1 **Title: A novel vaccine targeting the viral protease cleavage sites protects Mauritian cynomolgus**
2 **macaques against vaginal SIVmac251 infection**

3

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One Sentence Summary:

The anti-PCS T cell responses and the immune environment induced by the novel PCS vaccine are key correlates of vaccine efficacy

Abstract:

After over three decades of research, an effective anti-HIV vaccine remains elusive. Unconventional and novel vaccine strategies are needed. Here, we report that a vaccine focusing the immune response on the sequences surrounding the 12 viral protease cleavage sites (PCSs) provides greater than 80% protection of Mauritian cynomolgus macaques (MCMs) against repeated intravaginal SIVmac251 challenges. The PCS-specific T cell responses are correlated with vaccine efficacy. The PCS vaccine does not induce immune activation and inflammation known to be associated with increased susceptibility to HIV infection. Machine learning analyses revealed that the immune environment generated by the PCS vaccine predicts vaccine efficacy. Our study demonstrates for the first time that a novel vaccine which targets viral maturation, but lacks full Env and Gag proteins as immunogens, can prevent intravaginal infection in a highly stringent NHP/SIV challenge model. Targeting HIV maturation thus offers a novel approach to developing an effective HIV vaccine.

69 **Introduction**

70 HIV mutates rapidly and preferentially infects activated CD4+ T cells. Inflammation activates and
71 attracts HIV target cells, thereby enhancing the risk of infection[1-3]. These inherent characteristics of HIV
72 infection underscore the greater challenges compared to other pathogens when developing a
73 prophylactic vaccine [4,5]. Among the six HIV vaccine clinical efficacy trials to date, only the RV144 Thai
74 trial demonstrated any efficacy, and it was modest (31.2%)[4,6]. Since activated CD4 T cells are
75 preferential targets for HIV-1, an effective HIV vaccine would need to elicit protective anti-HIV
76 immunity while minimizing vaccine-induced inflammation and immune activation[5,7,8]. Our previous
77 studies of HIV highly exposed seronegative (HESN) Kenyan female sex workers showed that narrowly
78 targeted T cell responses were associated with protection against HIV infection[8-10]. Thus, an alternative
79 strategy for an effective HIV vaccine would be to generate immune response only to the essential part of
80 HIV-1[8,11]. Learning from natural immunity observed in these Kenyan HESN women, we tested a novel
81 strategy by focusing the host immune response on the sequences surrounding the 12 viral protease
82 cleavage sites (PCSs) (the PCS vaccine)[7,12]. The HIV protease cleaves Gag, Gag-Pol and Nef precursor
83 proteins at the 12 PCSs[7,13]. Studies have shown that the process of protease cleavage requires a tightly
84 controlled and ordered sequence of proteolytic processing events mediated by different rates of cleavage
85 at the different processing sites[14-20]. Even subtle disturbances may be sufficient to interrupt this
86 delicately balanced process and drive it toward a non-productive end[14,17,18,21]. We hypothesized that a
87 vaccine targeting the 12 PCSs would be effective against HIV infection. First, as the sequences
88 surrounding the PCSs are highly conserved[7] the vaccine can target multiple HIV subtypes. Second, the
89 focused immune response may drive viral mutations surrounding the PCSs and impair the proteolytic
90 processing of HIV polyproteins. As a result, non-infectious viral progenies will be generated. Third,
91 restricting the immune response to a smaller number of T-cell epitopes surrounding the PCS may result
92 in lower vaccine-induced inflammation and fewer activated CD4 T target cells[7]. We tested this

93 hypothesis in a non-human primate (NHP)/simian immunodeficiency virus (SIV) infection model. We
94 demonstrate here that the PCS vaccine, without full Gag and Env immunogens, protects female
95 Mauritian Cynomolgus macaques (MCMs)[22] from acquiring highly pathogenic and heterogenic
96 SIVmac251[23] infections.

97 **Results**

98 ***Vaccine protection against vaginal SIVmac251 infection***

99 The PCS vaccine delivers twelve 20-amino acid peptides overlapping each of the 12 viral protease
100 cleavage sites with recombinant vesicular stomatitis viruses (rVSV) and nanoformulations (NANO) [7,12].
101 To evaluate the efficacy of the PCS vaccine (Figure 1A), 16 female MCMs that did not express known
102 SIV controller MHC haplotypes[24] (Table S1) were divided into two groups (the PCS vaccine and the
103 vaccine vector control; n=8 per group). MHC haplotypes were balanced between the two groups (Table
104 S2). The vaccination scheme consisted of prime with rVSVpcs or rVSV control vector (intramuscularly)
105 and four boosts with combinations of rVSVpcs or rVSV vector (intramuscularly) and NANOpcs
106 (intranasally)[12] (Figure 1B).

107 Approximately six months after the final boost, repeated low-dose intravaginal SIVmac251 challenges
108 (the Desrosiers 2010-Day 8 stock at 250 TCID50/animal/challenge) were carried out every two weeks
109 and infection status was monitored by quantification of plasma viral load on days 6, 10 and 14 after each
110 of the challenges (Figure 1B). The vaginal challenge route was chosen to mimic vaginal HIV exposure
111 in women, which accounts for about half of all HIV infections worldwide[25]. According to our study
112 design (detailed in Methods), the end point of the challenge study for evaluating vaccine efficacy was at
113 challenge number six (Figure 1C), which has also been used by many other studies[23,26-28]. At the end of
114 this standard challenge procedure, the majority (six of eight, 75%) of control animals were infected,
115 whereas only two of the PCS-vaccinated animals (25%) were infected (Figure 1C). To determine

116 whether continued challenges can lead to infection of the two uninfected control animals and to
117 determine the numbers of challenges needed to infect 50% of the vaccinated animals because of
118 biological variations among the monkeys, we extend the challenges to thirteen times (Table S3).
119 Following the extended challenges three additional animals in the PCS vaccine group became infected
120 (after 9, 11 and 13 challenges, respectively). However, the two previously uninfected control animals
121 remained uninfected (Table S3). This observation was consistent with findings from several previous
122 studies in rhesus macaques that a proportion of control animals were refractory to acquisition of
123 SIV/SHIV infections[28-36].

124 Survival analysis demonstrated that the PCS vaccine significantly increased the number of challenges
125 required for acquisition of SIVmac251 infection ($p = 0.046$, log-rank test) (Fig. 1C). It provided an
126 80.3% reduction in the per-exposure risk of viral acquisition (vaccine efficacy, VE = 1 – hazard ratio)
127 (proportional hazards regression) (Figure 1C). Furthermore, while it took only two SIVmac251
128 challenges to infect 50% animals in the control group (Figure 1C and 1D), extended challenges showed
129 that it took 11 challenges to infect 50% animals in the PCS vaccine group (Figure 1D). The observed
130 vaccine protection was not related to the menstrual phases of these female animals during the
131 SIVmac251 challenges (Figures. S1 and S2) or their MHC haplotypes (Tables S4 and S5).

132 These data demonstrate for the first time that a candidate prophylactic PCS HIV vaccine, without
133 traditional types of immunogens such as full Gag and Env proteins, significantly protected female
134 monkeys against highly pathogenic and heterogenic SIVmac251 challenges.

135 ***The PCS vaccine did not elicit significant inflammatory responses in the cervico-vaginal mucosa***

136 Inflammation can activate and attract HIV CD4 T target cells to the portal of entry. Elevated
137 cervicovaginal mucosal (CVM) inflammation is associated with increased susceptibility to HIV/SIV
138 infection and reduced efficacy of anti-HIV microbicides[37-39]. We examined vaginal mucosal

139 inflammation following vaccination. The vaccine-induced mucosal inflammation was analyzed by
140 quantifying inflammatory cytokine responses in cervicovaginal lavage (CVL) samples (Figure 2 A-G
141 and Figure S3). Thirteen pro-inflammatory cytokines (TNF-α, IFN-γ, IL-6, RANTES/CCL5, GM-CSF,
142 IL-1β, MCP-1/CCL2, IL-8, MIP-1α/CCL3, MIP-1β/CCL4, IP-10/CXCL10, IL-17A and IL-1α) and one
143 anti-inflammatory cytokine (IL-10) were analyzed. Vaccine-induced fold changes in cytokine levels at
144 multiple time points were determined and shown as heat maps (Figure 2A). No apparent induction of
145 inflammatory cytokine responses, apart from some sporadic fluctuations, was observed in the control or
146 the PCS vaccine group. The short SIV PCS peptide immunogens in the PCS vaccine did not lead to the
147 persistent increase in cervicovaginal inflammatory responses in the PCS vaccine group in comparison to
148 the control group, except an initial increase after prime in IFN-γ, IL-6, RANTES, GM-CSF, MCP-1, and
149 IL-17A, which were dampened at later boost points (Figure 2A). When the absolute cytokine levels
150 were compared, the PCS vaccine group had significantly lower levels of IFN-γ and MIP-1β at week 73
151 (one week after the final boost), and IL-8 and MIP-1β at week 90 (18 weeks after the final boost), than
152 that of the control group (Figure 2B-G). These data indicate that the PCS vaccine did not elicit
153 significant inflammatory responses in the cervico-vaginal mucosa.

