Role of human Pegivirus infections in whole *P. falciparum* sporozoite vaccination and controlled human malaria infection in African volunteers

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Abstract

Background: Diverse vaccination outcomes and protection levels pose a serious challenge to the development of an effective malaria vaccine. Co-infections are among many factors associated with immune dysfunction and sub-optimal vaccination outcomes. Chronic, asymptomatic viral infections can contribute to the modulation of vaccine efficacy through various mechanisms. Human Pegivirus-1 (HPgV-1) persists in immune cells thereby potentially modulating immune responses. We investigated whether Pegivirus infection influences vaccine-induced responses and protection in African volunteers undergoing whole *P. falciparum* sporozoites-based malaria vaccination and controlled human malaria infections (CHMI).

Methods: HPgV-1 prevalence was quantified by RT-qPCR in plasma samples of 96 individuals before, during and post vaccination with PfSPZ Vaccine in cohorts from Tanzania and Equatorial Guinea. The impact of HPgV-1 infection was evaluated on (1) systemic cytokine and chemokine levels measured by Luminex, (2) PfCSP-specific antibody titers quantified by ELISA (3) asexual blood stage parasitemia and pre-patent periods with HPgV-1 infection status and (4) HPgV-1 RNA levels upon asexual blood stage parasitemia induced by CHMI.

Results: The prevalence of HPgV-1 was 29.2% (28/96) and sequence analysis of the 5`UTR and E2 region revealed the predominance of genotypes 1, 2 and 5 in the positive volunteers. HPgV-1 infection was associated with elevated systemic levels of IL-2 and IL-17A. Comparable vaccine-induced anti-PfCSP antibody titers, asexual blood stage multiplication rates and pre-patent periods were observed in HPgV-1 positive and negative individuals. However, higher level of protection was detected in the HPgV-1 positive group (62.5%) than negative one (51.6%) following CHMI. Overall, HPgV-1 viremia levels were not significantly altered after CHMI.

Conclusions: Although HPgV-1 infection did not alter vaccine-elicited levels of PfCSP-specific antibody responses and parasite multiplication rates, an ongoing infection appears to improve some degree of protection against CHMI in PfSPZ-vaccinated individuals. This is likely through modulation of immune system activation and systemic cytokines as higher levels of IL-2 and IL17A were observed in HPgV-1 infected individuals. CHMI is safe and well tolerated in HPgV-1 infected individuals. Identification of cell types and mechanisms of both silent and productive infection in individuals will help to unravel the biology of this widely present but largely under-researched virus.

Keywords: Malaria, vaccination, human pegivirus, HPgV-1, CHMI, immune activation

BACKGROUND

Vaccination is an invaluable tool in public health that has contributed to control of many, and in some cases, to the elimination of infectious disease like smallpox [1]. Malaria, a disease caused by *Plasmodium* species remains a major public health burden particularly in the tropics and sub-tropical regions where it accounted for approximately 405,000 deaths in 2018 [2]. Currently, one major goal in malaria research is to develop an efficacious vaccine that complements currently used control tools based on vector control and treatment of clinical malaria infections [3] . An efficacious malaria vaccine could alleviate malaria disease burden, prevent malaria related deaths, contain the spread of drug resistant malaria parasites and might even support the aim of malaria elimination [3]–[5].However, these vaccine development efforts are challenged by an incomplete understanding of the immune mediators leading to highly protective, long-lasting vaccine induced immunity in the field [6]. A number of studies testing metabolically active, purified, cryopreserved and radiation-attenuated whole sporozoites of *P. falciparum* as vaccine approach (PfSPZ Vaccine) have been published recently [7]–[11]. Strikingly, the comparison of PfSPZ vaccine-induced antibody titers specific for the *P. falciparum* circumsporozoite protein (PfCSP) showed significantly lower titers in malaria pre-exposed than malaria-naive individuals immunized with the PfSPZ Vaccine using comparable regimen [8]–[11]. These differences in PfSPZ vaccine-induced immunity was also observed between vaccinees residing in malaria endemic countries including Tanzania, Mali and Equatorial Guinea [11]–[13].

