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High background in ELISpot assays is associated with elevated levels of immune activation in HIV-1-seronegative individuals in Nairobi

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1 **High background in ELISpot assays is associated with elevated levels of immune**
2 **activation in HIV-1-seronegative individuals in Nairobi**

3
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17
18 Written informed consent was obtained from all study participants. This study received
19 ethical approval from the institutional review boards of the University of Washington and
20 the University of Nairobi and was conducted according to the guidelines set forth by the
21 United States Department of Health and Human Services.

22
23 Conflict of interest: None

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25 BLG, CF and BLP revised the manuscript. SCD, BLG and BAR verified data analyses
26 and interpretation. RYC and RB conducted the clinical study. JK provided clinical study
27 space and coordinated the clinical study. CF and BLP conceived of the study and
28 obtained funding.

29

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35

36 Summary:

37 Spontaneous interferon- γ (IFN γ) released detected by enzyme-linked immunospot
38 (ELISpot) assays may be a biological phenomenon. Markers of immune activation
39 levels were assessed as correlates of high background among individuals in Kenya.
40 Couples concordantly seronegative for HIV-1 were enrolled. IFN- γ ELISpot assays were
41 conducted and negative control wells were categorized as having either high or low
42 background (≥ 50 and < 50 SFU/ 10^6 peripheral blood mononuclear cells (PBMC),
43 respectively). PBMC were stained for CD4, CD8, and immune activation markers (CD38
44 and HLA-DR) and analyzed using flow cytometry. Proportions of activated T-cells were
45 compared between those with low and high background by Mann-Whitney U test.
46 Correlates of background SFU and immune activation were assessed using regression
47 models. Among 58 individuals, 14 (24%) had high background. Frequencies of
48 CD4⁺CD38⁺HLA-DR⁺ and CD8⁺CD38⁺HLA-DR⁺ cells were higher in individuals with
49 high background compared to those with low background ($p=0.02$). Higher background
50 SFU was associated with history of sexually transmitted infections ($p=0.03$), and illness
51 in the past 3 months ($p=0.005$), in addition to increased levels of activated CD4⁺ and
52 CD8⁺ cells (p range= $0.008-0.03$). Female gender and male circumcision decreased
53 levels of CD4⁺ and CD8⁺ immune activation (p range= $0.002-0.03$). Additionally, higher
54 background SFU and activated CD4⁺ and CD8⁺ cells were individually associated with
55 positive ELISpot responses to HIV-1 peptide pools (p range= $0.01-0.03$). These findings
56 suggest that increased basal immune responses may be a biological mechanism
57 contributing to higher background ELISpot SFU. Systematic exclusion of data from

58 individuals with increased background in IFN- γ release assays may bias results in
59 population-based studies.

60

61 **Keywords:** ELISpot; interferon gamma; IGRA, immune activation; flow cytometry, CD4,
62 CD8, HLA-DR, CD38

63 Introduction:

64 The IFN- γ enzyme-linked immunospot (ELISpot) assay is widely used to quantify
65 viral, tumor, allo- and auto-antigenic cellular immune responses in clinical and vaccine
66 trials. This assay is quick, cost-effective and one of the most sensitive methods to
67 detect antigen-specific T cell responses {Schmittel, 1997 #1657;Schmittel, 2001 #235}.
68 Quality of ELISpot assay results depends on accurate detection of spot forming units
69 (SFU) through good staining that allows discrimination of specific spots (signal) from
70 non-specific background spots (noise). Therefore, it is necessary to minimize
71 background SFU in negative control wells and to maximize antigen-induced spots in
72 experimental wells to optimize the signal-to-noise ratio.

