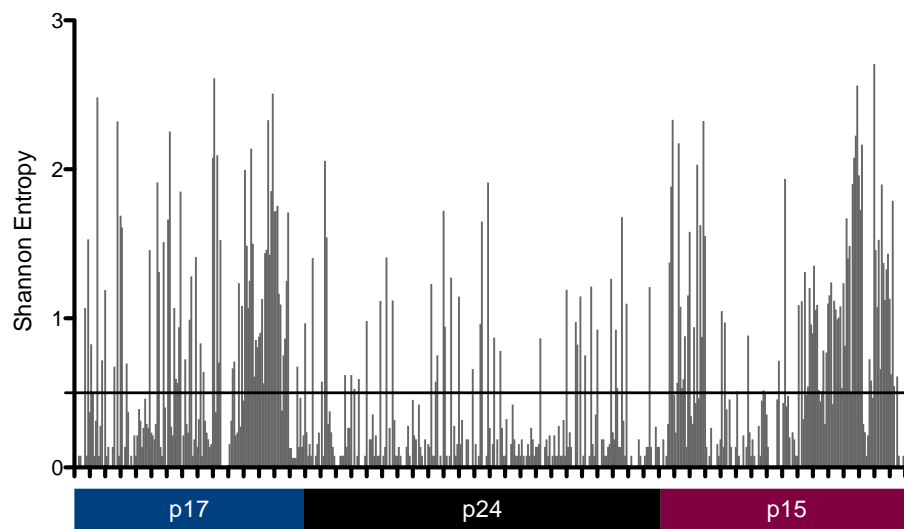
704 **Figure 1**

705

a



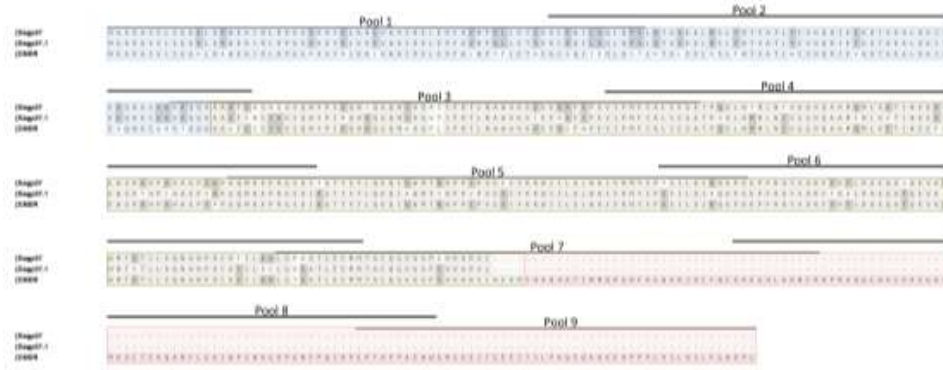
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708 **Figure 2**