154

155 ***The PCS vaccine induced antibody and cellular responses***

156 Mucosal antibodies to PCS peptide antigens were measured in CVL (reported previously)[12] and plasma
157 samples (Figure S4A and Fig. S5). The patterns of anti-PCS peptide antibody (IgG) responses in CVL
158 and plasma were similar. A notable trend of increase in antibodies to individual PCS antigens was
159 observed at week 73 (one week after the last boost, defined as the "peak" immune response time point)
160 (Fig. S5). The level of combined plasma antibodies to all 12 PCS peptides at this time point in the PCS
161 vaccine group was approximately two fold of that in the control group (Figure S4A). Thus, the PCS
162 vaccine induced antibodies to PCS peptides. The PCS peptides are not located in the Env, and the pre-

163 challenge plasma samples of the PCS vaccine group did not have neutralizing activities against tier 1
164 and tier 2 SIVmac251 (data not shown). The moderate magnitude of anti-PCS antibodies may reflect the
165 existence of B cell epitopes in the sequences surrounding the PCS, and indicate that immunization with
166 the PCS vaccine was effective in inducing antibodies to the PCS peptides. However, the contribution of
167 these antibodies to the protection is currently unknown.

168 To examine cellular immune responses, peripheral blood mononuclear cells (PBMCs) from the "peak"
169 time point were quantified by ELISPOT for frequency of IFNγ-producing cells in response to a pool of
170 SIV peptide antigens (PCS1-4, PCS5-8 or PCS9-12) (Figure S4 B-D). Slightly higher numbers of
171 animals in the PCS vaccine group than those in the control group showed above background responses
172 to PCS5-8 and PCS9-12 peptide pools, but these differences were not statistically significant (Figure S4
173 B-D). Although IFNγ ELISPOT has been traditionally used to evaluate T cell responses to vaccines,
174 many cytokines other than IFNγ are known to be secreted by activated T cells[40-42]. Thus, simply
175 assessing the IFNγ response by ELISPOT may give a very limited view of vaccine-induced T cell
176 responses[43]. Furthermore, in some vaccine studies, IFNγ secretion was a poor correlate of protection
177 against HIV, and was not the best indicator of vaccine-induced responses[43]. We therefore expanded the
178 analysis of cellular responses by measuring 14 cytokines using our customized Bio-Plex multiplexed
179 cytokine assay.

180 PBMCs of the PCS vaccine group from the "peak" time point were stimulated with PCS peptide pools
181 and quantified for cytokines secreted into the culture supernatants (Table S6). Between nine to fourteen
182 cytokines were detected following stimulations with different PCS peptide pools (PCS1-4: nine
183 cytokines, PCS5-8 and PCS9-12: fourteen cytokines). Among these cytokines, IFNγ was at a low level
184 and detectable only in less than half of the animals. Six other cytokines, including RANTES, MIP-1α,
185 MIP-1β, TNF-α, IL-6 and IL-8, were detected in 50% or higher percentage of animals (Table S6). Some

186 of these cytokines are currently known to have potential functions in directly inhibiting HIV infection or
187 regulating inflammation. RANTES, MIP-1α and MIP-1β are HIV-suppressive factors produced by $CD8^+$
188 T cells possibly through competitive binding of HIV co-receptor CCR5[44]. IL-6, produced by various
189 cell types including T cells, B cells and monocytes[45], can have an anti-inflammatory role in controlling
190 local or systemic acute inflammatory responses[46,47]. A source of IL-8 is $CD4^+FOXP3^+$ regulatory T
191 cells[48], which could play a role in regulating antiviral response. In summary, these data indicated that
192 several types of cellular immune responses to PCS antigens can be generated following vaccination.

193 ***The PCS vaccine generated fewer viral target cells and higher PCS peptide specific $CD8^+$ T memory***
194 ***and $CD4^+$ T_{reg} responses***

195 We characterized the T cell subsets after immunization with the PCS vaccine. PBMCs from week 90 (a
196 pre-challenge time point) without antigen stimulation (*ex vivo*) or stimulated with a pool of PCS
197 peptides (PCS1-12) were analyzed by flow cytometry (Figure 3, 4 and Figure S6). Different cellular
198 markers[49-51] were chosen to differentiate $CD4^+$ and $CD8^+$ T cell subsets including naive and memory T
199 cells (CCR7 and CD45RA)[52,53], Th17 cells[4,54] (IL-17A and CCR5) and Treg cells (CD25, FoxP3 and
200 CD127)[54], together with activation and differentiation markers including CD38, CD69, CD107a, Ki-67,
201 PD-1, IL-2, TNF-α, IFN-γ, MIP-1β and IL-10.

202 Under *Ex vivo* conditions, compared to the control group the monkeys of the PCS vaccine group had
203 lower frequency of $CD4^+CCR7^-CD45RA^+$ terminal effector CD4 T cells ($CD4^+$ T_{EMRA}) and lower
204 frequency of TNF-α+$CD4^+$ T_{EMRA} cells. These $CD4^+$ T_{EMRA} cells expressed lower amounts of
205 proliferation marker Ki67 and pro-inflammatory cytokine TNF-α (Figure 3A-D). Similarly, $CD8^+CCR7^-$
206 $CD45RA^+$ terminal effector CD8 T cells ($CD8^+$ T_{EMRA}) of the PCS vaccine group expressed lower levels
207 of TNF-α (Figure 3E). These suggest that the monkeys of the PCS vaccine group had an overall lower
208 level of immune activation.

209 After PCS peptide stimulation the monkeys in the PCS vaccine group had a lower frequency of
210 $CD4^+CCR7^-CD45RA^+$ terminal effector CD4 T cells ($CD4^+$ T_{EMRA}) cells (Figure 4A). While the surface
211 expression of CD45RA on $CD4^+$ T_{EMRA}, and CCR7 expression on $CD4^+CCR7^+CD45RA^-$ T central
212 memory ($CD4^+$ T_{CM}) were significantly higher in the PCS vaccine group (Figure 4B, 4C). The PCS-
213 vaccinated animals also had lower CD107a expression in naive CD4+CCR7+CD45RA+ T cells (CD4+
214 Tnaive) (Figure 4D), but significantly higher frequency of $CD8^+CCR7^+CD45RA^-$ T central memory
215 cells ($CD8^+$ T_{CM}) (Figure 4E) and higher proportions of them expressing CD107a (Figure 4F). However,
216 the PCS-vaccinated monkeys had fewer $CD8^+$ T_{CM} cells expressing IFN-γ^+ (Figure 4G).
217 Thus, the frequency of PCS specific $CD4^+$ T_{EM} was low, but the surface expression of CD45RA on
218 $CD4^+$ T_{EMRA} cells and CCR7 on $CD4^+$ T_{CM} cells were higher in the PCS vaccine group. Consistent with
219 lower immune activation status, the PCS peptide specific CD4+ T naive cells of the PCS vaccine group
220 had lower level of CD107a expression. The higher frequency and higher expression level of CD107a+
221 $CD8^+$ T_{CM} cells in the PCS vaccine group demonstrated that immunization with the PCS vaccine
222 generated PCS specific CD8+ central memory T cells that have better potential in killing viral infected
223 cells.
224 We conducted phenotypic analysis of Th17 and CD8+Th17 cells that were considered to be susceptible
225 target cells for HIV or inflammatory disease[55-57]. Under *ex vivo* conditions, monkeys of the PCS vaccine
226 group had a lower proportion of naive Th17 cells ($CD4^+$IL-$17A^+CCR7^+CD45RA^+$) and naive $CD8^+$ IL-
227 $17A^+$ T cells expressing SIV/HIV co-receptor CCR5 (Figure 3G-H). They also had a lower proportion of
228 naive $CD8^+$IL-$17A^+$ T cells expressing activation markers CD38 and CD69 (Figure 3I, 3J). With PCS
229 peptide stimulation the monkeys in the PCS vaccine group also had a lower proportion of PCS peptide
230 specific $Th17_{EMRA}$ and $CD8^+$IL-$17A^+$ T_{CM} cells expressed CD107a (Figure 3H, 3I). Thus, immunization
231 with the PCS vaccine generated fewer viral susceptible target cells and fewer $CD8^+$IL-$17A^+$ T cells that

232 were involved in inflammatory disease[55-57].

233 Antigen-specific regulatory T cells protect against aberrant immune responses[51]. We analyzed vaccine
234 induced T regulatory cells. The monkeys in the PCS vaccine group had a higher frequency of PCS
235 peptide specific $CD4^+CD25^+FOXP3^+CD45RA^+CD127^-$ ($Treg_{EMRA}$) cells and a higher frequency of PCS
236 peptide specific $CD4^+CD25^+FOXP3^+CD45RA^+CD127^+$ (naive Treg) cells expressing immunoregulatory
237 cytokine IL-10 compared to that of the control group (Figure 4J, 4K). Taken together, the monkeys of
238 the PCS vaccine group had lower immune activation, lower activated PCS peptide specific $CD4^+$ T cells
239 - the primary SIV target cells, but higher PCS peptide specific $CD8^+$ T memory and $CD4^+$ Treg
240 responses.

241 ***Immunologic correlates of protection (CoPs) induced by vaccination***

242 We conducted regression analysis to identify potential immunologic factors that might be positively
243 correlated with protection against acquisition of SIVmac251 infection in the vaccinated group. We
244 assessed immunological measurements, including CVL mucosal inflammatory cytokines, CVL and
245 plasma antibodies, PBMC cytokine-producing antigen recall responses and $CD4^+$ or $CD8^+$ T cell subsets
246 (memory, IL-$17A^+$ and Treg, *ex vivo* or Ag recall) from the peak (week 73) and pre-challenge (week 90)
247 time points. The Spearman rank correlation was first used to assess individual immunological correlate
248 of protection (CoP) observed in the PCS vaccine group, defined as the number of challenges required for
249 SIVmac251 infection. The identified CoPs (Table 1) (positive Spearman's rho with $p<0.05$, unadjusted
250 for multiple inference) include (1) cytokines secreted by PBMCs (peak time point) after PCS peptide
251 stimulations, including RANTES and IL-6 (to PCS1-4 peptides), and MIP-1α (to PCS9-12 peptides); (2)
252 the frequency of CCR5 expressing Th17 T_{EM} ($CD4^+IL17A^+CCR7^-CD45RA^-$) cells (pre-challenge time
253 point, *ex vivo*); (3) the frequency of CCR5 expressing $CD8^+IL17A^+$ T_{EM} cells
254 ($CD8^+IL17A^+CCR7^+CD45RA^-$) and the intensity of CCR5 expression on these cells (pre-challenge time

255 point, *ex vivo*); and (4) the frequency of PD-1 expressing PCS peptide specific Treg effector memory
256 cell population ($CD4^+CD25^+FoxP3^+CD45RA^+CD127^-$) (pre-challenge time point). These results
257 suggested that protection was significantly correlated with T-cell responses. However, no significant
258 associations with antibody response were observed.

