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14

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Abstract

Objectives

Human papillomavirus (HPV)-associated cancers disproportionately affect those infected with human immunodeficiency virus (HIV) despite effective combination antiretroviral therapy (cART). The primary aim of this study was to quantify HPV16 and 52 E6-specific interferon (IFN)-γ ELISPOT T-cell responses, a correlate of protective immunity in the first year following cART and subsequently in those patients with suboptimal (sIR) and optimal immune reconstitution (oIR).

Methods

94 HIV-infected patients were recruited; a longitudinal cohort just prior to commencing cART and followed-up for 48 weeks (n=27), and a cross-sectional cohort (n= 67) consisting of patients with sIR (CD4+ T-cell <350cells/μl) and oIR (CD4+ T-cell >500cells/μl) after a minimum of two years on cART. Controls (n=29) consisted of HIV-negative individuals. Interferon-γ ELISPOT responses against HPV16 and 52 E6 were correlated to clinical

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characteristics, anal and oral HPV carriage, T-cell maturational subsets, markers of activation, senescence and T-regulatory cells.

Results

HPV16 and 52 E6-specific T-cell responses were detected in only 1/27 (3.7%) patients during the initial phase of immune recovery. After at least 2 years of cART, those who achieved oIR had significantly higher responses (9/34, 26.5%) compared to sIR (2/32, 6.3%) (p=0.029). Apart from higher CD4+ T-cell counts and lower CD4+ T-cell activation, no other immunological correlates were associated with the detection of HPV16 and 52 E6-specific responses.

Conclusions

HPV16 and 52 E6-specific IFN-γ T-cell responses, a correlate of protective immunity, was detected more frequently among HIV patients who achieved optimal immune recovery on cART (26.5%) compared to those with suboptimal recovery (6.3%).

Word count: 248 words

Key words

Human papillomavirus (HPV), HPV-specific immune responses, human immunodeficiency virus (HIV), immune recovery, cancer, combination antiretroviral therapy (cART)

BACKGROUND

Effective combination antiretroviral therapy (cART) has led to a dramatic increase in the life expectancy among individuals living with human immunodeficiency virus (HIV) (1, 2). While opportunistic infections have reduced significantly among those living with HIV, the incidence of human papillomavirus (HPV)-associated diseases including cancers are increasing (3, 4). This is consistent with the rising burden of HPV-associated cancers even among those without HIV (5, 6). When compared to the general population in the USA, those living with HIV had a 38-fold higher risk of developing anal cancer and 2.7-fold increase risk of invasive cervical cancer (7). In addition, HPV-associated oral cancer is also 3-fold more common in HIV-positive men than in the general population (8). It is estimated that 15% of

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96 all new cancer diagnosis among those living with HIV can be attributed to HPV infection (7)
97 compared to 4.8% among the general population (9).
98
99 Cellular immune responses are central in balancing health and disease states associated
100 with HPV. The role of cellular immune responses to HPV have been demonstrated in
101 immune-histological studies where the regression of cervical intraepithelial neoplasia and
102 genital warts have been associated with an influx of CD4+ T-cells, CD8+ cytotoxic T-cells
103 and macrophages (10, 11), while HPV-specific T-cell responses to HPV E6 antigens have
104 been consistently shown to correlate with virological clearance or clinical regression of HPV-
105 associated diseases (12-15). This in turn has been exploited in the development of therapeutic
106 vaccines where immunological responses to E6 was found to correlate with clinical outcomes
107 (16-18). Therefore, systemic HPV-specific E6 responses can be used to identify those who
108 will likely mount a protective cellular immune response against HPV infection and its related
109 diseases compared to those who may fail. The higher prevalence of HPV infection (19) and
110 cancers among individuals living with HIV suggest that despite apparently optimal immune
111 reconstitution as assessed by CD4+ T-cell count and viral suppression, a protective
112 immunological response against oncogenic HPV may either never or inadequately be
113 mounted. A better understanding of the natural history of HPV infection and functional
114 reconstitution of HPV-specific immunity in those with living with HIV would inform
115 prevention and treatment strategies against HPV-associated diseases.
116
117 We therefore explored the association of HPV immunity in relation to CD4+ T-cell
118 reconstitution among HIV-infected individuals receiving suppressive cART. Our primary aim
119 was to define immunological correlates of HPV16 and 52 E6-specific immune responses
120 following cART. We also explored the prevalence and risk factors associated with HPV
121 DNA detection in the anogenital tract and oral cavity among this multi-ethnic Asian HIV-
122 infected cohort receiving suppressive cART.
123

124 **METHODS**

125

126 **Study population**

127 Two cohorts of HIV-infected patients (longitudinal and cross-sectional) were recruited from
128 the Infectious Diseases Unit (IDU), University Malaya Medical Centre (UMMC). The
129 longitudinal cohort (n=27) comprised of patients commencing cART (t=0 weeks) and were

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followed-up for 48 weeks (t=12, 24 and 48 weeks). The inclusion criteria were: men or women >18 years old, naïve to cART, no acute illness and not pregnant at recruitment. The cross-sectional cohort consisted of patients with suboptimal (sIR) and optimal (oIR) immune recovery. The inclusion criteria were: men >18 years old, receiving cART for a minimum of two years, CD4+ T-cell count persistently <350cells/μl (sIR) or >500cells/μl (oIR) on suppressive (HIV RNA <50copies/ml) cART. HIV-negative controls were recruited from a local community-based clinic. Samples collected were 40mls of blood, anal swab and oral rinse. Peripheral blood mononuclear cells (PBMCs) were isolated for interferon-γ (IFN-γ) ELISPOT assay and immunophenotyping. Clinical and detailed socio-demographic parameters were extracted from medical records and a self-administered questionnaire. All participants provided written informed consent and this study was approved by UMMC ethics review board (Medical Ethics Committee Reference: 865.18).