A range of factors have been described to be associated with immune dysfunction in infectious diseases and suboptimal vaccine-induced responses [14] . Recently our group demonstrated that age, location and iron status influence the immune system development of children as well as vaccine-induced responses to the most advanced malaria vaccine candidate, the RTS,S [15]. Co-infections have also been implicated in reduced responses to vaccination [14]. Chronic,

asymptomatic viral infections at the time of immunization might contribute to reduced magnitude and longevity of vaccine-induced immune responses [16]–[18]. To date, the number of human viruses investigated in this context is limited and their mechanisms in modulation of vaccine-induced responses remain unclear.

Human Pegivirus-1 (HPgV-1) is a positive strand RNA virus and a member of the Flaviviridae family [19]. HPgV-1 is common in Africa where an estimated 18-28 % of its 750 million global infections occur [20]. HPgV-1 is thought to cause a clinically silent, chronic infection [21] . The virus can persist with high viremia in serum and potentially by replicating in immune cells including T cells, B cells, monocytes, and natural killer (NK) cells [21, 22]. Interestingly, seminal studies in the field have linked HPgV-1 co-infection status to significant survival advantage in HIV-1 and Ebola infected humans [23]–[26]. These favourable outcomes are thought to be based on immune-modulatory properties of HPgV-1 such as activation of T cells, B cells and NK cells [21, 27] and the altered regulation of cytokine and chemokine expression [28]–[30]. Different HPgV-1 genotypes might influence the extent of immune modulation resulting in varied disease outcomes [23]–[25]

Given the high prevalence of HPgV-1 infection in *P. falciparum* endemic areas we suggest not only potential overlapping geographical distribution but also within-host interactions between the two infections [20] . We were therefore interested to study whether HPgV-1 infection status might influence PfSPZ vaccine-induced immune responses. We characterized prevalence and genotype distribution of HPgV-1 in three cohorts of adult volunteers participating in PfSPZ Vaccine studies [31, 32] (manuscript submitted),(manuscript in preparation). We explored the influence of HPgV-1 infection on cytokine and chemokine levels in serum samples and tried to correlate HPgV-1 infection on vaccine-induced anti-PfCSP-antibody titers and protection against CHMI. We also aimed to characterize for the first time the potential impact of a CHMI study on HPgV-1 viremia in these volunteers.

METHODS

Study population

Volunteers were enrolled in the BSPZV1 (NCT03420053), BSPZV2 (NCT02613520) BSPZV3 (NCT03420053) and EGSPZV2 studies (NCT02859350) that evaluated the safety, immunogenicity and efficacy of live, cryopreserved, purified, irradiated-attenuated *P. falciparum* sporozoites in malaria pre-exposed volunteers. Vaccine efficacy was evaluated by homologous CHMI based on direct intravenous inoculation of 3200 fully infectious, aseptic purified cryopreserved *P. falciparum* sporozoites. These whole sporozoites were suspended in 0.5 ml human serum and administered as a single dose 3 weeks after last vaccination.

Identification of Human Pegivirus RNA in RNA-Seq data from whole blood

Whole blood samples were used from a subset of participants (n=28) (Supplementary Figure 1A) that were enrolled into the BSPZV1 trial (NCT03420053). All volunteers were healthy males, aged 18 to 35 years and confirmed as negative for HIV-1, Hepatitis B and C before enrolment into the trial. Blood was collected and stored in Paxgene RNA tubes at different timepoints including before vaccination (baseline), 2 days after first vaccination, 7 days after the first and second vaccination as well as before CHMI, 2 and 9 days after CHMI. Each of the placebo (n=6) and the vaccinees (n=22) had a total of 3 and 7 blood sampling timepoints respectively resulting in 172 samples in total. All available samples (n=172) were subjected to RNA-Seq analysis performed by Stuart Lab in Seattle. Briefly, RNAseq data was generated and made from globin/rRNA cleared whole blood RNA that was fragmented and first strand cDNA synthesis was done by random priming and dTTP was used whereas 2nd strand cDNA synthesis used dUTP which eliminates 2nd strands in the downstream PCR amplification that enabled strand specific RNAseq sequencing [33]. From the RNA seq sample set (n=172), 800 million