259 Several immune correlates of risk (CoRs) (defined as negative Spearman's rho, with $p<0.05$ unadjusted
260 for multiple inference) were also identified, including CVL mucosal IL-1α level at the peak and pre-
261 challenge time points, CVL mucosal antibodies to PCS1, 3, 4, 5 and 9 at the peak time point, as well as
262 the frequency or intensity of several activation or differentiation makers on subsets of $CD4^+$ or $CD8^+$
263 memory, $IL17A^+$ or Treg cells at the pre-challenge time point (Table S7). These CoRs appear to relate to
264 inflammation, immune activation and SIV target cell availability.

265 To explore more potential CoP/CoRs, especially combinations of immunological predictors which might
266 act together to protect the vaccinated animals, we performed a multiple regression analysis of the
267 immunological data using the LASSO (least absolute shrinkage and selection operator) (Figure 5).
268 Although the risk of overfitting is large, and R^2 optimistic ($R^2 = 0.964$) in this situation, the LASSO
269 regression model has nevertheless identified 6 potential predictors from pre-challenge T cell subsets, 5
270 of which were not identified via the single predictor Spearman correlation analysis (Figure 5A, 5B and
271 Table S8). Individually these predictors also positively correlated with many other immunological
272 variants by Spearman rank correlation analysis (Figure 5C and Table S8). The three predictors with
273 positive coefficients in the LASSO model were positively correlated with several cytokine-producing
274 antigen recall responses at the peak or pre-challenge time point. They were also positively correlated
275 with the frequency or expression intensity of CD107a, TNF-α, MIP-1β, CD38, IFN-γ, IL-2 or CD69 in
276 several T cell subsets ($Th17_{EM}$, $Th17_{CM}$, $CD8^+IL\text{-}17A^+$ $T_{CM/TEM}$, $CD8^+$ T $_{naive/TEM}$, $CD4^+$ T_{EMRA},
277 $CD4^+/CD8^+$ Treg naive or $CD4^+$Treg RM) either under *ex vivo* conditions or after PCS peptide

278 stimulations at the pre-challenge time point (Fig. 5C and Figs. S7-9). The predictor CD4TregEmPD1,
279 strongly correlated with 14 variants. Seven of the 14 variants plus the predictor itself also significantly
280 correlated with vaccine protection, individually. The three predictors with negative coefficients in the
281 LASSO model positively correlated with the fold change of two CVL mucosal cytokines, RANTES and
282 IL-17A, at the pre-challenge time point, and with CVL antibodies to PCS4 and PCS5 at the peak time
283 point (Figure 5C and Figs. S10-12). The predictor CD4TreNPD1m appeared to play an important role in
284 regulating proliferation of several T cell subsets, as shown by its significant correlation with Ki67
285 frequency or/and expression intensity in $CD4^+$ and $CD8^+$ Tnaive, T_{EM}, T_{CM}, and T_{EMRA} cells under *ex*
286 *vivo* conditions or after PCS peptide stimulations. CD4TreNPD1m also significantly correlated with the
287 frequency or/and expression intensity of CD38, IFNγ, PD-1 in $CD8^+IL17A^+$ Tnaive, $CD8^+$ T_{EMRA}, and
288 $CD4^+$ Treg EM under *ex vivo* conditions and/or after PCS peptide stimulations. The other two predictors
289 with negative coefficients in the LASSO model, CD8TregCmIL10 and CD4TreCmCD38 significantly
290 correlated with the frequencies of $CCR5^+$ Th17 naive cells after PCS peptide stimulation. The
291 frequencies of these two central memory Treg cells significantly correlated with the frequency or
292 expression intensity of IL-10, CD38, CD69, MIP-1β, and PD-1 in $CD4^+$ Treg naive, $CD4^+$ Treg EM,
293 $CD8^+$Treg naive, $CD8^+$ Treg RM, $CD8^+$ Treg EM, or $CD8^+$ Treg CM cells under *ex vivo* conditions or
294 after PCS peptide stimulations. These two features also correlated with the expression intensity of
295 CD107a in $CD8^+IL17A^+$ T naïve, $CD8^+IL17A^+$ T_{EM} and Th17 T_{EMRA} cells, as well as the expression
296 intensity of CD38 and IFNγ in Th17 T_{EMRA} cells.

297 Of the six predictors identified by the LASSO model, four are PCS peptide-stimulated Treg cells. These
298 antigen-specific Treg cells appear to act together with PCS peptide specific IL2+CD8+ T central
299 memory cells and PCS peptide specific CD107a+$CD8^+$IL-17^+ T effector memory cells to regulate the

300 immunological environment in the vaccinated macaques and contribute to the effective protection
301 against pathogenic SIVmac251 acquisition.
302
303 **Discussion**
304 The development of an effective HIV vaccine is faced with great challenges. HIV has the formidable
305 capacity to mutate its genome and evolves rapidly to evade immune recognition[58-60]. It targets $CD4^+$ T
306 cells and thrives in an activated immune system, a unique character distinguishing it from many other
307 infectious pathogens[4,5]. The knowledge gained from the past failures and modest success in HIV vaccine
308 development[61] and from the natural immunity of highly exposed seronegative individuals[8] are important
309 in developing an effective HIV vaccine. Traditional candidate vaccines have been based on full HIV
310 proteins such as Env and Gag, which contain multiple immunodominant decoy epitopes that divert
311 immune responses away from targeting protective epitopes[62,63]. Furthermore, most of these vaccines
312 may not have considered inflammation and immune activation associated with the strong and broad
313 immune responses aimed by these vaccines. The potential impact of which may be an increase in
314 activation of CD4 T cells, the preferred HIV targets, thus enhancing viral propagation and diminishing
315 the benefit of anti-HIV immune responses. It has been proposed that the increased risk of infection
316 observed in the vaccinees of the Step (HVTN 502) and Phambili (HVTN 503) trials, may have resulted
317 from increased activation of mucosal CD4 T cell following vaccination[6,64,65]. The hypothesis is
318 supported by preclinical data of similar vaccine studies in nonhuman primates (NHP)[6,64,65]. In the RV144
319 trial, the ALVAC vaccine used for prime was found to have much lower immunogenicity than the highly
320 immunogenic Ad5 vaccine in the Step trial[66,67]. An NHP study mimicking the RV144 trial and its
321 follow-up African trial observed that replacing the vaccine adjuvant alum with more immunostimulatory
322 MF59 resulted in loss of the protective effect[68]. These data need to be considered in development and
323 evaluation of anti-HIV vaccines.

324 Our study showed that the PCS vaccine addressed these concerns and provided greater than 80%
325 protection of Mauritian cynomolgus macaques (MCM) against each of the
326 repeatedly intravaginal SIVmac251 challenges. Cynomolgus macaques and Mauritian Cynomolgus
327 macaques are susceptible to SIV and SHIV infection, its natural course of infection leading to simian
328 AIDS more closely recapitulates the HIV infection in humans, and is a valid vaccine evaluating model
329 [22,24,69-81]. A study showed that there was no difference in terms of infectivity and peak viral load levels
330 between rhesus macaques and Cynomolgus macaques inoculated through oral and rectal mucosal
331 routes[82].

332 Furthermore, the SIVmac251 is a swarm of viruses with high pathogenicity and significant genetic
333 heterogeneity[81]. We believe that the MCM/SIVmac251 vaccine challenge model is stringent in
334 evaluating the vaccine efficacy. The efficacy of the PCS vaccine is among the best protective vaccines
335 thus far tested in the NHP models[23,27,83]. The PCS vaccine focused immune responses on the functionally
336 critical part of the virus - sequences surrounding the protease cleavage sites. Anti-PCS peptide $CD8^+$
337 central memory T cells with higher potential of killing viral infected cells were among the T cell
338 responses correlated with vaccine efficacy. The mucosal inflammation induced by the PCS vaccine is
339 low. The amount and activation of viral target T cells in the blood was significantly lower in the PCS
340 vaccine group than that in the control group. The PCS vaccine immunized monkeys had significantly
341 lower CCR5 expression in Th17 cells, lower frequencies of $CCR5^+IL\text{-}17A^+CD8^+$ T naive cells, and
342 lower expression of activation markers CD69 and CD38. These are relevant to HIV infection since Th17
343 cells are preferential targets for productive infection[55,56] and $IL17A^+CD8^+$ T cells also are thought to
344 play a role in immune pathology that contributes to the maintenance of target cell populations for HIV
345 infection[57]. Thus, the PCS vaccine limited the availability of viral target cells. Furthermore, the PCS-
346 vaccinated monkeys had higher frequency of PCS-specific $CD4^+$ Treg EMRA and $CD4^+IL\text{-}10^+$ Treg naive

347 cells than controls, thus they may be better able to regulate anti-viral immune responses. Six predictors
348 identified using the LASSO model that predicted vaccine efficacy are all PCS peptide specific T cell
349 responses including four PCS peptide-specific Treg cell populations and two PCS peptide specific CD8
350 T central/effector memory cell populations. It suggests that regulation of anti-SIV CD8 T cell responses
351 is important.