HPV DNA testing

Anal swabs were collected using a pre-moistened cervical brush (Hybribio Limited, China). Oral rinse was obtained by gargling 10ml of Listerine® mouthwash. DNA was extracted from both anal and oral samples, and HPV DNA typing was performed using the Hybribio Rapid GenoArray test kit (Hybribio Limited) (20). This kit detects 21 HPV genotypes, including 13 high-risk (HR) genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), 6 low-risk genotypes (6, 11, 42, 43, 44, and CP8304 [81]), and 2 probable high-risk types (53 and 66). The tests were performed according to the manufacturer's protocol (20). Briefly, the extracted DNA was subjected to PCR amplification using HPV L1 consensus PCR primers. The PCR products were subsequently added to a probed membrane followed by flow-through hybridisation and colourimetric reaction for detection of amplified HPV DNA.

Anal cytology

The anal cytological samples were collected without anoscopic guidance using the pre-moistened brush into ThinPrep (Hologic, Inc., USA) liquid-based specimen and Papanicolaou stained. All slides were read by a single experienced pathologist, utilising the Bethesda System 2001 criteria and terminology (21).

IFN-γ ELISPOT

IFN-γ producing HPV-specific T-cells were quantified using ELISPOT as previously described (13, 22, 23). Briefly, PBMCs were suspended in Isocove's modified Dulbecco's

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media enriched with 10% fetal bovine serum and seeded at $2x10^6$ cells/ml/well in a 24-well plate. A set of HPV16 E6 peptides consisting of 37 15-mer peptides that overlap by 11 amino acids (Supplementary data. CTL, Germany) at 1μg/ml/peptide (24) were pooled with a set of HPV52 E6 peptides consisting of 10 9-mer non-overlapping peptides (supplementary data) at 10μg/ml/peptide (25). Memory recall mix containing 2 peptides from tetanus and 11 peptides from human Cytomegalovirus pp65 antigen (supplementary data)) at 10μg/ml/peptide were used as positive controls. Following four days of incubation at $37^{o}C$, PBMCs were harvested, washed and seeded in triplicate at a density of 10^5 cells/well in a Multiscreen 96-well plate (Millipore, USA) coated with an IFN-γ capture antibody (Mabtech, Ireland). Further antibody incubations and development of the ELISPOT was performed as described. The measured HPV E6-specific IFN-γ responses were from a combination of CD4 and/ or CD8 T-cells against types HPV16 or 52. Spots were counted with a fully automated computer-assisted-video-imaging analysis system (CTL ImmunoSpot®, USA). Specific spots were calculated by subtracting the mean number of spots plus two times standard deviation of the medium control from the mean number of spots in experimental wells. Antigen-specific T-cell frequencies were considered to be positive when specific T-cell frequencies were $>1/10^4$ PBMCs.

T-cell immunophenotyping by flow cytometry

Briefly, $1x10^6$ thawed PBMC were stained in two separate panels: 1) CD3 (PerCP-Cy5.5), CD4 (PE-Cy7), CD8 (APC-H7), CD28 (APC), CD57 (FITC), CD45RA (PE), CCR7 (BV421) and viability dye (FVS510) and 2) CD3, CD4, CD8, CD38, HLA-DR (BV421, clone G46-6), CD25 (BB515, clone 2A3), Foxp3 (Alexa Fluor® 647, clone 259D/C7) and viability stain. All antibodies were purchased from BD Pharmigen. PBMCs were stained for surface markers for 20 minutes at room temperature and washed twice before acquisition. PBMCs in the second panel were further fixed and permeabilized with Foxp3 staining buffer kit prior to intracellularly staining with anti-Foxp3 antibodies or isotype control IgG1 (Alexa Fluor® 647, clone MOP-21) for 30 mins. All samples were re-suspended in stabilizing fixative and acquired on BD FACS Canto II (BD Biosciences, USA) and analysed using FACS Diva v6. Following gating to exclude doublets and dead cells (FVS510+), CD3+ cells were selected and sequentially gated for CD4+ and CD8+ T-cells and subsequently for the maturational subsets of naïve (CD45RA+CCR7+), central memory, CM (CD45RA-CCR7+), effector memory, EM (CD45RA-CCR7-) and terminally-differentiated effector memory, TdEM (CD45RA+CCR7-) cells. The proportion of activation and senescence markers on