73 Methodological issues are commonly cited as possible reasons for spot
74 production in negative control wells {Streeck, 2009 #1677}. Although the general
75 consensus is that high SFU in unstimulated wells are due to assay-specific problems
76 and should be excluded from analyses, ELISpot assays are not standardized and
77 exclusion criteria based on high background varies depending on protocols {Streeck,
78 2009 #1677;Cox, 2005 #1489;Immunotec, 2013 #1704}. However, biological factors
79 may also influence basal IFN- γ production detected *ex vivo*. Our group has repeatedly
80 observed dramatic differences in negative control IFN- γ responses from PBMC obtained
81 from two individuals tested on one ELISpot plate, where the only variation in assay
82 protocol occurred at the level of the sample. This observation propelled us to examine
83 correlates of spontaneous IFN- γ secretion in greater detail.

84 Studies have observed elevated immune activation among populations from
85 developing countries compared to North American or European cohorts and

86 environmentally-driven factors, such as chronic infections, limited nutrition, and poor
87 hygienic conditions, have been proposed as possible explanations for these
88 findings{Rizzardini, 1998 #1639;Clerici, 2000 #1479;Lukwiya, 2001 #1582;Messele,
89 1999 #1404;Borkow, 2000 #1459;Koesters, 2004 #1400}. We hypothesized that there
90 may be a biological phenomenon mediated by increased immune activation that results
91 in increased IFN- γ secretion *ex vivo*, read out as higher SFU (background) in negative
92 controls wells of ELISpot assays. We conducted a cross-sectional study to assess the
93 prevalence of high background among HIV-1-seronegative couples and to identify
94 potentially modifiable biological correlates driving these background responses.

95

96 **Materials and Methods**

97 Study population

98 Couples in which both partners tested HIV-1-seronegative were recruited from
99 voluntary counseling and testing centers in Nairobi, Kenya from 2007-2009. Enrolled
100 couples reported having sex with their partner ≥ 3 times in the 3 months prior to
101 screening and did not report any outside sexual partnerships, and women participants
102 were not pregnant. Written informed consent was obtained from all study participants,
103 and ethical approval was received from the Institutional Review Boards at the University
104 of Washington and Kenyatta National Hospital. Couples were seen once in clinic at
105 enrollment. During this visit, clinical staff collected blood and genital specimens and
106 administered questionnaires to collect sociodemographic, sexual behavior, and self
107 reported medical history data.

108

109 Laboratory procedures

110 Couples were determined to be concordantly HIV-1-seronegative using the
111 Determine HIV-1/2 rapid test (Abbott, Japan) and the Bioline HIV 1/2 rapid test
112 (Standard Diagnostics, Korea). HSV-2 serology was determined using the HerpeSelect
113 IgG ELISA kit (Focus Technologies, USA). Equivocal HSV-2 test results were analyzed
114 as either a negative or a positive result. Syphilis was tested using a rapid plasma reagin
115 (RPR) test (Becton Dickinson (BD), USA); reactive tests were confirmed by *T. pallidum*
116 haemagglutination assay (Randox, UK). For female participants the following tests were
117 conducted: urine pregnancy tests (Quick Vue One Step hCG Urine Pregnancy kit,
118 Quidel Corporation, USA), *Trichomonas vaginalis* cultures (In-Pouch TV, Biomed
119 Diagnostics, USA), and bacterial vaginosis (BV).

120

121 IFN- γ ELISpot assays

122 Blood samples from the couples were collected on the same day and batch
123 processed to reduce introduction of within-couple variability. ELISpot assays were
124 conducted to evaluate the frequency of background and antigen stimulated SFU with a
125 previously described protocol using Millipore plates (Millipore, USA) and Mabtech
126 (Mabtech, Sweden) reagents {Lohman, 2005 #1579}. One $\times 10^5$ freshly isolated
127 peripheral blood mononuclear cells (PBMC)/well were stimulated with
128 phytohemagglutinin (PHA) (Murex Biotech Ltd, UK) in triplicate as positive control wells,
129 media alone in 9 negative control wells, or HIV-1 peptide pools in triplicate experimental
130 wells. Twenty-two peptide pools of 15-mers overlapping by 10 amino acids spanning the
131 HIV-1 genome were derived from the HIV-1 subtype A consensus sequence (Sigma-

132 Gynosys, USA). Plates were read on a CTL ImmunoSpot S4 Core Analyzer, and
133 analyzed using ImmunoSpot Software (Cellular Technology Ltd, USA).