352 The PCS vaccine generated a focused and modest magnitude of protective immune responses. These
353 antiviral immune responses in combination with low inflammation and reduced $CD4^{+}CCR5^{+}$ viral target
354 cells appear to be sufficient to prevent pathogenic SIVmac251 acquisition. Since most sexual HIV
355 transmission was initiated by a single founder virus[84,85], a focused, modest-magnitude and well-
356 regulated antiviral immune response might be enough to prevent the viral establishment and propagation
357 as demonstrated in this study.

358 In conclusion, this study showed that a novel HIV vaccine candidate focusing immune response to
359 sequences surrounding the 12 protease cleavage sites can protect female macaques from pathogenic
360 SIVmac251 intravaginal acquisition. For the first time, a candidate HIV vaccine without full Gag and
361 Env proteins as immunogens has demonstrated high efficacy of protection. Our study also showed that
362 the PCS immunogen-specific $CD8^{+}$ T central and effector memory cells, and PCS immunogen-specific
363 $CD4^{+}$ and $CD8^{+}$ regulatory T cells correlated with the vaccine efficacy. Thus, an effective prophylactic
364 HIV vaccine needs to not only generate effective antiviral immune responses to kill infected cells, but
365 also regulate the immune responses and control immune activation and inflammation. These can be
366 achieved by the PCS vaccine, a vaccine targeting HIV maturation.

367 In order to observe within a reasonable time that the vaccine was effective, the repetitive low-dose
368 SIVmac251 intravaginal challenges using the NHP model employed virus titres far exceeding the
369 average HIV viral load in semen[86-88]. This ensured that the NHP/SIVmac251 model used in this study be

370 highly stringent. Although this study shows that the PCS vaccine is effective in preventing infection in
371 the setting of a highly stringent NHP/SIVmac251 intravaginal challenge model, human clinical trials
372 will constitute the ultimate test. The detailed available information of T cell epitopes surrounding the 12
373 HIV protease cleavage sites of major HLA class I alleles in the world populations[11], and the immune
374 correlates of protection identified in this study will facilitate future clinical trials.

375 **Materials and Methods**

376 **Study design**

377 Vaccine efficacy against acquisition of infection was evaluated in female MCMs receiving repeated
378 low-dose (RLD) intravaginal challenges by SIVmac251, which mimics natural HIV infection through
379 the female genital tract. Sample sizes were determined using the parameters described in previous
380 studies[89]. An infection rate of 0.4 among control macaques and an interval of two weeks among each
381 challenge of mucosal RLD were estimated from our pilot study. Statistical power was then calculated
382 based on assuming an infection rate of 40% per exposure and a susceptibility to infection of 85% in
383 control animals at the end-point of the 6th viral challenge when majority of control macaques become
384 infected. To achieve 80% statistical power with a 2-sided type I error rate < 0.05, a total of 16 female
385 MCMs were required and assigned into two groups (eight per group): the PCS and sham control vaccine
386 groups.

387 **Experimental animals and ethics statement**

388 Female Mauritian cynomolgus macaques (MCMs) were pair-housed within the same experimental
389 group during the immunization phase of the study with visual and auditory access to other conspecifics.
390 Paired monkeys lived in two adjacent standard stainless-steel primate cages (27”L x 27”W x 32”H per
391 cage). Rooms were maintained at 65–75°F, 30–70% humidity, and on a 12:12 light-dark cycle (ON:
392 0600, OFF: 1800). Standard nonhuman primate chow with fruit or vegetables was provided daily. In

393 addition, we provided foraging activities and physical environmental enrichment at least weekly for both
394 activities. All animals were observed at least twice daily for health or welfare issues. Sedation (ketamine
395 alone, or ketamine/dexmedetomidine, atipamezole for reversal) was provided during the experimental
396 procedures. The experiments were approved by the University of Wisconsin IACUC protocol
397 (G005765) in accordance with the US Animal Welfare Act and following the recommendations of the
398 National Research Council Guide for the Care and Use of Laboratory Animals, 8th Edition and the
399 Weatherall report, The Use of Nonhuman Primates in Research. The Wisconsin National Primate
400 Research Center is fully accredited by AAALAC under the University of Wisconsin, Division of Vice-
401 Chancellor for Research and Graduate Education.
402 All animal work was carried out at Wisconsin National Primate Research Center. We conducted vaccine
403 evaluation using the Mauritian cynomolgus macaque (MCM)/SIVmac251 infection model. MCMs are
404 abundant in supply, and have simple major histocompatibility complex (MHC) genotypes (with only
405 seven MHC haplotypes[22,69,90-94]). Their infection by SIVmac251 leads to peak and set point viral loads
406 and disease progression pattern that closely mimic human HIV infection[22,95,96]. This infection model has
407 proven stringent for testing prophylactic vaccines: None of the vaccines previously tested in this model
408 has demonstrated significant efficacy against acquisition of SIVmac251 (or SIVmac239) infection[22,24,72-
409 76,78-80,97-107]. Additional animal information, concerning grouping, ID, gender, age, weight and MHC
410 haplotypes, is summarized (Table S1-S2).

411 **Immunization scheme**

412 The PCS vaccine contained twelve 20-mer peptides, derived from the twelve protease cleavage sites
413 (PCSs) of SIVmac239 (ten amino acids flanking each side of the cleavage site)[12,108]. These peptide
414 immunogens were delivered as recombinant vesicular stomatitis viruses (rVSVpcs) or biodegradable
415 nanoparticles (NANOpcs)[12]. The rVSVpcs consisted of twelve different rVSV viruses each delivering

416 one PCS peptide (PCS1, PCS2, …, or PCS12) and the NANOpcs was formed by a pool of twelve
417 formulations, each made of chitosan/dextran sulfate nanoparticles containing one different PCS
418 peptide[12]. The rVSVpcs and NANOpcs in combination were referred to as the PCS vaccine. The sham
419 control vaccine was rVSV vector (rVSVvt) virus (without SIV immunogen insert) and sterile water
420 (NANO vehicle)[12]. The vaccination scheme was described previously[12], consisting of a prime (with
421 rVSV at week 0) and four boosts (1st boost: rVSV + NANO at week 6, 2nd boost: NANO at week 16, 3rd
422 boost: rVSV + NANO at week 51, and 4th boost: rVSV at week 72). rVSVs were administered
423 intramuscularly at a dose of 2×10^7 pfu/animal (i.e. 1.67×10^6 pfu/rVSVpcs type/animal × 12 PCS types =
424 2×10^7 pfu/animal for the PCS vaccine group, or 2×10^7 pfu of rVSVvt/animal for the control group),
425 except that, in the 4th boost, 1×10^8 pfu of each rVSVpcs or rVSVvt was used. NANOpcs was
426 administered intranasally at a dose of 50 μg peptide/PCS type/animal × 12 PCS types = 600 μg/animal.

427 **Repetitive low-dose intravaginal SIVmac251 challenges**

428 Challenges were carried out every two weeks starting 24 weeks after the last vaccination boost. The
429 vast majority of SIV challenges in previous studies were performed weekly[23,26,109] while viral
430 acquisition status was monitored by quantification of plasma viral load (VL). However, establishment of
431 systemic infection can take up to 10 – 14 days[54]. Potentially some slowly establishing infections may not
432 be detectable within one week post challenge by the routine method of plasma VL measurement. This
433 was also confirmed in our study. Therefore, using the weekly challenge protocol, an infection identified
434 during a later challenge round may actually have been acquired in an earlier round, leading to an over-
435 estimation of vaccine efficacy. In this regard, we carried out challenges every two weeks, and plasma
436 VL was quantified on days 6, 10 and 14 of each challenge round (any animal found infected was not
437 challenged further). Indeed, we found that in some cases an infection could remain below detectable
438 level until as late as 14 days following a challenge (Table S3). In addition, we took into consideration

439 that in females, susceptibility to vaginal HIV infection could be affected by phases of the menstrual
440 cycle. It was reported that the susceptibility is higher in the luteal phase than in non-luteal phases[110-114].
441 Our 2-week challenge cycle was also designed to maximize the chance for even distribution of
442 challenges in the luteal versus non-luteal phases, since the luteal phase constitutes approximately half of
443 a 4-week menstrual cycle. It was confirmed that in both the control and PCS vaccine groups challenges
444 fell in the luteal and non-luteal phases nearly equally, without any significant difference between the two
445 groups (Figs. S1 and S2).
446 Intravaginal challenges were performed by atraumatically delivering 250 times 50% tissue culture
447 infectious doses (TCID50) of SIVmac251 in 1 ml of saline or tissue culture medium to the vagina while
448 the animal was anesthetized as described below. The virus was delivered using a TB syringe with the
449 needle removed (or using a TB syringe with a rounded end gavage tube). The syringe was gently
450 inserted into the vagina about 4cm, then withdrawn slightly and the dose was injected slowly over 1
451 minute, then the animal's pelvic region kept elevated up to 30 minutes before returning the animal to its
452 cage. When withdrawn, the syringe was examined to ensure that there was no trauma to the vagina
453 inflicted by the syringe (in which case blood would be visible).
454 For anesthesia, animals were immobilized against the front of the cage by a squeeze back mechanism,
455 and anesthetized using up to 7 mg/kg ketamine (i.m.) and up to 0.03 mg/kg dexmedetomidine (i.m.) to
456 be reversed at the conclusion of a procedure by up to 0.3 mg/kg atipamezole (i.v. or i.m.). Any additional
457 anesthesia was administered only in consultation with a WNPRC veterinarian. Alternative anesthesia
458 would only be used as directed by a WNPRC veterinarian. The duration of anesthesia was usually less
459 than 45 minutes. Monitoring of anesthesia recovery was documented every 15 min until the animal was
460 sitting upright, then every 30 minutes until the animal was fully recovered from the anesthesia.