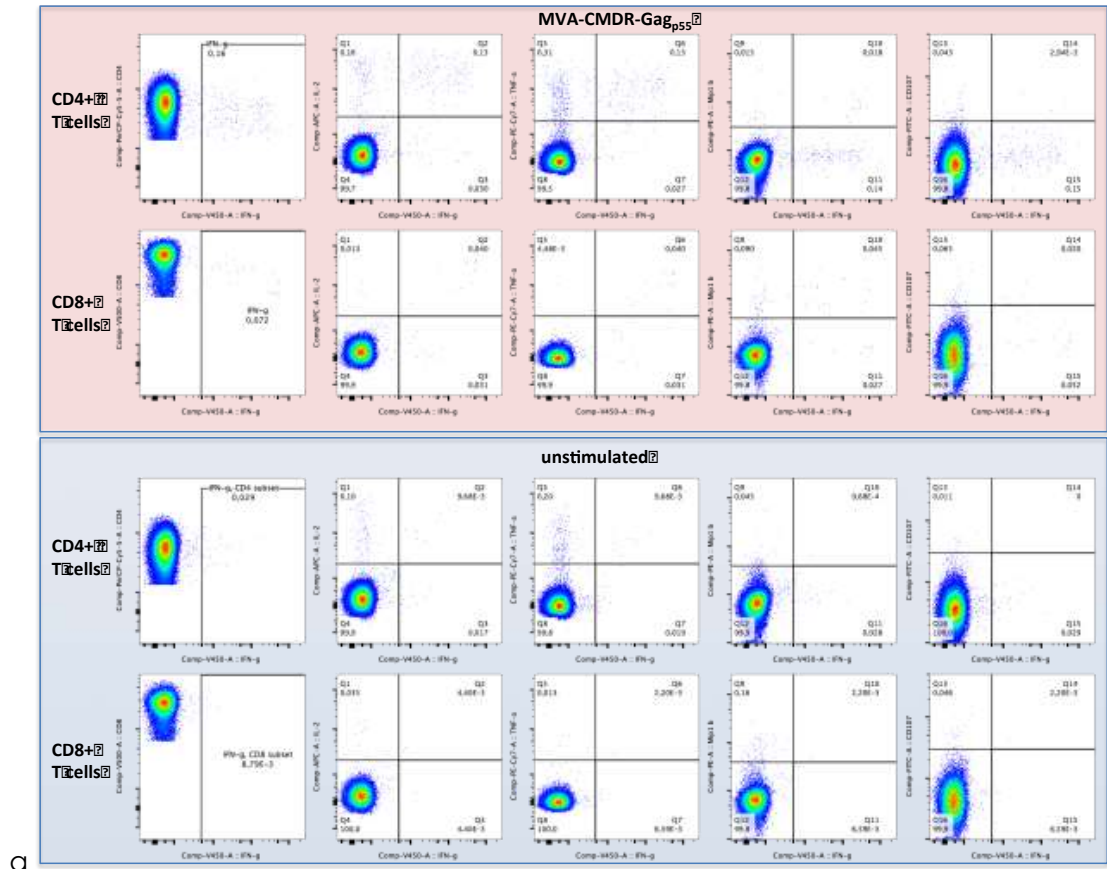
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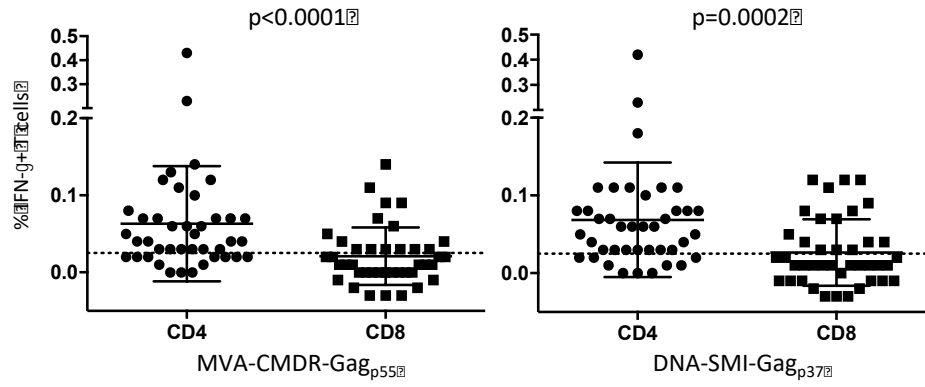
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712 **Figure 3**