134 The background response, defined as the mean SFU in the 9 negative control
135 wells, was categorized as low (<50 SFU/ 10^6 PBMC) or high (≥ 50 SFU/ 10^6 PBMC).
136 Background responses were examined both as a dichotomous (pre-defined cutoffs
137 above) and continuous (magnitude of responses) variable. HIV-1-stimulated SFU were
138 defined as the average number of spots in triplicate wells minus background. IFN- γ
139 ELISpot responses were considered positive if experimental wells had ≥ 50 HIV-1-
140 stimulated SFU/ 10^6 PBMC and more than twice the background response. Individuals
141 were defined as positive ELISpot responders if they had ≥ 1 peptide pool with a positive
142 response.

143

144 Immune activation assays

145 Immune activation markers were measured on fresh whole blood specimens.
146 Specimens were stained with the following pre-mixed 4-color fluorochrome-conjugated
147 antibody combinations: anti-CD4-FITC, anti-CD38-PE, anti-CD3-PerCP, anti-HLA-DR-
148 APC and anti-CD8-FITC, anti-CD38-PE, anti-CD3-PerCP, anti-HLA-DR-APC (BD).
149 Specimens were run on a 4-color FACSCalibur flow cytometer (BD), and flow cytometry
150 data was analyzed and quality controlled using FlowJo software (Treestar, USA). Gates
151 were set conservatively to capture high-level expression of CD38/HLADR. Percentages
152 of activated cells, defined as CD38⁺HLA-DR⁺, CD38⁻HLA-DR⁺, or HLA-DR⁺ (CD38⁺ or
153 CD38⁻), in both CD4⁺ and CD8⁺ subsets were used in analyses.

154

155 Statistical methods

156 Activated cells were compared between individuals with high and low
157 background responses and between positive and negative ELISpot responders using
158 Mann-Whitney U tests. Linear regression with robust standard errors was used to
159 assess correlates of background response. Data was \log_{10} transformed when examining
160 magnitude of background SFU as a continuous variable to achieve a normal distribution.
161 A set of characteristics was selected *a priori* as potential correlates of immune activation.
162 Both partners of the couple reported number of sex acts with their study partner in the
163 past month, and the mean number of acts reported by the couple was used for analysis.
164 Also, any individual who self-reported having a fever, diarrhea, vomiting or a cough in
165 the past 3 months was considered to have a recent illness. To determine correlates of
166 immune activation, immune activation data was modeled as a proportion, and
167 multivariate analyses were performed for each characteristic adjusting for gender and
168 age using generalized linear models with logit link and robust standard errors. All
169 analyses were conducted using Stata version 11.2 statistical software (College Station,
170 USA).

171

172 **Results**

173 Study population characteristics

174 Fifty-eight individuals in monogamous, concordant HIV-1-seronegative
175 relationships were included in the analyses. Among these individuals, 29 (50%) were
176 females and the median age for all participants was 27 (interquartile range [IQR] 23-31)
177 (Table 1). Median length of cohabitation was 1 year (IQR 0-7). Participants reported a

178 median of 4 lifetime sexual partners (IQR 3-6) and 5 sex acts (IQR 3-12) with their study
179 partner in the past month. From self-reporting, 43 (74%) had unprotected sex in the past
180 month, 12 (21%) had a history of sexually transmitted infections (STI), and 5 (9%) had
181 an illness (fever, diarrhea, vomiting, or cough) in the past 3 months. Twenty (35%)
182 individuals were HSV-2 seropositive or had an equivocal result, and 24 (83%) of the
183 men were circumcised. Among the women, 20 (69%) used birth control, the most
184 common form was oral, injectable or implanted hormonal contraceptives.