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198 CD4+ and CD8+ T-cells were defined from the co-expression of CD38+HLA-DR+ and
199 CD57+CD28- cells, respectively. T-regulatory cells were reported as percentage of
200 CD3+CD4+ T-cells expressing CD25+Foxp3+ (Figure 1).
201
202 **Data analysis**
203 Clinical and immunological characteristics were compared using Mann-Whitney U and Chi-
204 square/Fisher's exact test. Logistic regression analysis was used to assess immunological
205 factors associated with HPV carriage and HPV-specific responses. Co-variates with p<0.25
206 and well established risk factors were included in the multivariate model where a *p*-value of
207 <0.05 was considered significant. Statistical analyses were performed using SPSS Statistics
208 version 22 (IBM).
209
210 **RESULTS**
211
212 **Patient characteristics**
213 In total, 94 HIV-infected patients were recruited; 27 in the longitudinal arm and 67 in the
214 cross-sectional arm, and 29 HIV-negative controls.
215
216 In the cross-sectional cohort, 32 patients displayed suboptimal immune recovery (sIR)
217 while 35 had optimal immune recovery (oIR). The median age was 41 (interquartile range
218 [IQR]: 39-48) years, with 40/63 (63.5%) smokers, 16/50 (32.0%) circumcised and 27/64
219 (42.2%) men who had sex with men (MSMs) (Table 1). The median duration on suppressive
220 cART was 5 (IQR: 3-9) years (Table 2). The age, smoking history, circumcision rates, and
221 sexual orientation, were not significantly different between sIR and oIR (Table 1) but
222 baseline CD4+ T-cell counts was significantly higher in oIR compared to sIR (190 [IQR: 66-
223 314] vs 28 [IQR: 10-59] cells/µl, p<0.001) (Table 2).
224
225 In the longitudinal cohort, the median age was 35 (IQR: 30-41) years, with all other
226 parameters including smoking history, circumcision rates and sexual orientation being
227 comparable to the cross-sectional cohort (Table 1). The median baseline CD4+ T-cell count
228 was 221 (IQR: 63-319) cells/µl (Table 2). Of interest, comparison of T-cell subsets between
229 hetero- and homosexuals, demonstrated a significantly higher proportion of activated CD8+
230 T-cells (CD8+CD38+HLADR+) among the homosexuals. However, there were no

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231 differences observed in proportions of T-regs, senescent CD4+ and CD8+ T-cells or activated
232 CD4+ T-cells.
233
234 In the HIV-negative controls, the median age was 39 (IQR: 29-53) years, comparable to
235 the HIV-positives (p=0.314). There were more individuals who were circumcised 17/28
236 (60.7%, p=0.014) and notably, 24/25 (96.0%) being heterosexuals compared to the HIV-
237 infected individuals (Table 1).
238
239 **Prevalence and risk factors associated with HPV DNA detection**
240 Overall, 25/44 (56.8%) and 7/51 (13.7%) of the cross-sectional cohort had HPV DNA
241 detected in the anal and oral cavity respectively, with no significant differences between sIR
242 and oIR (p=0.604 and p=0.432, respectively) (Table 3). In anal samples with detectable HPV
243 DNA, 17/25 (68.0%) had two or more HPV types, with 21/25 (84.0%) having a HR-HPV.
244 HPV16 and HPV52 were detected in 6/21 (28%) and 5/21 (23%) of the anal samples with
245 multiple infections detected in all the samples carrying HPV16. The other commonly
246 detected HPV types were 6,11,16,31 and 58. In oral samples with detectable HPV DNA, 6/7
247 (85.7%) had single HPV types, with 4/7 (57.1%) being HR-HPV.
248
249 In the longitudinal cohort, anal HPV DNA was detected in 19/26 (73.1%) at recruitment
250 and went up to 16/20 (80.0%) at 48 weeks with 4/27 (14.8%) and 6/21 (28.6%) detected in
251 the oral cavity during the same time-points (Table 3). 21/24 (87.5%) of the anal samples had
252 two or more HPV types, with 23/24 (95.8%) having a HR-HPV type at any single time-point.
253 The most common HR-HPV types present in this cohort at any single time-point were
254 HPV16 (45.8%) and HPV52 (37.7%). For oral samples with detectable HPV DNA, 4/10
255 (40.0%) had two or more HPV types detected at any single time-point, all of whom had a
256 HR-HPV type. The most common HR-HPV types present at any single time-point were
257 HPV18 (50.0%) and HPV16 (40.0%). No decrease in anal and oral HPV carriage was
258 observed over the 48-week period despite cART-induced CD4+ T-cell count increase.
259
260 There was a correlation between alcohol consumption with oral HPV carriage in the
261 HIV-infected individuals (p=0.042) and a tendency of greater HPV anal carriage with
262 engagement of anal sex (p=0.055) (Table 4). Both circumcision (p=0.255) and smoking
263 history (p=0.358) did not influence anal and oral HPV carriage respectively. However, no