461 **Viral load analysis**

462 Viral RNA was isolated from plasma samples using the Maxwell 16 Viral Total Nucleic Acid
463 Purification kit on the Maxwell 16MDx instrument (Promega, Madison WI). Viral RNA was then
464 quantified using a highly sensitive qRT-PCR assay based on a previously published protocol[115].

465 **Bio-Plex multiplexed cytokine/chemokine assay**

466 We used a customized cytokine/chemokine panel targeting 14 (pro- or anti-) inflammatory
467 cytokines/chemokines including Granulocyte Macrophage Colony Stimulating Factor (GM-CSF),
468 Macrophage Chemo-attractant Protein 1 (MCP-1), Monocyte Inflammatory Protein 1 alpha (MIP-1α),
469 Monocyte Inflammatory Protein 1 beta (MIP-1β), CCL5/RANTES, IFN-γ inducible protein 10 (IP-
470 10/CXCL-10), Tumor Necrosis Factor alpha (TNF-α), Interferon gamma (IFN-γ), IL-1α, IL-8, IL-10,
471 IL-17a, IL-1β, and IL-6. Mucosal inflammatory responses and cellular recall responses (see below) were
472 measured using cervicovaginal lavage (CVL) and PBMC culture supernatant samples, respectively.
473 Antibodies and protein standards used in these assays are listed in Table S9. The CVL sample collection
474 method was described previously[12]. For the CVL assay, PBS with 1% BSA (Assay buffer) was used
475 both as general diluent and blank controls, while RPMI-1640 Culture Medium (Sigma, R8758-
476 6X500ML) was used as diluent for standards and blank controls for the PBMC supernatant samples. In
477 order to generate the standard curves, we prepared a cytokine/chemokine protein standard pool with
478 final concentrations listed in Table S10, using PBS with 1% BSA as diluent. We then prepared eight
479 four-fold serial dilutions of this cytokine/chemokine protein standard pool and transferred 50 μL of each
480 dilution to a 96-well Bio-Plex Pro™ Flat bottom plate (BioRad, Catalog# 171025001). Final standard
481 curves were optimized and generated using Bio-Plex Manager 6.1 software, where coefficient of
482 variation (%CV) was no more than 30% and percentage of recovery was at least 70. 20 μg of capture
483 antibody for each cytokine/chemokine type was coupled to 1.25×10^{7} Bio-Plex Pro™ Magnetic COOH
484 Beads (BioRad, Catalog# MC10053-01) using Bio-Plex Amine Coupling Kit (Bio-Rad, Catalog# 171-

485 406001) according to the manufacturer's suggestions and diluted to a final concentration of 5×10^6
486 beads/mL. 50 µL of undiluted sample was added to each well containing ~420,000 coupled magnetic
487 beads/capture antibody type (1 in 600 dilutions) and incubated while shaking at high speed (~1000rpm)
488 for 30 seconds, then at medium speed (~300rpm) for one hour on a plate shaker at room temperature.
489 After washing the plate 3× (MAG3× setting on BioRad wash station) with wash buffer (PBS with 0.05%
490 Tween20), 30 µl of biotinylated detection antibody at 1µg/ml was added to each well and again
491 incubated while shaking for 30 minutes at room temperature. After another routine wash, 50 µl of
492 Streptavidin-PE in 1:100 dilution of BioRad 1× stock (Catalog# 171304501) was added per well and
493 incubated while shaking for 10 min at room temperature. A final wash was performed and afterwards
494 125 µl of assay buffer was added to each well. After shaking the plate for 10 seconds, we ran the plate
495 on a Bio-Rad Bio-Plex™ 200 System. Each bead fluorescence intensity and corresponding protein
496 concentration (pg/ml) were generated by Bio-Plex Manager 6.1 software. To take into account dilution
497 variations during CVL sample collections, cytokine/chemokine concentrations were all normalized to
498 total protein concentrations in the same sample. CVL total protein concentration was quantified using
499 NanoOrange® protein quantitation kit (Thermo Fisher Scientific) according to the supplier's protocol.

500 **MHC typing and Bio-Plex multiplexed antibody assay**

501 These were described previously[12,108].

502 **IFN-γ enzyme-linked immunospot (ELISPOT) assay**

503 Freshly isolated PBMCs ($2\text{-}2.5 \times 10^5$/well) were stimulated for 16-18 hours in a 96-well pre-coated plate
504 (ELISpotPLUS kit, MABTECH Inc., Mariemont, OH) at 37°C in a 5% CO_2 incubator. Stimulation was
505 performed with PCS peptide pool, targeting subsets of PCSs (PCS1-4, PCS5-8 and PCS9-12,
506 respectively), at 2.5µg/ml per individual peptide. The sequences of these peptides (Sigma-Aldrich) were
507 listed in Table S11. Cells stimulated with 5.0 µg/ml of concanavalin A (Sigma Chemical, St. Louis,

508 MO) were used as positive control. All tests were performed in duplicate. Wells were imaged with an
509 AID ELISPOT reader (AID, Strassberg, Germany), and counted by AID EliSpot Reader version 3.2.3,
510 with set parameters for spot size, intensity and gradient. Background (mean of wells without peptide)
511 levels were subtracted from each well on the plate. A response was considered positive if the mean
512 number of SFCs of duplicate sample wells exceeded twice the background and was >50 SFC per 1×10^6
513 cells.

514 **Cell culture of PBMCs and PCS peptide stimulation for analysis of multiple cytokine secretions or**
515 **T cell subsets**

516 2.0×10^6 PBMCs/ml in R10 media (RPMI1640-HyClone + 10% FBS + 2% antibiotic/antimycotic,
517 Thermo Fisher Scientific) were added into each well of a 96-well plate, and cultured in media only (no-
518 stimulation negative control), in the presence of 0.001µg/ml PMA with 0.01µg/ml Ionomycin (positive
519 control), or with 1.5µM SIV PCS peptide pools (Sigma-Aldrich, Table S11), overnight at 37°C with 5%
520 CO_2. Cytokines secreted into the culture supernatants were analyzed by the Bio-Plex multiplexed
521 cytokine assay as described above. For flow cytometry analysis (with intracellular staining) of T cell
522 subsets, PBMC culture and stimulation were performed in the presence of protein transport inhibitors,
523 50nL GolgiStop^{TM} (monensin) and 200nL GolgiPlug (brefeldin A).

524 **Flow Cytometry**

525 PBMCs with minimal manipulation (ex vivo) or with stimulation by a total PCS peptide pool (PCS1-12,
526 Table S11) were transferred to 12 x 75mm^3 polystyrene tubes (BD Falcon) and washed with FACS wash
527 (PBS supplemented with 2% FBS). Three sets of fluorochrome-labeled antibody cocktails (Table S9)
528 were prepared, and added to the washed PBMCs, and incubated at room temperature for 30 minutes in
529 the dark. The extracellular antibody cocktails panels included: Panel 1 - LIVE/DEAD^{TM} fixable Blue
530 stain (ThermoFisher Scientific), CD3 V500 SP34-2, CD4 BV605 L200, CD8 V450 RP8-T4, CCR7

531 A700 15053, CD127 PE-CF594 HIL-7R-M2 and CD45RA/RO APC-H7 UCHL1; Panel 2 (T cell
532 regulatory and activation) - LIVE/DEAD^{TM} fixable Blue stain, CD3 V500 SP34-2, CD4 BV605 L200,
533 CD8 APC-Cy7 RP8-T4, CCR7 A700 15053, CD127 PE-CF594 HIL-7R-M2, CD38 FITC HB7, PD-1
534 BV650 M1H4, CD69 APC FN50, CD25 PE-Cy5 and CD45RA PE-Cy7 L48; and Panel 3 (Th17) -
535 LIVE/DEAD^{TM} fixable Blue stain, CD3 V500 SP34-2, CD4 BV605 L200, CD8 V450 RP8-T4, CD38
536 FITC HB7, CD69 APC FN50, CD45RA PE-Cy7 L48, CCR7 A700 15053, CD127 PE-CF594 HIL-7R-
537 M2 and CD195 PE-Cy72 D7/CCR5 (all antibodies from BD Biosciences). Following the surface
538 staining 500µl FACS wash was added to each tube to wash the cells followed by centrifugation at
539 1500rpm for 5 min with low brakes. Next, the cells stained with extracellular antibodies for panels 1 and
540 3 were permeabilized using 150µl Cytoperm/Cytofix^{TM} (BD Biosciences) for 10-15 min, while those for
541 panel 2 ICS staining were fixed using 2ml of 1x FoxP3 buffer (BD Pharmingen) for 10-15 min. The
542 cells (panels 1-3) were then washed using FACS wash, and incubated with ICS panel 1 and 3 antibodies
543 for 30 minutes. Alternatively, the cells (panel 2) were further permeabilized using 1X Human FoxP3
544 Buffer C (BD Pharmingen) for 30 min, then washed using FACS wash, and finally incubated with a
545 cocktail of ICS antibodies. The panels of ICS antibodies used were as follows: Panel 1 ICS - CD107a
546 PE-Cy5 H4:H3, IFN-γ BV786 4S:B3, IL-2 FITC MQ1-17H12, TNFα APC Mab11, Ki67 PE-Cy7 B56,
547 MIP1α PE D21-1351 and IL-17A BV650 N49-653; Panel 2 - FoxP3 V450 259D/C7, IL-10 BV711
548 JES3-9D7 and MIP1α PE D21-1351. Panel 3 - CD107a PE-Cy5 H4:H3, IL-17A BV650 N49-653, IFN-γ
549 BV786 4S:B3, and MIP1α PE D21-1351. Following the ICS antibody incubation, the cells (panels 1-3)
550 were washed using FACS wash, and 300µl 1% formaldehyde with 1X PBS was added to each tube.
551 Flow cytometry analysis was done on a multicolor BD LSRII cytometer with data acquisition using BD
552 FACS DIVA software. Data analysis was carried out using FlowJo (courtesy of African HIV/AIDS
553 program -Treestar).