185

186 ELISpot background response and immune activation

187 Of the 58 low-risk participants, the median magnitude of background response
188 was 24 SFU/10⁶ PBMC (IQR 10-44). Replicate spot counts per well based on input cell
189 number of 1 × 10⁵ cells are shown in Figure 1a, demonstrating the range of individual
190 variability within the cohort. When ELISpot background responses were dichotomously
191 categorized, the majority (n=44,76%) individuals had low background secretion of INF- γ ,
192 however 14 (24%) individuals were categorized as having high background with a
193 median of 61 SFU/10⁶ PBMC (IQR 54-121), shown in detail in Figure 1b as partners
194 within each couple. Approximately half the individuals were in partnerships with other
195 'high background' individuals, while the other half were in partnerships with individuals
196 characterized as low background on the same plate, as shown in Figure 1c.

197 T cells were surface stained with immune activation markers CD38⁺ and HLA-
198 DR⁺ and analyzed to determine the level of immune activation. Depending on
199 differential CD38⁺ and HLA-DR⁺ phenotypes, 10-40% more CD8⁺ T cells expressed
200 markers of activation compared to CD4⁺ T cells (Figure 2). When compared by ELISpot

201 background response, frequency of CD4⁺ T cells co-expressing CD38⁺ and HLA-DR⁺
202 was significantly elevated in individuals with high background compared to those with
203 low background ($p=0.02$) (Figure 3a). Similarly, compared to individuals with low
204 background, those with high background had higher frequencies of CD8⁺ HLA DR⁺ T
205 cells with and without CD38 expression ($p=0.02$ and $p=0.05$, respectively) (Figure 3b).

206

207 Correlates of IFN- γ ELISpot background response

208 Univariate and multivariate models were used to evaluate the association
209 between ELISpot background, immune activation and sociodemographic variables.
210 ELISpot background was modeled as a continuous variable to increase the power of
211 detecting an association and because a biologically meaningful cutoff is not known.
212 Variables linked to infectious processes were associated with increased background. In
213 univariate models, individuals who reported a history of STI or recent illness had higher
214 magnitudes of \log_{10} background SFU compared to those who did not ($\beta=0.82$, 95%
215 confidence interval [CI]=0.07-1.56, $p=0.03$; $\beta=0.98$, 95% CI=0.31-1.65, $p=0.005$,
216 respectively) (Table 2). There was also a trend toward higher \log_{10} background SFU for
217 individuals who were HSV-2 positive or equivocal ($p=0.07$).

218 Furthermore, T cell activation was associated with higher magnitude of
219 background response, similar to the previous associations when background response
220 was examined as a dichotomous variable. Among CD4⁺ T cells, an increase in
221 proportions of CD38⁻HLA-DR⁺ and total HLA-DR⁺ cells were associated with an
222 increase in \log_{10} background SFU ($\beta=0.42$, 95% CI=0.06-0.79, $p=0.03$; $\beta=0.39$, 95%
223 CI=0.08-0.71, $p=0.02$, respectively). Analogous associations were found with higher

224 CD8⁺ T cell activation (CD38⁺HLA-DR⁺: $\beta=0.16$, 95% CI=0.04-0.28, $p=0.008$; HLA-DR⁺:
225 $\beta=0.13$, 95% CI=0.03-0.23, $p=0.01$). To determine whether immune activation or
226 infectious processes was driving the increased background responses, multivariate
227 analyses were conducted. Infectious processes and immune activation both remained
228 statistically significant correlates of magnitude of background SFU (range of p-values:
229 $<0.001 - 0.05$), except for the model including CD4⁺CD38⁺HLA-DR⁺ cells.

230

231 Correlates of immune activation

232 Having seen an association between T cell activation and IFN- γ ELISpot
233 background response, additional analyses were conducted to examine correlates of
234 immune activation. These analyses were adjusted for gender and age. Females were
235 more likely to have activated CD4⁺CD38⁺HLA-DR⁺ (aOR=0.73, 95% CI=0.58-0.91,
236 $p=0.006$) and total HLA-DR⁺ (aOR=0.68, 95% CI=0.52-0.87, $p=0.002$) compared to
237 males (Table 3). Additionally, trends were observed indicating that women who reported
238 any unprotected sex in the past month were more likely to have activated CD38⁺HLA-
239 DR⁺ cells ($p=0.10$), and those who had BV were more likely to have a higher proportion
240 of cells expressing HLA-DR⁺ ($p=0.09$).