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264 significant risk factors were found to be independently associated with either anal or oral
265 HPV carriage in the multivariate analysis.
266
267 In the HIV-negative controls, detection of HPV DNA in the anal canal was 1/16 (6.3%)
268 which was significantly lower than the HIV-infected cohorts (p<0.001), while the detection
269 in the oral cavity was comparable at 3/29 (10.3%), p=0.337.
270
271 **Correlation between anal cytology and HPV DNA detection**
272 In the cross-sectional cohort, only 44/67 samples had sufficient cells for cytological
273 assessment. When comparing the sIR and oIR groups, there was no difference in those with
274 cytology ≥atypical squamous cells of undetermined significance (ASC-US) (38.9% vs 25.0%,
275 p=0.358). While presence of anal HPV DNA carriage was positively associated with
276 cytology ≥ASC-US (p=0.001), no correlation between multiple HPV types and the presence
277 of HR-HPV was associated with cytology ≥ASC-US (p=0.13 and p=0.23 respectively). No
278 cytological trends were observed in the longitudinal cohort.
279
280 **Immunological and clinical correlates of HPV16 and 52 E6-specific responses**
281 Overall, 12/91 (13.2%) from the HIV-infected cohort exhibited HPV16 and 52 E6-specific
282 responses (Table 3) compared to 2/28 (7.1%) in the HIV-negative cohort (p=0.385).
283
284 In the cross-sectional cohort, regardless of HPV carriage and anal cytology, significantly
285 lower HPV16 and 52 E6-specific responses were observed among the sIR (2/32, 6.3%)
286 compared to oIR (9/34, 26.5%) (p=0.029) (Table 3). The magnitude of the E6-specific
287 responses between oIR (17.4, IQR: 13.2-18.9) were comparable to the sIR (19.3, IQR: 15.2-
288 23.8) group (*p*= 0.637) The presence of HPV16 and/or 52 at anal and/or oral sites at the time
289 of recruitment did not correspond to the HPV16 and 52 E6-specific immunity detected
290 (p=0.339). Baseline CD4+ T-cell counts and duration on cART were not different in
291 individuals with and without HPV16 and 52 E6-specific responses (p=0.282 and 0.965,
292 respectively). However, individuals with higher CD4+ T-cell counts at recruitment had
293 significantly greater magnitude of HPV16 and 52 E6-specific responses (p=0.028). While
294 there were significant differences in the E6-specific responses between oIR and sIR,
295 responses to MRM only trended towards significance (p = 0.569) (Table 3).
296

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In the longitudinal cohort (n=25), only one patient displayed HPV16 and 52 E6-specific responses at weeks 12 and 24 after cART. This individual had HPV52 detected in the anal canal at weeks 12 and 24. While the immune responses to E6-peptides were barely detectable, approximately 50% of the patients mounted a response to MRM despite the low CD4+ T-cell counts (Table 3).

In the analysis of immune correlates of HPV16 and 52 E6-specific responses in cART-treated HIV-infected individuals, both the data for the cross-sectional and longitudinal (week 48) were combined (Table 5). In the univariate analysis, differences in T-cell maturational subsets (naïve, CM, EM and TdEM) were not associated with HPV16 and 52 E6-specific responses. However, higher CD4+ T-cell activation (p=0.030) was significantly associated with the lack of HPV16 and 52 E6-specific responses with only a tendency for higher CD8+ T-cell activation exhibiting a similar trend (p=0.067). Neither the proportion of CD4+ T-regulatory cells (p=0.959) nor the proportion of senescent CD4+ T-cells (p=0.718) and CD8+ T-cells (p=0.433) were associated with HPV16 and 52 E6-specific responses. Besides CD4+ T-cell counts at recruitment (p=0.008), there was a tendency for patients with a lower CD4:CD8 T-cell ratio (p=0.072) to fail in mounting a HPV16 and 52 E6-specific responses (Table 5).

In the multivariate analysis, none of the immunological parameters were independently associated with HPV16 and 52 E6-specific immune responses.

DISCUSSION

To date this is the largest study to have assessed the correlates of systemic HPV-specific immune responses to immune recovery following cART among those with HIV infection. We quantified HPV-specific immunity during the initial phase of immune recovery following cART and subsequently in patients with sub-optimal and optimal immune recovery (sIR and oIR). We showed that HPV-specific immune responses were rarely detected during the first year following cART but in individuals on cART for a longer duration, an optimal immune recovery >500cells/µl was associated with significantly higher frequencies of HPV-specific responses compared to individuals with suboptimal recovery (<350cells/µl). When assessing the immunological correlates of HPV-specific responses, only higher CD4+ T-cell counts and lower CD4+ T-cell activation were found to be associated with HPV-specific responses.

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331

332 The anal and oral HPV carriage rates in our HIV-infected cohort of 64.1% and 18.1%, 333 respectively are consistent with the high prevalence of HPV infection reported among HIV-334 infected individuals (42-96% anal and 9.6-38.5% oral HPV). These studies also demonstrate 335 HPV16 and 52 being the most common HPV types (26-29). The consistently higher 336 prevalence of HR-HPV observed globally, despite effective cART and CD4+ T-cell recovery 337 point towards an inadequate immune-mediated resolution of and protection against HPV 338 infection.

339

340 We investigated HPV-specific immune responses during the different phases of immune 341 recovery following cART. In the longitudinal cohort, only 1/25 patient exhibited HPV-342 specific responses within the first 48 weeks of suppressive cART. In contrast, higher 343 frequencies of HPV-specific responses were detected in the cross-sectional cohort with a 344 median duration of 5 years on cART. More strikingly, individuals in the oIR group had 345 significantly higher HPV-specific responses (26.5%) compared to the sIR group (6.3%). The 346 lack of antigen-specific responses against pathogens during early recovery has previously 347 been observed in other HIV co-infections (30, 31). It was therefore not unexpected for HPV-348 specific responses to be infrequently detected during the first year of treatment. Furthermore, 349 HPV is exclusively epitheliotrophic where the majority of antigen-specific immune cells 350 reside locally (reviewed in (32)) with low but detectable levels of circulating HPV–specific 351 T-cells. The recovering immune system during cART initiation is alsochallenged by a larger 352 load of other systemic antigens including human herpes viruses (33) as evidencedby a 353 detectable MRM response in nearly half of the longitudinal cohort, 12 weeks after 354 commencing cART, Taken together, the lack of HPV-specific responses in early phase of 355 CD4+ T-cell recovery and subsequently, increased responses seen in oIR implies that a 356 specific threshold of CD4+ T-cells is required before HPV-specific immunity can be 357 detected. The concept of immune-competence as assessed by CD4+ T-cell counts and its 358 association with virus-specific cellular immunity has also been observed in HIV/hepatitis B 359 virus and CMV co-infected individuals (30, 34). Alternatively, the lack of HPV-specific 360 responses in the early phase of immune recovery could be driven by the loss of memory-361 specific cells due to greater turnover as we observed a significant inverse correlation between 362 CD4+ T-cell activation and HPV-specific responses in this cohort.