554 **General statistical analysis**

555 Time to infection in each group, measured by the number of challenges required to acquire infection and
556 censored following challenge 6 on day 84, was summarized using the Kaplan-Meier survival curves and
557 compared using the log-rank test; vaccine efficacy was evaluated as (1 – Hazard Ratio)*100% in a
558 proportional hazard regression model. Spearman rank correlations were used to assess the associations
559 between each immune factor and the number of challenges to infection. Immune responses were
560 compared between the vaccine and control groups using Mann-Whitney's U. For each of the above tests,
561 $p < 0.05$ was considered significant, without adjustment for multiple inference.

562 **LASSO analysis for a predictive model of vaccine efficacy**

563 Our Spearman rank analysis considered each immunologic measure individually. To identify potential
564 combinations of immunological predictors of resistance or susceptibility, we also performed a multiple
565 regression analysis. We used a linear model for the number of challenges survived versus the available
566 biological measurements (features) due to the small numbers of monkeys in each group. The linear
567 model was fitted using the LASSO, a variant of least-squares regression with an L1 regularization term,
568 implemented in the Python scikit-learn library v0.20.3[116]. This regularization forces most of the
569 coefficients in the final model to be zero and selects only the most important features, to a maximum of
570 7 features for a group of 8 animals, before estimating the final model. The regularization parameter was
571 selected using a leave-one-out cross-validation strategy to achieve the lowest squared prediction error
572 averaged across the fitted models. Each feature was scaled to range from 0 to 1 to facilitate the
573 interpretation of the regression coefficients.

574

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Authors contributions:

M.L. designed and coordinated the study with N.S., M.J.A., Q.L., J.W., D.S., J.C., H.L., interpreted the data and wrote the manuscript. B.L., R.F.S., A.B.L., X.Q.L. analyzed the vaccine efficacy data, performed statistical analysis, LASSO analysis, and the correlates of protection/risk analyses, prepared the figures and helped to write the manuscript. R.W.O. performed the flow cytometry analysis for PBMCs in the blood, FACS data analysis, and helped to write the manuscript. H.L. prepared the rVSVs for vaccinations, performed the studies on antibodies together with L.L. and Y.H. and analyzed the antibody data and prepared some of the figures and tables, and drafted manuscript together with M.L.. N.T. performed the analysis of cytokines in the CVL and performed cytokine analysis with R.W.O. and

926 M.K. and helped for data analysis. N.S. coordinated animal study with D.S. (who performed sampling,
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931 **Competing interests**: M.L. is the inventor of patents owned by Her Majesty The Queen In Right of
932 Canada as represented by The Minister of Health: Canada patent no. 2,833,425 "Protease cleavage site
933 peptides as an HIV vaccine," United States Patent no. 10,285,942 "Methods of inducing an immune
934 response against HIV by administering immunogenic peptides obtained from protease cleavage sites,"
935 and European Patent no. 2,694,654 "Protease cleavage site peptides as an HIV vaccine.

936 **Data and materials availability:** The authors declare that the data supporting the findings of this study
937 are available within the paper and its supplementary information files. The data that support the findings
938 of this study are available from the corresponding author upon reasonable request. The materials can be
939 made available upon request through an MTA.

Table 1 Immune correlates of Protection (CoPs)

Immune response				Correlation with No. of challenges (Spearman rank)	
Type	**Sub-type**	**Ag**	**Time point**	**Rho (r)**	**p**
Cytokine secretion (Ag recall)	**RANTES**	**PCS1-4 peptide pool**	**Peak**	**0.8500**	**0.0075**
Cytokine secretion (Ag recall)	**MIP-1α**	**PCS9-12 peptide pool**	**Peak**	**0.7798**	**0.0225**
Cytokine secretion (Ag recall)	**IL-6**	**PCS1-4 peptide pool**	**Peak**	**0.7148**	**0.0463**
CD4+IL17+ T cells (ex vivo)	**CCR5+% of CD4+IL17+CCR7-CD45RA-**	**N/A**	**Pre-challenge**	**0.7750**	**0.0239**
CD8+IL17+ T cells (ex vivo)	**CCR5+% of CD8+IL17+CCR7+CD45RA-**	**N/A**	**Pre-challenge**	**0.8539**	**0.0070**
CD8+IL17+ T cells (ex vivo)	**CCR5 MFI of CD8+IL17+CCR7+CD45RA-**	**N/A**	**Pre-challenge**	**0.7563**	**0.0299**
CD4+CD25+FoxP3+ T cells (Ag recall)	**PD-1+% of CD4+CD25+FoxP3+CD45RA+CD127-**	**PCS1-12 peptide pool**	**Pre-challenge**	**0.8660**	**0.0054**

Immune factors significantly correlating with number of challenges needed to cause infection were determined by Spearman rank correlation analysis. Ag: antigen (used to quantify antibody or to stimulate cells). CVL: cervicovaginal lavage wash. N/A: not applicable. Peak time point: Week 73 of vaccination schedule (1 week after final vaccination boost). Pre-challenge time point: Week 90 of vaccination schedule. Ab: antibody. Ex vivo: peripheral blood mononuclear cells (PBMCs) were not stimulated with Ag. Ag recall: PBMCs were stimulated with Ag in culture. MFI: mean fluorescent intensity. All analyses were conducted in the PCS vaccine group (n=8).

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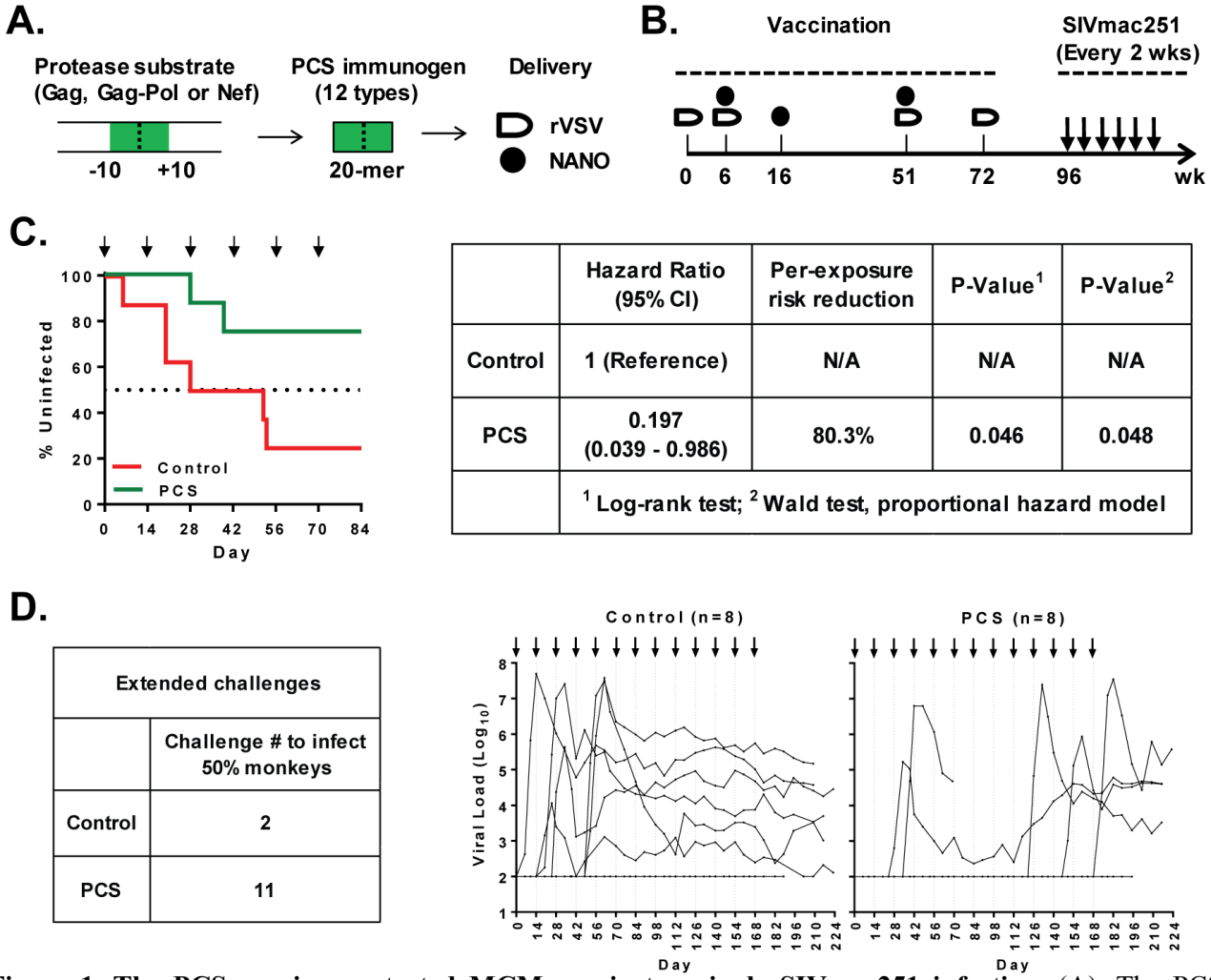

	Hazard Ratio (95% CI)	Per-exposure risk reduction	P-Value[1]	P-Value[2]
Control	1 (Reference)	N/A	N/A	N/A
PCS	0.197 (0.039 - 0.986)	80.3%	0.046	0.048
	[1] Log-rank test; [2] Wald test, proportional hazard model			

Extended challenges	
	Challenge # to infect 50% monkeys
Control	2
PCS	11

Figure 1. The PCS vaccine protected MCMs against vaginal SIVmac251 infection. (A). The PCS vaccine. Twelve 20-mer peptides derived from the twelve protease cleavage site (PCS) regions of SIVmac239 (between -10 and +10 positions flanking each cleavage site) were delivered as recombinant vesicular stomatitis viruses (rVSV) and nanoparticles (NANO). (B). Immunization and challenge scheme. Two groups of animals were involved, the PCS vaccine group (n = 8) and the sham vaccine control group (n = 8). (C) Vaccine efficacy. Left: Kaplan-Meier plot showing the percentage of uninfected animals following challenges. Right: Statistical analyses of vaccine efficacy. (D). Extended challenges beyond the pre-determined, standard challenge protocol. Left: Numbers of challenges required to infect 50% animals in each group. Right: Viral load time course of each animal (note: n = 8 per group while data of some animals at baseline levels overlap and are not visually distinguishable on graph).