241 A different set of characteristics was observed to be associated with CD8⁺
242 immune activation. Circumcised men were less likely to have activated CD38⁺HLA-DR⁺
243 cells (aOR=0.55, 95% CI=0.32-0.94, $p=0.03$). Furthermore, there were trends for
244 females who were positive for BV to have increased proportions of total HLA-DR⁺ cells
245 ($p=0.07$) and for those who reported hormonal contraceptive use to have decreased
246 frequency of total HLA-DR⁺ cells ($p=0.08$).

247

248 Background and immune activation by antigen-stimulated IFN- γ ELISpot response

249 To assess the association between immune activation and ELISpot response to
250 antigen-specific stimulation, levels of activated cells were compared between individuals
251 who had positive HIV-1 ELISpot results and those who had negative results. The
252 majority (50/58, 86%) had negative IFN- γ responses to HIV-1 peptides and 9 of these
253 50 (18%) had high background. In comparison 8 participants (14%) were categorized as
254 positive responders and among those 63% (5/8) had a high background response. Thus,
255 individuals with a high background response were ~7 times more likely to have a
256 positive IFN- γ ELISpot response to HIV peptide pools (OR=7.6, $p=0.01$). Additionally, a
257 strong positive correlation was seen between magnitude of background response and
258 magnitude of HIV-1-stimulated SFU (Spearman's $\rho=0.43$, $p<0.001$, data not shown).
259 Furthermore, CD38+HLADR+ expression in both CD4+ and CD8+ T cell subsets were
260 significantly elevated in individuals who had a positive ELISpot response compared to
261 those who did not (Figure 4). The association between presence of a positive response
262 and magnitude of background response remained statistically significant ($p\leq 0.05$) after
263 adjusting for frequency of CD8+HLADR+ activated cells, except when adjusting for the
264 CD4+HLA-DR+ phenotype, which showed a trend ($p=0.08$, data not shown).

265

266 Discussion

267 In this study we found of high spontaneous IFN- γ release in ELISpot assays
268 conducted on PBMC from ~25% of individuals and these individuals were more likely to
269 have elevated levels of immune activation in both CD4+ and CD8+ T cells. Naïve T cells

270 differentiate into memory T cells after being activated {Hazenberg, 2000 #1530}, and
271 the activated effector cells are then responsible for producing cytokines *in vivo*, most
272 typically only during the acute immune response before the cells die or differentiate into
273 resting memory cells. As other studies in developing countries have speculated, our
274 participants are likely exposed to a wide spectrum of pathogens that may result in
275 persistent infections that cause chronic immune activation. Thus, these findings support
276 the hypothesis that highly activated T cells contribute to spontaneous IFN- γ production
277 *ex vivo* detected in IFN- γ release assays.

278 We found that a history of having a STI or an illness in the past 3 months was
279 significantly associated with high background SFU, even after adjusting for immune
280 activation. We expected these markers of infection to lead to an increase in activated
281 cells, and subsequently, elevated background response; however, the immune
282 activation markers that were measured may not have captured the whole T cell
283 activation pathway. In addition, since unfractionated PBMC were added to our ELISpot
284 assays, the cells of the innate immune system, particularly NK cells, may also be
285 responsible for IFN- γ production detected in the negative control wells {Dittrich, 2012
286 #1496}.

287 Interestingly, different correlates were found for high background response and
288 immune activation. Women had lower median frequencies of activated CD4⁺CD38-
289 HLADR⁺ cell phenotypes compared to men, which is consistent with a previous study
290 that found African American women had decreased levels of the immune activation
291 marker neopterin compared to African American men {Spencer, 2010 #1672re}. The
292 preponderance of HLA-DR⁺ cells relative to CD38⁺ populations was unexpected. Male

293 circumcision was associated with decreased immune activation. Since results from
294 large randomized clinical trials showed a strong association between male circumcision
295 and protection against HIV-1 infection {Auvert, 2005 #1437;Bailey, 2007 #1440;Gray,
296 2007 #1521}, it is not surprising that circumcised men have lower levels of activated T
297 cells. Furthermore, this finding may provide an explanation as to why uncircumcised
298 men had a higher risk for HIV-1 infection in the Step Study {McElrath, 2008 #1595}.