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364 In our cohort, neither the extent of immune recovery nor the development of HPV16 and
365 52 E6-specific responses were associated with differences in HPV carriage or abnormal anal
366 cytology. This is not surprising as histological outcomes (biopsies) have been shown to
367 correlate better to the immunological markers compared to virological or cytological
368 outcomes (13) particularly since this study was limited by its small numbers.

369

370 The largest study to date identifying HPV16 E6-specific responses was in a mixed HIV-
371 infected/negative population of MSMs using a flow cytometry-based assay(24). In that study,
372 50% displayed HPV16 E6-specific responses, but this did not show any correlations with age,
373 HIV status, or anal HPV16 status. However, a trend between recent anal high-grade
374 squamous intraepithelial lesion (HSIL) regression and systemic E6-specific CD4+ T-cell
375 responses was demonstrated, confirming the superiority of tissue biopsies over HPV DNA
376 detection as a surrogate marker for clinical outcomes.

377

378 The observed HPV16 and 52 E6-specific responses among the HIV-negative controls
379 (7.1%) was lower than that expected of around 30-50%. The discrepancy could be explained
380 by differences in the study populations. Historically, studies demonstrating high proportions
381 of protective immunity associated with E6-specific responses have been predominantly in
382 women with cervical disease with regressing lesions or low-grade disease that did not
383 progress to high-grade lesions (12-15). In contrast, our controls were healthy males with low
384 anal HPV carriage rates.

385

386 The strength of the study is its well-characterized clinical population and correlating it to
387 immunological outcomes in HIV-infected individuals. It also utilises a sensitive T-cell assay
388 (IFN-γ ELISPOT) that has been validated and used in various populations (12, 13, 22, 23) to
389 detect low levels of antigen-specific immunity. A major limitation of this assay is that IFN-γ
390 was the only parameter measured, discounting the effects of other regulatory cytokines. Other
391 limitations of the study include small sample numbers in the longitudinal cohort and the lack
392 of immunological correlates to long-term clinical outcomes.

393

394 Despite effective viral suppression on cART, 1/3 of patients will not achieve optimal
395 CD4+ T-cell recovery, especially in those starting treatment late (35, 36). They are also
396 particularly susceptible to non-AIDS associated co-morbidities (37, 38). This study has
397 demonstrated that those with suboptimal immune recovery also lacks HPV protective

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immunity and are possibly at greater risk of developing serious HPV-associated diseases. We propose that this group should be considered formore intensive screening and should be encouraged to consider HPV vaccination. There is good evidence to suggest that HPV vaccination among those who have previously received treatment for pre-malignant disease of the cervix (cervical intra-epithelial neoplasia) significantly reduced the incidence of subsequent HPV-related disease (39). This suggests that those infected with HIV on cART exhibiting HPV-associated diseases may also potentially benefit. This is supported by immunogenicity studies among those who are HIV-infected (40)and studies that have shown that vaccinating men with a history of high-grade intraepithelial neoplasia reduces the recurrence rates by up to 50% (41), and that this strategy is cost effective even among the HIV-infected (42).

CONCLUSIONS

HPV-related pathology remains a significant cause of morbidity and mortality among those living with HIV despite effective viral suppressive therapy. The failure to mount an effective HPV-immune response among those with suboptimal CD4+ T-cell recovery may increase their susceptibility to HPV-associated cancers. Patients on cART with suboptimal CD4+ T-cell recovery should therefore be considered for increased surveillance and HPV vaccination in the prevention of HPV-associated cancers.

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Author contributions: RR, AK and YLW designed this study. MLC, HS and IA recruited patients, collected samples and data. CYL, HCL, LLC, RR, JMR and YLW analysed patient samples, generated, compiled and analysed the results. All authors have reviewed, commented, and approved the approved the final manuscript. All authors report no conflicts of interest.

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564 **Table 1** Demographic characteristics of HIV-infected patients and HIV-negative controls

565

	Cross-sectional		Longitudinal (n=27)	Control (n=29)
	sIR (n=32)	oIR (n=35)		
Age, median (IQR)	42 (39-50)	40 (38-46)	35 (30-41)	39 (29-53)
Ethnicity (%)				
Malay	2 (6.2)	7 (20.0)	6 (22.2)	12 (41.4)
Chinese	29 (90.6)	21 (60.0)	15 (55.6)	9 (31.0)
Indian	1 (3.2)	7 (20.0)	1 (3.7)	8 (27.6)
Others	0 (0)	0 (0)	2 (7.4)	0 (0)
Smoking history (%)				
Yes	20/30 (66.7)	20/33 (60.6)	12 (44.4)	18/28 (64.3)
Never	10/30 (33.3)	13/33 (39.4)	15 (55.6)	10/28 (35.7)
Circumcised (%)				
Yes	6/24 (25.0)	10/26 (38.5)	5/14 (35.7)	17/28 (60.7)
Sexual orientation (%)				
Heterosexual	18/30 (60.0)	16/34 (47.1)	6/26 (23.1)	24/25 (96.0)
Homosexual	11/30 (36.7)	16/34 (47.1)	19/26 (73.1)	0/25 (0)
Bisexual	1/30 (3.3)	2/34 (5.8)	1/26 (3.8)	1/25 (4.0)
Average number of lifetime sexual partners (%)				
1	2/24 (8.3)	4/26 (15.4)	1/15 (6.7)	6/25 (24.0)