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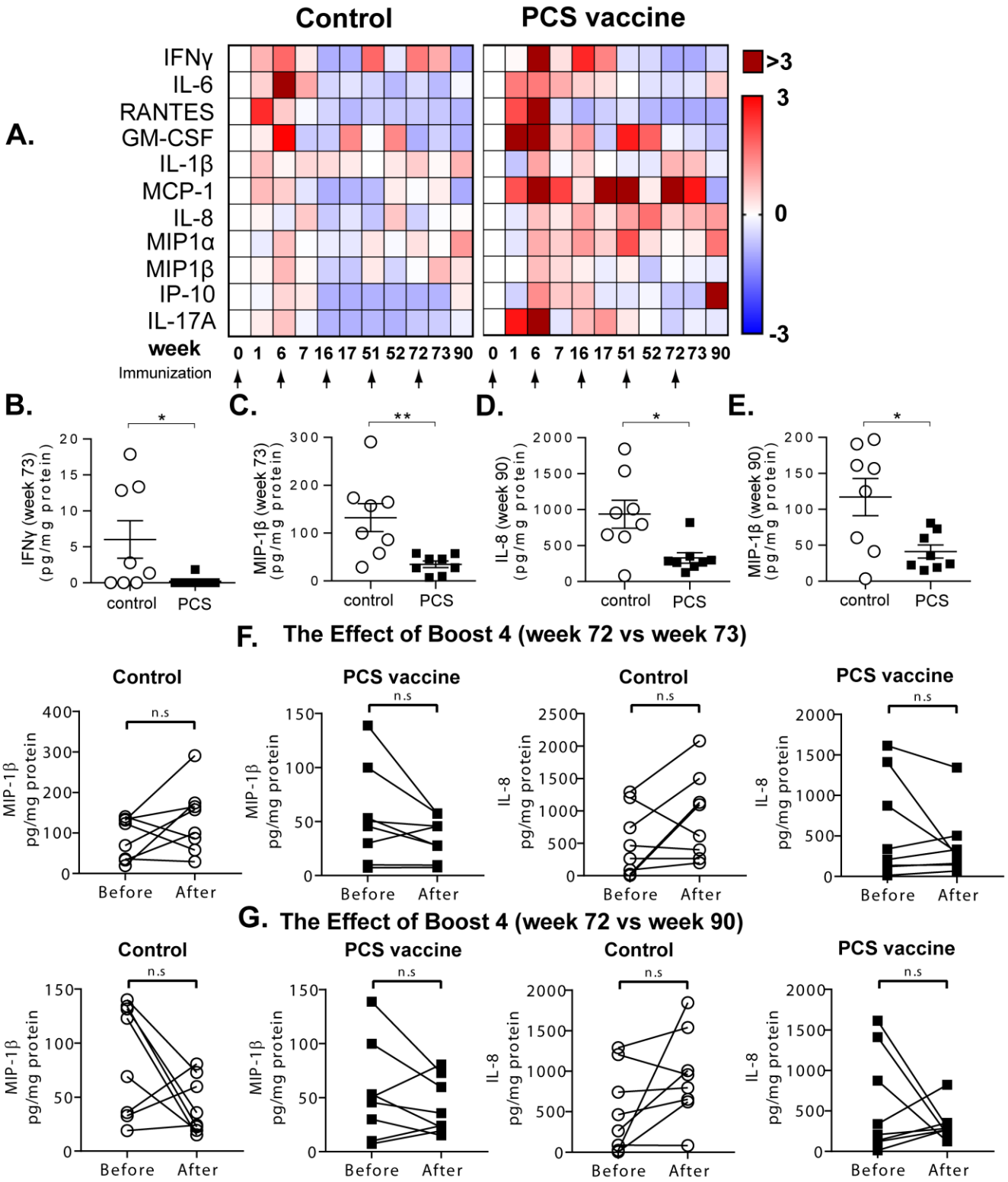

Figure 2. The PCS vaccine and mucosal inflammatory cytokines. (A). Heat maps showing net fold change (relative to pre-immunization baseline) in group median inflammatory cytokine levels at the cervicovaginal mucosa at different time points of the vaccination scheme. (B-E). The PCS vaccine group showed significantly lower levels of several cytokines at indicated time points than the control group. Data are presented as values from individual animals with mean ± SEM. *$p < 0.05$ and **$p < 0.01$ (Mann-Whitney's test). (F). Comparisons of the cytokines in B-E at 4th boost (week 72) and a week after (week 73). (G). Comparisons of the cytokines in B-E at 4th boost (week 72) and 18 weeks after (week 90).

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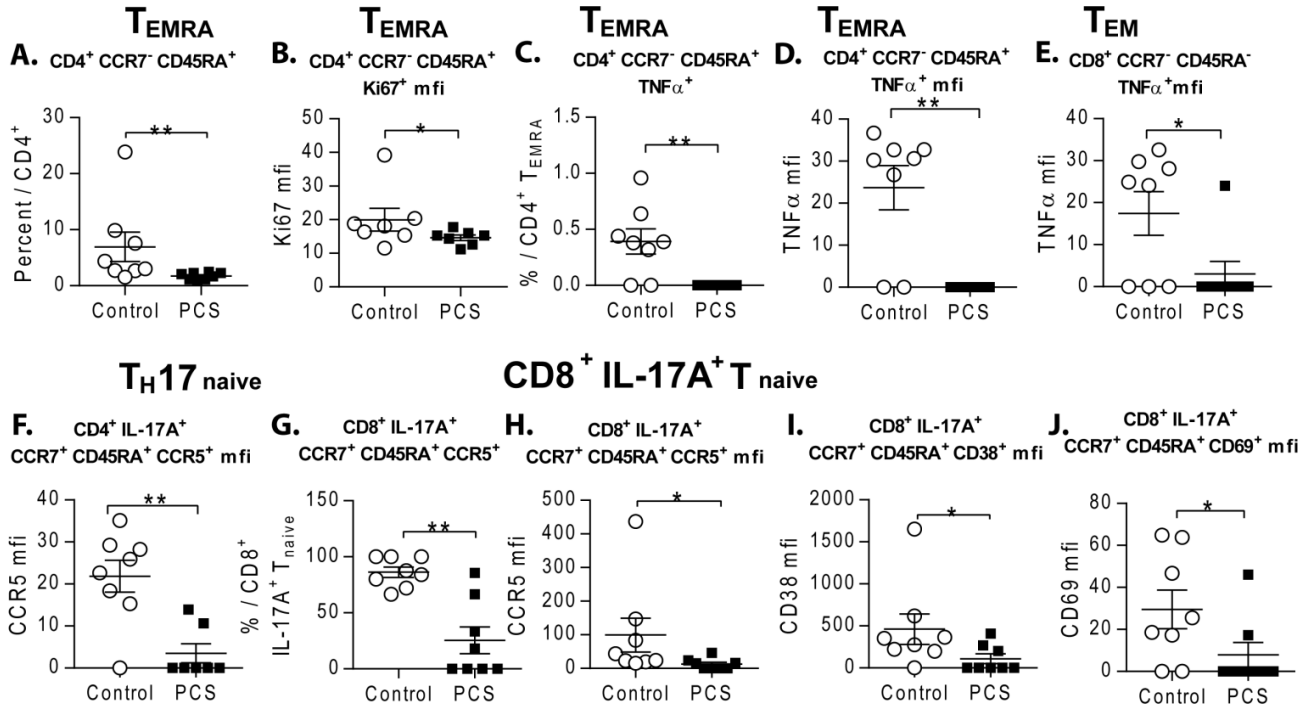

Figure 3. The PCS vaccine induced CD4 and CD8 T cell responses (Ex vivo). PBMCs from the pre-challenge time point (week 90) without antigen stimulation (ex vivo) were analyzed by multicolor flow cytometry. Definition of T cell subsets based on cellular markers. TCM: central memory T cells, Tnaive: naïve T cells, TEM: effector memory T cells and TEMRA: CD45RA+ effector memory T cells. Gating strategy is shown in Fig. S6. A-J: Comparisons of cellular subsets between the control and PCS vaccine group. Data are presented as values from individual animals with median lines. Statistical significance of difference was determined by Mann-Whitney's test: *$p < 0.05$.