non-human reads were identified and were analysed in a custom-established in-house viral metagenomics analysis pipeline (Bagamoyo Virome analysis pipeline: (**Supplementary Fig 1 B–C)**. The pipeline is a combination of several algorithms adapted from commonly used viral metagenomic analytical tools [34]–[38]. The analyses were carried out in three main steps: viral identification, *in silico* validation and RT-PCR confirmation. In the viral identification step, approximately 3 million initial non-human unmapped paired end reads from each volunteer were analysed. The unmapped reads were first searched for "suspected" viral hits by running bowtie2 against the NCBI database containing more than 7424 viral genomes. Thereafter, low quality and complexity reads as well as reads mapping to human genome, transcriptome and repeat regions were removed from the resulting "suspected" viral reads using bowtie 2, knead data and tandem repeat finder algorithms respectively. The "clean" viral reads were then comprehensively searched for viral hits using virome scan [34] and Taxonomer [35] and for viral proteins using adapted Diamond tool containing a custom made database with more than 100,000 viral proteins [37]. The initial unmapped reads were also analysed by Fast virome explorer without filtering for host reads to allow the identification of endogenous retroviral elements and other viruses that may have been missed previously [36]. Only viral hits associated with human host were selected, and viral contaminants such as lymphotrophic murine virus and synthetic constructs with either HIV-1 or hepatitis B were removed based on documented literature [39]. In a following in-silico confirmation step, the suspected viral hits were blasted and mapped against specific viral whole genomes using a Geneious bioinformatics tool [40]. The presence of RNA coding for the most prevalent virus (HPgV) was confirmed by reverse transcription polymerase chain reaction (RT-PCR) in plasma samples.

RT-qPCR for detection and quantification of HPgV-1 and HPgV-2

Samples from male and female individuals (n=96), aged 18-45 years, and participating in the BSPZV2 and BSPZV3 studies in Tanzania and in the EGSPZV2 study in Equatorial Guinea were included. Cryopreserved plasma prepared by density gradient centrifugation of whole blood was used for detection of HPgV-1 and HPgV-2 RNA in each of the study participants. Here, plasma samples collected at 3 different time points for each volunteer were included, namely before vaccination (baseline), before CHMI and 28 days after CHMI. Presence or absence of HPgV-1 and HPgV-2 was determined in parallel using RT-qPCR based on published methods[27] . Briefly, total nucleic acids were extracted from 300 ul plasma using Zymo quick DNA/RNA viral kit (Zymo Research, Irvine, USA) and eluted in 50 ul of DNase/RNase free water and 5 ul of the recovered RNA was used as template together with 2X Lunar universal one step qPCR master mix (10 ul, 1X) , Luna warm start reverse transcriptase enzyme mix (1 ul, 1X) and primers corresponding to 5` untranslated region of HPgV-1 and HPgV-2 (each at 2 ul, 0.4 uM) [27], [41] . In addition, human RNase P primers were also added as internal control. Each sample was run in triplicate in a one-step multiplex RT-qPCR using the CFX96 real time PCR system (Bio-Rad, Hercules, CA, USA). The RT-qPCR thermo cycling conditions were 55^{o}C for 10 mins, 95^{o}C for 1 min, 45 cycles at 95^{o}C for 15 secs and 55^{o}C for 1 min. The generated data was uploaded to an in-house platform where quantification cycle values (Cq) were calculated automatically [42]. HPgV viral quantification was done as described by Stapleton et al. using *in vitro* transcribed (IVT) RNA for HPgV-1 [27]. Controls were included in each RT-qPCR experiment including HPgV-1 and HPgV-2 IVT RNA as positive controls and H2O as non-template controls.