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≥2	15/24 (62.5)	17/26 (65.4)	10/15 (66.7)	17/25 (68.0)
Do not remember	7/24 (29.2)	5/26 (19.2)	4/15 (26.7)	2/25 (8.0)
Sexual practices (%)				
Oral sex	17/24 (70.8)	22/26 (84.6)	13/15 (86.7)	12/25 (48.0)
Anal sex	10/24 (41.7)	12/26 (46.2)	12/15 (80.0)	2/25 (8.0)
HSV-1 antibody positive (%)	12/19 (63.2)	13/22 (59.1)	12/20 (60.0)	--
HSV-2 antibody positive (%)	13/19 (68.4)	9/22 (40.9)	4/20 (20.0)	--

566 HSV, herpes simplex virus; IQR, interquartile range; oIR, optimal immunological responder; sIR, suboptimal immunological responder.

567 **Table 2** Clinical and immunological parameters in the HIV-infected patients and HIV-negative controls

568

	Cross-sectional		Longitudinal (n=27)				Control
	sIR (n=32)	oIR (n=35)	Week 0	Week 12	Week 24	Week 48	(n=29)
Years on cART, median (IQR)	5 (3-9)	5 (3-9)	--	--	--	--	--
cART regimen							--
NNRTI-based	32 (100)	35 (100)	--	26 (96.3)	26 (96.3)	25 (92.6)	
PI-based	0 (0)	0 (0)	--	0 (0)	0 (0)	1 (3.7)	
Integrase inhibitor-based	0 (0)	0 (0)	--	1 (3.7)	1 (3.7)	1 (3.7)	
CD4+ T-cell count (cells/µl), median (IQR)							--
Baseline	28 (10-59)	190** (66-314)	221 (63-319)	--	--	--	
At recruitment	285	733**	--	291	310	410	

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	(224-332)	(671-945)		(186-406)	(207-462)	(268-481)	
CD8+ T-cell count (cells/µl) , median (IQR)							--
Baseline	473 (237-876)	710.5* (492.3-1391)	745 (414-1020)	--	--	--	
At recruitment	799 (560-982)	950* (723-1421)	--	883 (596-1271)	809 (539-1118)	952 (668-1297)	
CD4:CD8 ratio, median (IQR)							--
Baseline	0.07 (0.03-0.12)	0.22** (0.09-0.31)	0.29 (0.16-0.38)	--	--	--	
At recruitment	0.37 (0.31-0.45)	0.78** (0.57-1.09)	--	0.38 (0.25-0.54)	0.44 (0.29-0.61)	0.39 (0.29-0.55)	
HIV viral load (copies/ml), median (IQR)							
Baseline	194245.5 (97525-405805)	92461* (37599-233000)	54401 (27649-155050)	--	--	--	--
At recruitment	Below limit of detection	Below limit of detection	--	Below limit of detection	Below limit of detection	Below limit of detection	
Anal cytology (%)							
Technically unsatisfactory	3/21 (14.3)	3/23 (13.0)	11/24	4/16 (25.9)	5/20 (25.0)	3/14 (21.4)	26/29

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			(45.8)				(89.7)
Normal	11/21 (52.4)	15/23 (65.2)	4/24 (16.7)	4/16 (25.0)	9/20 (45.0)	6/14 (42.9)	3/29 (10.3)
ASC-US	2/21 (9.5)	1/23 (4.4)	3/24 (12.5)	0/16 (0)	0/20 (0)	2/14 (14.3)	0/29 (0)
LSIL	2/21 (9.5)	3/23 (13.0)	4/24 (16.7)	5/16 (31.3)	2/20 (10.0)	1/14 (7.1)	0/29 (0)
ASC-H	3/21 (14.3)	0/23 (0)	2/24 (8.3)	1/16 (6.3)	3/20 (15.0)	0/14 (0)	0/29 (0)
HSIL	0/21 (0)	1/23 (4.0)	0/24 (0)	2/16 (12.5)	1/20 (5.0)	1/14 (7.1)	0/29 (0)
T-cell immunophenotyping							
CD4+ T-cells, median % (IQR)							
Naïve	25 (15-31)	36 (24-50)	28 (14-39)	23 (6-34)	28 (17-41)	35 (22-46)	43 (35-47)**
CM	46 (39-51)	38 (34-48)	31 (23-41)	35 (28-45)	40 (33-44)	35 (31-38)	37 (31-43)
EM	27 (21-36)	19 (11-26)	28 (20-49)	27 (19-54)	22 (17-36)	23 (17-35)	15 (13-20)**
TdEM	1 (1-3)	2 (1-5)	4 (1-11)	2 (1-8)	2 (1-6)	1 (1-5)	1 (1-4)
Activation	8 (6-11)	5 (4-7)	29 (15-50)	25 (13-40)	16 (13-29)	13 (10-21)	3 (3-5)**
Senescence	3 (1-7)	4 (1-10)	11 (2-28)	8 (2-16)	6 (1-13)	5 (1-14)	1 (0-3)*
T-regulatory cells	7 (6-9)	6 (5-7)	7 (5-10)	6 (5-12)	7 (5-11)	7 (5-10)	6 (5-6)
CD8+ T-cells, median % (IQR)							
Naïve	8 (5-15)	16 (10-25)	8 (4-11)	9 (4-15)	12 (8-22)	14 (7-17)	21 (9-28)*
CM	5 (3-8)	7 (5-9)	2 (1-3)	3 (1-4)	4 (3-5)	3 (2-5)	6 (4-9)