299 To better understand the detection of HIV-1-stimulated responses in our HIV-1-
300 uninfected participants, we determined the effect of background SFU and immune
301 activation on the ELISpot response. We observed higher proportions of activated T cells
302 were associated with positive ELISpot responses. These results are similar to a
303 previous study that showed HIV-1-negative Kenyan women who had elevated T cell
304 immune activation expressed significantly increased IFN- γ production when stimulated
305 with superantigen staphylococcal enterotoxin B {Koesters, 2004 #1400}. Our results
306 suggest that either immune activation leads to non-specific IFN- γ release that may
307 interfere with the classification of having a positive response due to background (i.e.
308 false positives), or immune activation leads to higher levels of cross-reactivity to HIV-1
309 peptides. Furthermore, we found that individuals with higher background SFU were
310 more likely to have a higher magnitude HIV-1-stimulated response. When including both
311 background response and CD8+CD38+HLA-DR⁺ immune activation in the model for
312 predictors of a positive antigen stimulated ELISpot response, background remained the
313 only statistically significant predictor. The results from adjusted models suggest
314 activated T cells are hyper-responsive, secreting higher levels of cytokines when
315 stimulated, and thereby producing false positive ELISpot responses mediated through

316 higher background responses. While these findings support the idea that activated cells
317 may have contributed to false positive HIV-1-specific cellular responses through
318 background response, it does not preclude the possibility of the participants being
319 unwilling to acknowledge exposure to HIV-1 from outside partnerships, exposure that
320 can lead to HIV-1 specific responses{Guthrie, 2012 #1525}.

321 Our data suggest criteria for defining positive ELISpot responses balance the risk
322 of false positive responses with the risk of introducing significant bias through the
323 selective removal of data from individuals with high background. Chronic immune
324 activation can lead to immune dysfunctions such as anergy, activation-induced cell
325 death, cytokine dysregulation, and impaired signal transduction {Lukwiya, 2001
326 #1582;Bentwich, 1998 #1446;Alimonti, 2003 #1431;Leng, 2002 #1576}, and previous
327 studies have demonstrated the importance of the host's pre-existing immune status in
328 influencing the efficacy of immune responses to vaccine or pathogen challenge {Borkow,
329 2002 #1458;Black, 2002 #1455}. It has been proposed that the activation state of the
330 participants may be a possible explanation for the unexpected increase in HIV-1
331 acquisition found in the Step study {Sekaly, 2008 #1661;McElrath, 2008 #1595}.

332 Individuals who have elevated immune activation levels may have difficulty mounting an
333 effective immune response, so excluding these individuals may significantly bias study
334 findings, especially for vaccine trials.

335 While a strength of this study was performing ELISpot assays on fresh samples
336 to optimize sensitivity, rerunning the assays on frozen aliquots may yield different
337 findings. Due to limited numbers of PBMC, we were not able to compare responses
338 between fresh and frozen samples. Another limitation of this study is the limited data on

339 co-infections within this population. Helminth infections, malaria and TB are highly
340 endemic in Kenya and cause a chronic immune activation state, but we were unable to
341 diagnosis these conditions within this study.. Furthermore, unfractionated PBMC were
342 used in the assay, and together with the use of 15-mers peptides overlapping by 10
343 amino acids, the cell type responsible for IFN- γ secretion was not identified. While these
344 factors may limit the scope of our conclusions, they do not affect the foundational
345 findings.