Genotyping of HPgV-1

Fire Script cDNA kit was used to synthesize cDNA in accordance to manufacture instructions (Solis Biodyne, Tartu, Estonia). Briefly, 5 ul of extracted RNA was added into a master mix containing forward and reverse primers specific to 5 UTR of HPgV-1 (each at 1,1 uM**)**,

deoxribonucleotide Triphosphate mix (dNTP) (0.5 ul, 500 uM**)**, reverse transcription buffer with DTT (2 ul, X1) , RiboGrip Rnase inhibitor (0.5 ul, 1 U/ul), Fire script reverse transcriptase. (2 ul, 10 U/ul) and RNase free water (9 ul to 20 ul). Amplification conditions included 50 mins at 50ºC and 10 mins at 94ºC. 3 ul of cDNA generated by reverse transcription were used for the first round of PCR amplification using primers forward 5-AAAGGTGGTGGATGGGTGATG-3 and reverse–5-ATG CCACCCGCCCTCACCAGA A-3[43]. 1.2 ul of this amplification product was then used for the second, nested PCR amplification using the primers forward 5-AATCCC GGTCAYAYTGGTAGCCACT-3 and reverse 5-CCCCACTGGCZTTGYCAACT-3 [43]. Both PCR reactions included primers specific for HPgV-1 (1 ul, 1 uM**)**, firepol master mix (4 ul, X5) **(**Solis Biodyne, Tartu, Estonia**)** and RNase free water to final volume of 20 ul. Cycling conditions were 5 mins of initial denaturation at 95ºC, followed by 28 cycles of 95ºC for 30 sec, 56ºC for 30 sec and 72 for 30 sec with a final extension step at 72ºC for 10 min. The E2 region was amplified as described by Souza et al [44]. The final PCR products from 5`UTR amplification (size of 256 base pairs) and E2 amplification (size of 347 base pairs) were sequenced by the Sanger method.

HPgV-1 phylogenetic analysis

Nucleotide sequence analysis and phylogenetic relatedness was performed in Geneious software version 8.1.9. Chromatograms were examined for quality first, and only sequences with quality above 86% were included in analysis. CLUSTALW algorithm was used to align 5` UTR nucleotide sequences from volunteers to selected reference sequences corresponding to 5` UTR of HPgV-1 (genotype 1 to 7) available in the NCBI database. Thereafter phylogenetic trees were constructed by neighbour joining method and the Kimura two parameter models. The references sequences for 5` UTR included AF488786, AF488789, KC618399, KP710602, U36388, JX494177, Y16436, and MF398547 (Genotype 1); AB003289, AF104403, D90600, JX494179, MG229668, JX494180, U4402, U59518 (Genotype 2; 2a), MH000566, U59529,

U63715, MH053130 (Genotype 2; 2b); AB008335, KR108695, JX494176, D87714 (Genotype 3); AB0188667, AB021287, HQ3311721 (Genotype 4); DQ117844, AY949771, AF488796, AF488797 (Genotype 5); AB003292, AF177619 (Genotype 6), HQ331235, HQ 3312233 (Genotype 7) and Hepatitis C nucleotide sequences (AJ132997) was used as an out-group. For the E2 region the sequences were; KP701602.1, KM670109, U36380, KP710600, KC618399, AB003291 (Genotype 1); AF121950, MK686596, D90600 (Genotype 2a) U63715 (Genotype 2b); D87714 (Genotype 3); AB0188667 (Genotype 4); AY949771, KC618401, AY951979 (Genotype 5); AB003292 (Genotype 6). Chimpanzee HPgV-1 strain (AF70476, Black) was used as outgroup and U4402 (Genotype 2) was used for mapping of our sequences to identify regions of similarity.

Ex vivo cytokine and chemokine measurement

Serum samples of 44 volunteers collected at baseline, before vaccination, were used for the assessment of immune activation status. The cytokine and chemokine concentrations were measured using the Procartalex Human 45 plex kit (Cat no: EPX450-12171-901; Affymetrix Biosciences, USA) and acquired on a validated Luminex XMAP technology platform as described [45]. The investigated cytokines and chemokines included BDNF; Eotaxin/CCL11; EGF; FGF-2; GM-CSF; GRO alpha/CXCL1; HGF; NGF beta; LIF; IFN alpha; IFN gamma; IL-1 beta; IL-1 alpha; IL-1RA; IL-2; IL-4; IL-5; IL-6; IL-7; IL-8/CXCL8; IL-9; IL-10; IL-12 p70; IL-13; IL-15; IL-17A; IL-18; IL-21; IL-22; IL-23; IL-27; IL-31; IP-10/CXCL10; MCP-1/CCL2; MIP-1 alpha/CCL3; MIP-1 beta/CCL4; RANTES/CCL5; SDF-1 alpha/CXCL12; TNF alpha; TNF beta/LTA; PDGF-BB; PLGF; SCF; VEGF-A; VEGF-D. Only cytokines and chemokines with levels above the pre-defined lower detection limit of the specific standard curves were included in the group comparisons. Absolute concentrations were normalized to account for the inter-plate variations before analysis in R software version 3.5.1.

