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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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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.

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35

36 **Summary:**

37 Spontaneous interferon-γ (IFNγ) 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 $CD38^+HLA\text{-}DR^+$, $CD38^-HLA\text{-}DR^+$, or $HLA\text{-}DR^+$ ($CD38^+$ 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-sero*negative*

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/10^6 PBMC (IQR 10-44). Replicate spot counts per well based on input cell

189   number of 1×10^5 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/10^6 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 (β=0.82, 95%

215 confidence interval [CI]=0.07-1.56, p=0.03; β=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 (β=0.42, 95% CI=0.06-0.79, p=0.03; β=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$^+$: β=0.16, 95% CI=0.04-0.28, p=0.008; HLA-DR$^+$:

225 β=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 ρ=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≤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-stimuated 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 selectively 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

357 **Acknowledgements**

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 ± SEM are shown per person. Individuals scoring positive for HIV peptide responses (>2× 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
Univariate Analyses [a]			
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**
Multivariate Analyses [g]			
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** |

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 log$_{10}$ 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