346 In conclusion, our results suggest that elevated levels of immune activation,
347 along with previous infections, are associated with both higher background IFN- γ
348 secretion and positive HIV-1 stimulated ELISpot responses in a small but significant
349 percent of the general population. Additionally, different correlates were found to
350 contribute to T cell activation, highlighting the intricacies of the immune response. This
351 study indicates that conditions that lead to a persistently activated immune state in HIV-
352 1-seronegative individuals in Kenya can have a dramatic effect on immunological
353 assays, and as such, the pre-existing immune profile of populations should be
354 considered when developing and testing assays for measuring immunogenicity of
355 pathogens or potential vaccines.

356

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371

372 **Disclosure:**

373 The authors declare no conflicts of interest.

374

375 **References**

Figure Legends

Figure 1. Background response of ELISpot assays among HIV-1-uninfected

individuals. Negative control wells (replicates of 9) containing 10^5 PBMC and media only were cultured overnight for determination of background IFN- γ response. A reference line at 5 spots represents the cut-off number for categorizing a high (≥ 5 SFU/ 10^5 PBMC) vs. low (< 5 SFU/ 10^5 PBMC) response. Individual wells (squares) and mean \pm SEM are shown per person. Individuals scoring positive for HIV peptide responses ($> 2\times$ background and ≥ 50 spots) are indicated in red. a) Range of IFN- γ responses from 58 HIV-1-seronegative individuals. b) Couples in which one or both individuals scored as high background by IFN- γ response. c) ELISpot plate images from positive (columns 1-3) and negative control wells (columns 4-12) are shown for partners (A and B) of a couple run with the same assay conditions. Partner A was categorized as having a high background response and partner B as a low background response.

Figure 2. Example of staining profile for immune activation markers CD38⁺ and

HLA-DR⁺ on CD4⁺ and CD8⁺ T cells. CD38⁺ and HLA-DR⁺ staining among CD4⁺ and CD8⁺ T cells for an individual. Quadrants were placed conservatively high as activated cells generally have bright expression of these markers and this placement achieves a restrictive estimate of activated cells, reducing the likelihood of falsely categorizing cells as activated.

Figure 3. Immune activation markers by ELISpot background response.

Frequency of activated cells in (a) CD4⁺ T and (b) CD8⁺ T cell subsets are shown for

individuals with low (grey box plot, <50 SFU/ 10^6 PBMC) and high (open box plot, ≥ 50 SFU/ 10^6 PBMC) background response. Upper, middle, and lower lines of the box show group 75th percentile, median, and 25th percentile, respectively, individual outliers indicated by solid circles. P-values compare the distribution of the percent of activated cells for individuals with low background to those with high background, based on the Mann-Whitney U test.

Figure 4. Immune activation markers by ELISpot response. Frequency of activated cells in (a) CD4⁺ T and (b) CD8⁺ T cell subsets are shown for individuals with negative (filled box) and positive (open box) ELISpot response, defined as 2-fold over background and at least 50 SFU/ 10^6 cells. Upper, middle, and lower lines of the box show group 75th percentile, median, and 25th percentile, respectively. P-values compare the distribution of the percent of activated cells for individuals with negative response to those with positive response, based on the Mann-Whitney U test

Table 1. Cohort characteristics of concordant HIV-1-seronegative couples.

Characteristic	Median (IQR) or n (%) (N=58)*
Female	29 (50)
Age	27 (23 - 31)
Years living together	1 (0 - 7)
Lifetime sexual partners	4 (3 - 6)
Sex acts ^a	5 (3 -12)
Any unprotected sex ^a	43 (74)
History of STI	12 (21)
HSV-2 serostatus	
Negative	37 (65)
Equivocal	5 (9)
Positive	15 (26)
Bacterial vaginosis	5 (31)
Recent illness ^b	5 (9)
Male circumcision	24 (83)
Birth control use	20 (69)
Hormonal birth control use ^c	13 (45)

IQR, interquartile range; STI, sexually transmitted infection

* N=29 for characteristics unique to men or women. Bacterial vaginosis results were available for 16 women.