Serological analysis

Serum samples for anti-PfCSP antibody evaluation were collected before vaccination (baseline) and 14 days post last vaccination. Anti-PfCSP total IgG levels were measured by enzyme linked immunosorbent assay (ELISA) as described [11, 31, 32].

Quantitative detection of *Plasmodium falciparum*

During CHMI, malaria parasitemia was assessed using microscopy and retrospectively using qPCR as described in [11, 31, 32]. Whole blood samples for the assessment of parasitaemia were taken before CHMI and during the observation period following CHMI beginning at day 9 until volunteers became malaria positive or until day 21.Thick blood smears (TBS) were performed twice a day for days 9 to 14 and once a day for days 15 to 21. TBS were also performed on day 28. Pre-patent periods were calculated from the time of first positivity of qPCR and TBS following PfSPZ challenge as explained elsewhere [11, 31, 32]. Parasite multiplication rate (PMR) was assessed using a linear model fitted to log10-transformed qPCR data as previously published [46]. PMR was calculated for all volunteers that developed blood-stage parasitaemia which lasted for at least two 48-hour cycles [46].

Statistical analysis

Figures and statistical analyses were generated in R version 3.5.1 and GraphPad Software (Prism V5). Wilcoxon rank sum test or Mann-Whitney test used to compare continuous variables. Chi square test was used to compare categorical variables. Absolute values for antibody titers and concentrations of cytokines and chemokines were used. Data were log transformed only when investigating the anti-PfCSP antibody titres and viremia levels. Spearman correlation was used to investigate effect of HPgV-1 infection status and viremia with antibody titres and cytokine levels. Data for cytokines, chemokines and growth factors were not analysed for multiple correction as we considered this question as exploratory. P-value

296 ≤ 0.05 was considered significant. Differences in viral diversity and abundances and prevalence
297 were assessed using LEfSe (Linear discriminant analysis effect size) [47] and GraphPad
298 Software (Prism V5) respectively.
299
300 **RESULTS**
301
302 **Unbiased search for RNA molecules encoding human viruses in RNA-seq transcriptomics**
303 **data**
304 We aimed to identify viruses present in our volunteers participating in PfSPZ Vaccine studies
305 by using a metagenomics approach. Analyses included samples from 28 participants collected
306 at multiple time points including before vaccination (baseline), 2 days after first vaccination, 7
307 days after the first and second vaccination as well as before CHMI, 2 and 9 days after CHMI.
308 Viral sequences were identified from a pool of RNA-Seq data reads that did not map to the
309 human reference transcriptome. A total of 800 million non-human RNA-Seq reads derived from
310 172 whole blood samples were analysed with our virome discovery platform based on
311 previously established metagenomics pipelines and tools [34]–[37].
312 In total, RNA molecules encoding 9 human viruses were detectable including the Human
313 simplex virus (HSV-1), Cytomegalovirus (CMV), Epstein-Barr virus (EBV), Merkel cell
314 polyomavirus (MCV), Human mast adenovirus (HAdV), Astrovirus MBL2, Human
315 betaherpesvirus 7 (HHV-7), Human endogenous retrovirus K113 (HERV-K113), and HPgV-1
316 (**Fig. 1A**). The number of reads for each of the identified viruses was quantified and is given in
317 **Fig. 1B**. After identifying 9 viruses present in 172 whole blood samples, we further assessed
318 the distribution of viruses within each volunteer. HERV-K113 was detected with high number
319 of reads in all 28 individuals, while HSV-1 and CMV were present in seven and six individuals,
320 respectively (**Fig. 1C**). MBL2, HHV-7 and HAdV were present in low read counts in one
321 individual, respectively and MCV was present in two individuals. Eight individuals carried

HPgV RNA with read counts ranging from low to high (**Fig. 1C**). Three out of 8 HPgV-positive individuals were co-infected with CMV **(Fig. 1C)**. This result indicated that HPgV is highly prevalent (28.6%, 8/28) in Tanzanian adults. To reconfirm our findings, we extracted RNA from plasma samples collected from these 8 volunteers and amplified HPgV-1 by RT-qPCR. We were able to reconfirm in 2 out of 8 volunteers the in silico identified presence of HPgV RNA. Interestingly, these 2 volunteers had the highest RNA read counts for HPgV in our bioinformatics analysis.