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EM	56 (44-62)	44 (37-53)	57 (45-67)	49 (43-55)	43 (34-53)	47 (36-52)	46 (34-51)
TdEM	28 (20-35)	30 (20-38)	29 (23-41)	38 (29-44)	37 (29-45)	35 (27-44)	31 (23-40)
Activation	22 (13-30)	14 (11-19)	73 (58-77)	62 (50-71)	48 (45-53)	42 (29-51)	14 (8-21)*
Senescence	44 (32-55)	30 (22-43)	43 (37-51)	50 (43-58)	49 (37-57)	48 (43-58)	33 (26-43)*

ASC-H, atypical squamous cells cannot exclude high-grade squamous intraepithelial lesion; ASC-US, atypical squamous cells of undetermined significance; cART, combined antiretroviral therapy; CM, central memory; EM, effector memory; HIV, human immunodeficiency virus; HSIL, high-grade squamous intraepithelial lesion; IQR, interquartile range; LSIL, low-grade squamous intraepithelial lesion; NNRTI, non-nucleoside reverse transcriptase inhibitor; oIR, optimal immunological responder; PI, protease inhibitor; sIR, suboptimal immunological responder; TdEM, terminally-differentiated effector memory.

*p-value <0.05 and **p-value <0.001.

Table 3 Detection of HPV DNA and HPV16 and 52 E6-specific immune responses in the HIV-infected cohort

	Cross-sectional		Longitudinal (n=27)			
	sIR (n=32)	oIR (n=35)	Week 0	Week 12	Week 24	Week 48
Anal HPV carriage						
Any HPV positive (%)	12/21 (57.1)	13/23 (56.5)	19/26 (73.1)	14/19 (73.7)	16/21 (76.2)	16/20 (80.0)
Any HR-HPV positive (%)	10/12 (83.3)	11/13 (84.6)	19/19 (100)	14/14 (100)	15/16 (93.8)	14/16 (80.0)
HPV16 positive (%)	3/12 (75.0)	4/13 (30.8)	8/19 (42.1)	5/14 (35.7)	6/16 (37.5)	6/16 (37.5)
HPV52 positive (%)	4/12 (33.3)	1/13 (7.7)	4/19 (21.1)	6/14 (42.9)	5/16 (31.3)	5/16 (31.3)
Number of types detected per sample						
1	3/12 (25.0)	5/13 (38.5)	5/19 (26.3)	3/14 (21.4)	4/16 (25.0)	6/16 (37.5)

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≥2	9/12 (75.0)	8/13 (61.5)	14/19 (73.7)	11/14 (78.6)	12/16 (75.0)	10/16 (62.5)
Oral HPV carriage						
Any HPV positive (%)	4/24 (16.7)	3/27 (11.1)	4/27 (14.8)	4/20 (20.0)	5/22 (22.7)	6/21 (28.6)
Any HR-HPV positive (%)	2/4 (50.0)	2/3 (66.7)	4/4 (100)	4/4 (100)	5/5 (100)	6/6 (100)
HPV16 positive (%)	1/4 (25.0)	0/3 (0)	3/4 (75.0)	2/4 (50.0)	2/5 (40.0)	1/6 (16.7)
HPV52 positive (%)	0/4 (0)	0/3 (0)	0/4 (0)	0/4 (0)	0/5 (0)	0/6 (0)
Number of types detected per sample						
1	4/4 (100)	2/3 (66.7%)	2/4 (50.0)	3/4 (75.0)	3/5 (60.0)	6/6 (100)
≥2	0/4 (0)	1/3 (33.3%)	2/4 (50.0)	1/4 (25.0)	2/5 (40.0)	0/6 (0)
Cervical HPV carriage						
Any HPV positive (%)	--	--	2/2 (100)	2/2 (100)	2/2 (100)	1/2 (50.0)
Any HR-HPV positive (%)	--	--	2/2 (100)	2/2 (100)	2/2 (100)	1/1 (100)
HPV16 positive (%)	--	--	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	1/1 (100)
HPV52 positive (%)	--	--	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	0/1 (0)
Number of types detected per sample						
1	--	--	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	0/1 (0)
≥2	--	--	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	1/1 (100)
HPV16 and 52 E6-specific immune responses[1] (%)	2/32 (6.3)	9/34 (26.5)	0/25 (0)	1/19 (5.3)	1/20 (5.0)	0/20 (0)
Responses to memory recall mix (MRM) containing tetanus and CMV peptides (%)	21/32 (65.6)	20/34 (58.8)	10/25 (40.0)	8/19 (42.1)	10/20 (50.0)	9/20 (45)

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577 HIV, human immunodeficiency virus; HPV, human papillomavirus; HR-HPV, high-risk HPV genotypes; oIR, optimal immunological
578 responder; sIR, suboptimal immunological responder.[1]HPV16 and HPV52 E6 antigen-specific immune responses as determined by IFN-γ
579 ELISPOT.