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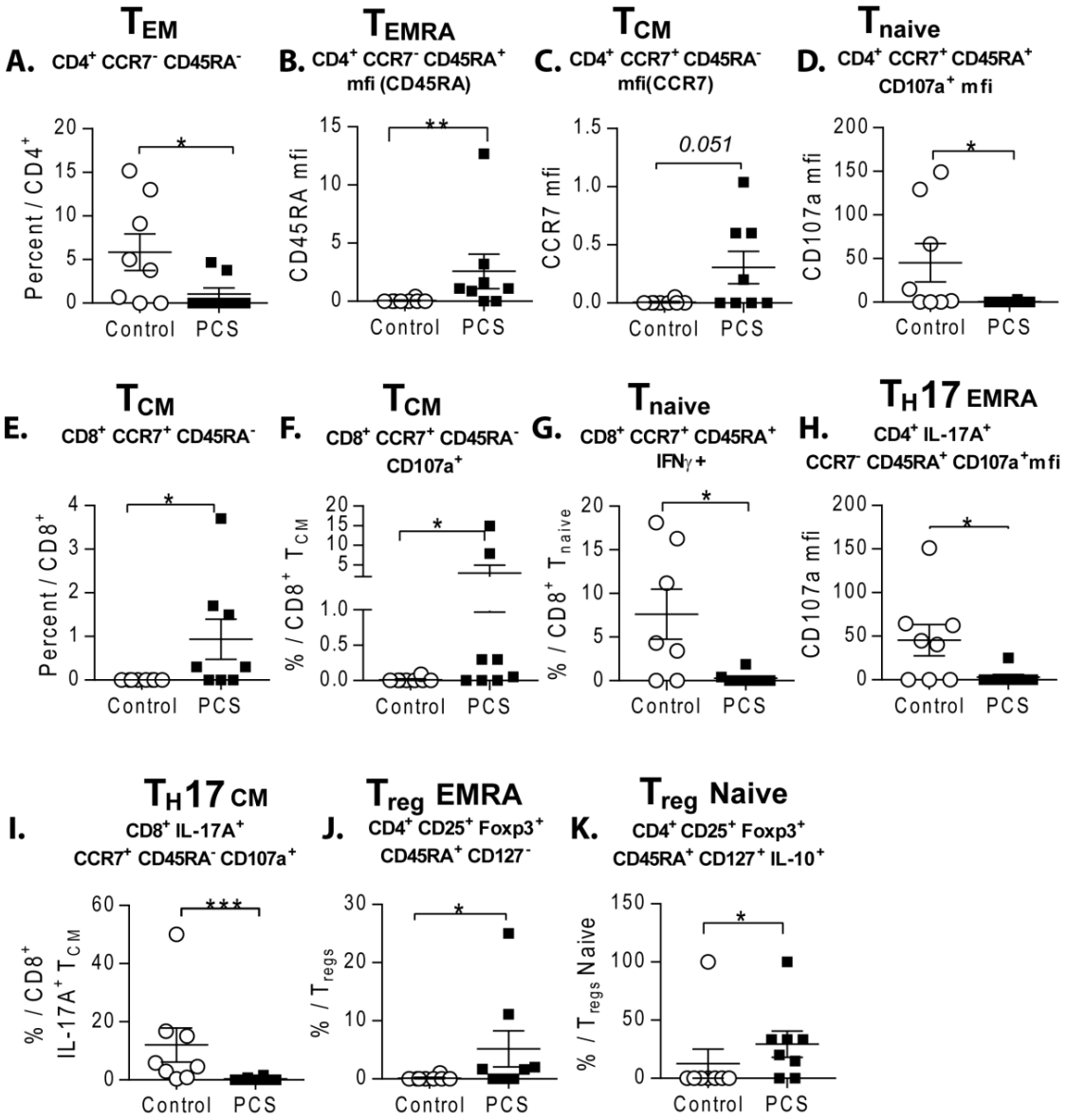

Figure 4. The PCS vaccine induced CD4 and CD8 T cell responses (recall) . PBMCs from the pre-challenge time point (week 90) stimulated with antigen (a total PCS peptide pool:PCS1-12), were analyzed by multicolor flow cytometry. Definition of T cell subsets based on cellular markers. TCM: central memory T cells, Tnaive: naïve T cells, TEM: effector memory T cells and TEMRA: CD45RA+ effector memory T cells. Gating strategy is shown in Fig. S6. A-K: Comparisons of cellular subsets between the control and PCS vaccine group. Data are presented as values from individual animals with median lines. Statistical significance of difference was determined by Mann-Whitney's test: *$p < 0.05$.

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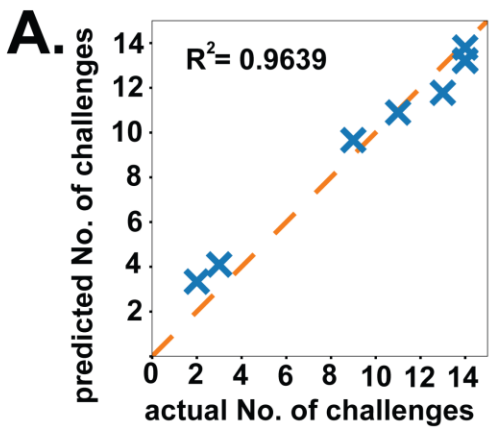

B.

Predictor No.	Predictor name	Immune variant	coefficients
P1	CD4TregEmPD1	CD4+Treg EM PD1+%	2.1849
P2	CD8IL17EmCD107	CD8+IL17A+ TEM CD107a+%	0.7559
P3	CD8TcmIL2	CD8+TCM IL2+%	0.1289
P4	CD4TreNPD1m	CD4+Treg Naïve PD1+ mfi	-1.2588
P5	CD8TregCmIL10	CD8+Treg CM IL10+%	-3.6970
P6	CD4TreCmCD38	CD4+Treg CM CD38+%	-5.3712

Y=10.8932+2.1849*P1+0.7559*P2+0.1289*P3-1.2588*P4-3.6970*P5-5.3712*P6

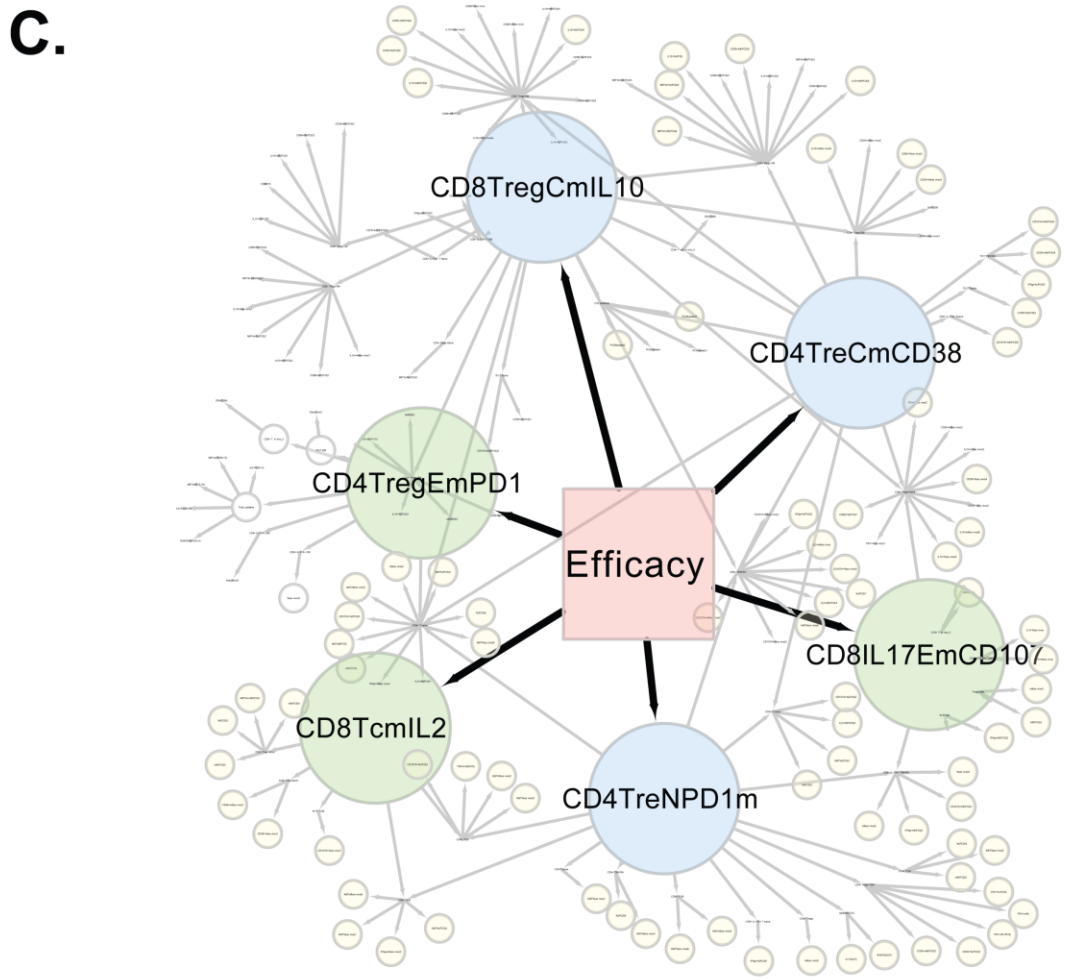

Figure 5. Immune predictors identified by LASSO analysis predict vaccine efficacy. a. LASSO model: Plot of actual versus predicted numbers of challenges from the identified immune predictors; b. The names of identified predictors and their coefficients by LASSO analysis. +/- signs indicate positive or negative association with protection, respectively. c. Networks of the identified immune predictors with other immune variants positively correlated the identified predictors by Spearman rank analysis (Table S8). The 6 identified predictors are labeled as big circled nodes in different colors. Green: predictors with positive co-efficient; Blue: predictors with negative co-efficient. Close-up view of the networks is available through the online version of this figure and in the Figs. S7-12.

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