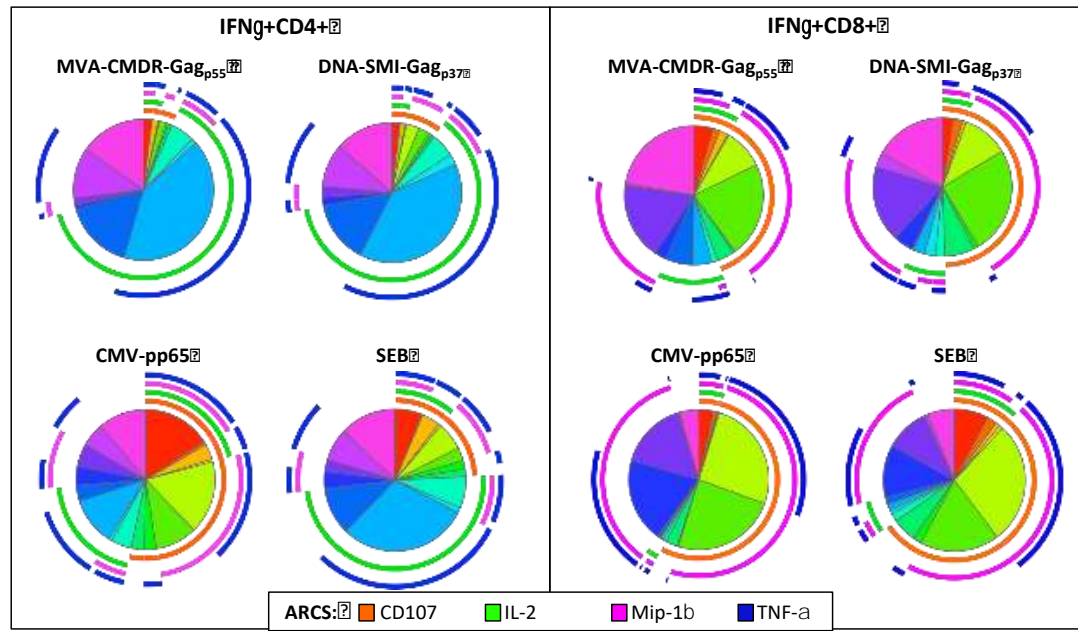


a

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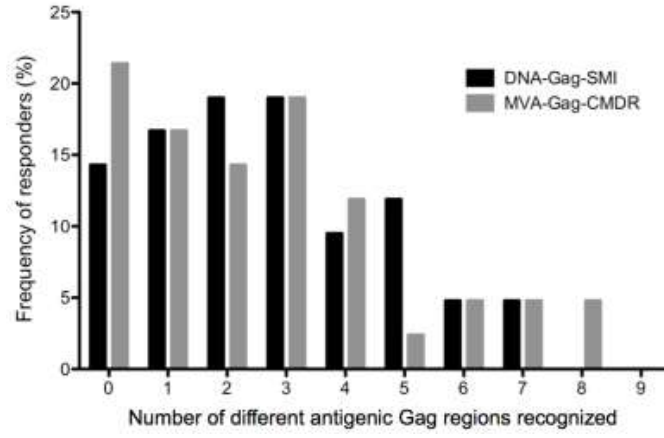


C



d

36

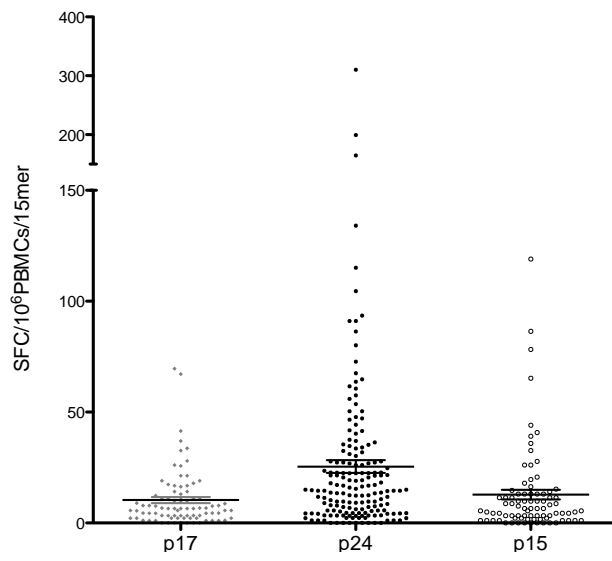


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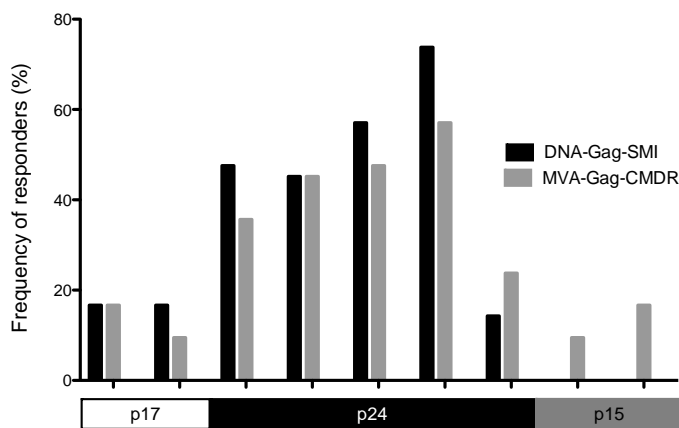
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714 **Figure 4**