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580 **Table 4** Univariate analysis correlating HPV carriage to risk factors and clinical parameters

581

Parameter	Coefficient	OR (95% CI)	*p*-value
Anal HPV carriage			
Age	-0.011	0.99 (0.93-1.06)	0.757
Smoking	-0.241	0.79 (0.28-2.23)	0.651
Engagement in anal sex	1.088	2.97 (0.98-9.02)	0.055
Number of lifetime sexual partners	0.272	1.31 (0.23-7.38)	0.758
1	0	1	
≥2	0.272	1.31 (0.23-7.38)	0.758
Sexual orientation			
Heterosexual	0	1	
Homosexual	0.981	2.7 (0.90-7.89)	0.077
Bisexual	0.693	2 (0.16-24.87)	0.590
Circumcised	0.713	2.04 (0.59-6.96)	0.255
HSV-1 antibody			
Not detected	0	1	
Detected	-0.229	0.80 (0.20-3.13)	0.743
HSV-2 antibody			
Not detected	0	1	
Detected	0.229	1.26 (0.32-4.94)	0.743
Oral HPV carriage			
Age	-0.057	0.94 (0.87-1.03)	0.173
Smoking history	0.603	1.83 (0.51-6.62)	0.358
Alcohol consumption	2.228	9.28 (1.09-79.40)	0.042
Engagement in oral sex	0.264	1.30 (0.32-5.26)	0.711
Number of lifetime sexual partners			
1	0	1	
≥2	19.439	276938527.31 (0)	0.999
Sexual orientation			
Heterosexual	0	1	
Homosexual	-2.708	5.19 (1.03-26.23)	0.046

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Bisexual	2.015	7.5 (0.46-122.70)	0.158
HSV-1 antibody			
Not detected	0	1	
Detected	-1.609	0.2 (0.034-1.19)	0.076
HSV-2 antibody			
Not detected	0	1	
Detected	-0.580	0.56 (0.096-3.28)	0.521

582 CI, confidence interval; HPV, human papillomavirus; HSV, herpes simplex virus; OR, odds
583 ratio.
584 *p*-value <0.05 is considered statistically significant different. *p*-value of >0.25 were included
585 into a multivariate analysis where no statistically significant relationship was demonstrated.
586 **Table 5** Univariate analysis correlating the presence of HPV16 and 52 E6-specific immune
587 responses to T-cell subsets
588

T-cell subsets	HPV16 and 52 E6-specific immune responses[1]		*p*-value
	Coefficient	OR (95% CI)	
CD4+ at recruitment (cells/µl)	0.002	1.00 (1.001-1.004)	0.01*
CD4:CD8 ratio	0.901	2.46 (0.65-9.34)	0.07
% CD4+CD45RA+CCR7+	0.005	1.01 (0.96-1.05)	0.83
% CD4+CD45RA-CCR7+	0.009	1.01 (0.95-1.08)	0.79
% CD4+CD45RA-CCR7-	-0.008	0.99 (0.94-1.05)	0.99
% CD4+CD45RA+CCR7-	-0.053	0.95 (0.80-1.13)	0.55
% CD8+CD45RA+CCR7+	0.002	1.00 (0.95-1.06)	0.94
% CD8+CD45RA-CCR7+	0.080	1.04 (0.89-1.22)	0.59
% CD8+CD45RA-CCR7-	-0.022	0.99 (0.93-1.03)	0.43
% CD8+CD45RA+CCR7-	0.017	1.02 (0.96-1.08)	0.54
% CD4+CD38+HLA-DR+	-0.248	0.78 (0.61-1.00)	0.03*
% CD8+CD38+HLA-DR+	-0.065	0.94 (0.88-1.00)	0.07
% CD4+CD28-CD57+	-0.032	0.97 (0.89-1.06)	0.72
% CD8+CD28-CD57+	-0.014	0.99 (0.95-1.03)	0.43
% CD4+CD25+Foxp3+	-0.059	0.94 (0.72-1.23)	0.96

589 CI, confidence interval; HPV, human papillomavirus; OR, odds ratio.

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590 *p-value <0.05 is considered statistically significant different. Univariate analysis showed a
591 positive correlation between HPV16 and 52-E6 responses and CD4+ T-cells at recruitment,
592 but inverse correlation with CD4+CD38+HLA-DR+ T-cells. p-value of >0.25 were included
593 into a multivariate analysis where no statistically significant relationship was demonstrated.
594 [1]HPV16 and HPV52 E6 antigen-specific immune responses as determined by IFN-γ
595 ELISPOT.

596 **Figure legends**

597

598 **Fig 1** Flow cytometry gating strategy for T-cell subsets. Panel A: Following gating to exclude
599 doublets, CD3+ cells were selected and sequentially gated for CD4+ and CD8+ T- cells and
600 subsequently for the maturational subsets of naïve (CD45RA+CCR7+), central memory, CM
601 (CD45RA-CCR7+), effector memory, EM (CD45RA-CCR7-) and terminally-differentiated
602 effector memory, TdEM (CD45RA+CCR7-). Panel B: Following gating to exclude doublets,
603 CD3+ cells were selected and sequentially gated for CD4+ and CD8+ T-cells and
604 subsequently for activation (CD38+HLA-DR+) and senescence markers (CD57+CD28-)
605 cells, respectively. T-regulatory cells were reported as percentage of CD3+CD4+ T-cells
606 expressing CD25+Foxp3+.

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