^a With study partner in the past month

^b Reported fever, diarrhea, vomiting, or cough in the past 3 months

^c Hormonal birth control use defined as oral, injectable, or implant contraceptives

Table 2. Correlates of magnitude of ELISpot background responses.

Characteristic (N=58)*	Coeff	95% CI	p-value
<i>Univariate Analyses^a</i>			
Female	-0.03	-0.68 – 0.61	0.92
Age (per 5 years)	0.03	-0.14 – 0.20	0.73
Years living together	-0.03	-0.18 – 0.11	0.64
High sexual partners ^b	0.22	-0.42 – 0.87	0.49
Sex acts ^c	-0.001	-0.05 – 0.05	0.97
Any unprotected sex ^c	0.54	-0.19 – 1.27	0.15
History of STI	0.82	0.07 – 1.56	0.03
HSV-2 seropositive (positive or equivocal) ^d	0.60	-0.05 – 1.25	0.07
Bacterial vaginosis	-0.31	-1.45 – 0.83	0.57
Recent illness ^e	0.98	0.31 – 1.65	0.005
Male circumcision	-0.35	-1.27 – 0.56	0.43
Hormonal birth control use ^f	-0.58	-1.51 – 0.36	0.22
CD4 ⁺ CD38 ⁺ HLA-DR ⁺	0.76	-0.44 – 1.96	0.21
CD4 ⁺ CD38 ⁻ HLA-DR ⁺	0.42	0.06 – 0.79	0.03
CD4 ⁺ HLA-DR ⁺	0.39	0.08 – 0.71	0.02
CD8 ⁺ CD38 ⁺ HLA-DR ⁺	0.23	-0.05 – 0.51	0.10
CD8 ⁺ CD38 ⁻ HLA-DR ⁺	0.16	0.04 – 0.28	0.008
CD8 ⁺ HLA-DR ⁺	0.13	0.03 – 0.23	0.01
<i>Multivariate Analyses^g</i>			
History of STI	0.70	0.07 – 1.33	0.03
Recent illness	0.95	0.51 – 1.39	<0.001
CD4 ⁺ CD38 ⁺ HLA-DR ⁺	0.82	-0.28 – 1.92	0.14
Recent illness	0.92	0.37 – 1.46	0.001
CD4 ⁺ CD38 ⁻ HLA-DR ⁺	0.40	0.05 – 0.75	0.03
Recent illness	0.96	0.45 – 1.48	<0.001
CD4 ⁺ HLA-DR ⁺	0.39	0.08 – 0.69	0.01
History of STI	0.73	0.06 – 1.40	0.03
Recent illness	0.95	0.48 – 1.42	<0.001
CD8 ⁺ CD38 ⁺ HLA-DR ⁺	0.27	0.00 – 0.53	0.05
History of STI	0.67	0.02 – 1.32	0.04
Recent illness	0.91	0.48 – 1.34	<0.001
CD8 ⁺ CD38 ⁻ HLA-DR ⁺	0.17	0.05 – 0.28	0.004
History of STI	0.68	0.05 – 1.32	0.04
Recent illness	0.95	0.52 – 1.39	<0.001

CD8 ⁺ HLA-DR ⁺	0.13	0.04 – 0.23	0.005
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Coeff = beta coefficient from regression model; CI = confidence interval; STI = sexually transmitted infections

* N=29 for characteristics unique to men or women. Bacterial vaginosis results were available for 16 women.

^a Association between log₁₀ background SFU and each covariate is examined separately in unadjusted models

^b Lifetime sexual partners dichotomized at median: <4 and ≥4

^c With study partner in the past month

^d HSV-2 seropositive defined as positive or equivocal HSV-2 test results

^e Reported fever, diarrhea, vomiting, or cough in the past 3 months

^f Hormonal birth control use defined as oral contraceptives, injectables, or implants

^g Multivariate analyses included history of STI, recent illness, and immune activation as covariates; models with CD4⁺C38⁺HLA-DR⁺ and CD4⁺HLA-DR⁺ cells only included recent illness due to co-linearity with history of STI

BOLD indicates p≤0.05