a

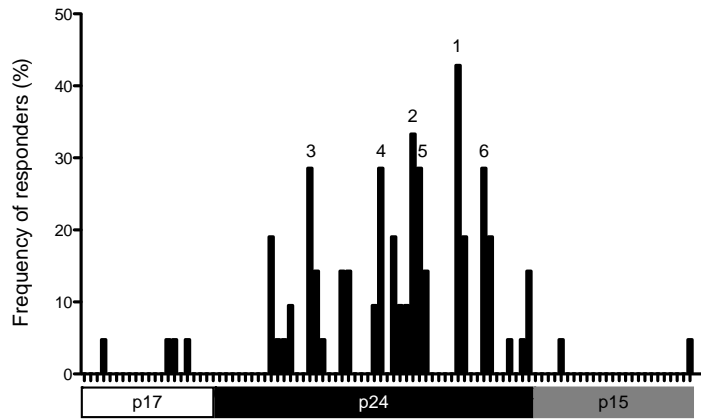


b



38

c



d

No.	Peptide sequence	HxB2 position	Frequency of recognition	aa mismatch MVA vs DNA B/DNA BA [%]
1	YVDRFYKTLRAEQAT	296-310	39.13%	6.67/13.33
2	YKRWIIILGLNKIVRMY	262-277	30.43%	0/0
3	GATPQDLNMMLNIVGG	178-193	26.09%	12.5/6.25

39

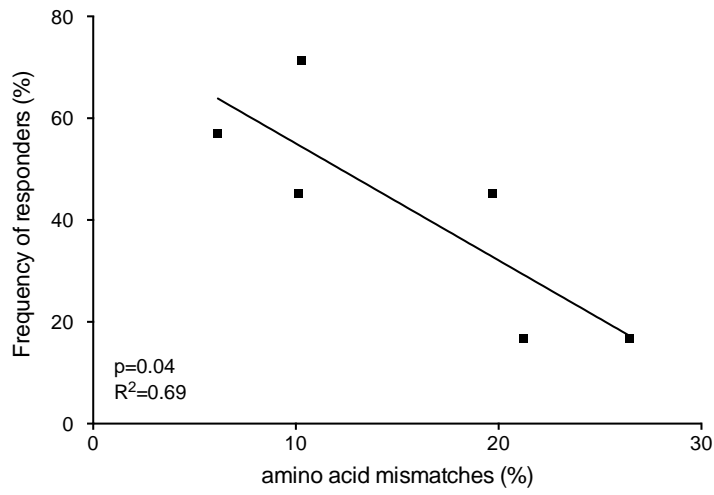
4	IAGTTSTLQEQIGWMT	236-251	26.09%	6.25/6.25
5	ILGLNKIVRMYSVSI	267-282	26.09%	6.25/0
6	WMTETLLVQNaNPDCK	316-331	26.09%	0/6.25

715

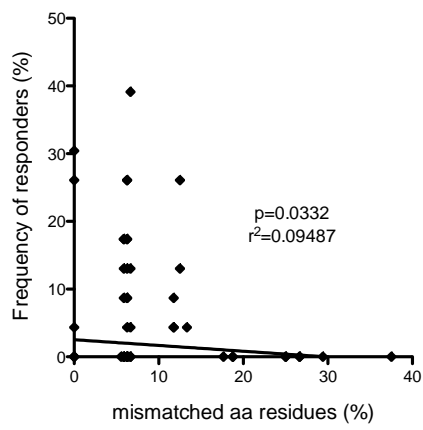
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716 **Figure 5**

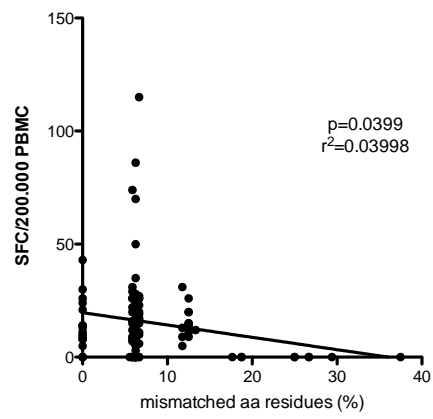
a



b



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