Detection of HPgV-1 in East and West African volunteers

After having established that HPgV is present in our vaccine cohort, we further aimed to explore the presence of HPgV-1 and HPgV-2 in two larger cohorts from Equatorial Guinea in addition. Plasma samples collected from 96 participants, including 12 HIV-1 positive individuals, were analysed for presence of HPgV-1 and HPgV-2 using RT-qPCR. The overall prevalence of HPgV-1 was 29.2 % (28/96) (**Fig. 2A)**, while HPgV-2 was not detected. The proportion of HPgV-1 positive individuals by gender and geographic location were comparable, with slightly more HPgV-1-positive individuals in Equatorial Guinea (31.4%) than Tanzania (26.7%) **(Fig. 2, B-C)**. Of the 12 HIV-1 positive individuals from Tanzania, two (16.7%, 2/12) were positive for HPgV-1 **(Fig. 2D)**.

HPgV-1 viral loads and distribution

Next, we quantified HPgV-1 viral load in plasma samples using RT-qPCR. HPgV-1 viral loads were comparable between individuals from the two countries **(Fig. 3A)**. However, based on viral loads with a defined threshold of 10^6 viral RNA copies/ml of plasma, both cohorts were subdivided into HPgV-1 low and high viremic individuals (**Fig. 3B)**. High and low HPgV-1 viremia were found in 17 (60%) and 11 (40%) of the 28 HPgV-1 positive volunteers respectively (**Fig. 3B**). Of the 17 high viremic individuals, 8 were from Tanzania and 9 from

Equatorial Guinea. Of the 11 low viremic individuals, 4 were Tanzanians and 7 Equatorial Guineans.

Genotyping of HPgV-1 isolates

Seven different genotypes of HPgV-1 have been described so far with genotype 1 and 5 being highly prevalent in Sub-Saharan Africa [20]. Therefore, we determined the phylogenetic relatedness of the isolates by amplifying and sequencing the 5´ UTRs. From the 28 positive individuals, 2 were excluded due to poor quality of the sequences. Genotype 1 was found in only 2 volunteers (7.7%). Surprisingly, genotype 2, described as dominating in Europe and America, was found in 24 of 26 volunteers (92.3%) **(Fig. 4)**. Most genotype 2 strains clustered closely with the related genotype 2a sequences described from Venezuela (**Fig. 4**). To further increase the resolution of the genetic relatedness of our isolates, we amplified in addition the polymorphic E2 region of HPgV-1 virus. E2 RNA was successfully amplified and sequenced in 9 out of 28 volunteers (32%). According to the E2-sequences of our HPgV-1 isolates, our strains clustered within genotype 1, 2, and 5 **(Fig. 5).** In summary, these results show that a range of HPgV-1 genotypes are circulating in Tanzania and Equatorial Guinea, clustering to published genotypes 1, 2 and 5.

Effect of HPgV-1 positivity on systemic cytokine and chemokine levels

To dissect whether ongoing HPgV-1 infection affects cytokine and chemokine levels in serum, we determined 45 cytokines, chemokines and growth factors in 44 volunteers. 23 cytokines, chemokines and growth factors were detected above their pre-defined lowest detection limits (**Supplementary Fig. 2**). Although there was a trend of overall higher cytokine levels in HPgV-1 infected individuals, only IL-2 and IL-17A reached significance levels **(Fig. 6).** There was no statistically significant difference in cytokine and chemokine levels when high and low viremic individuals were compared. Also, we could not find differences in chemokine and cytokine

levels when comparing the different HPgV-1 genotypes. Taken together these data suggest that the presence of HPgV-1 infection increases IL-2 and IL-17A levels in circulation.

Effect of HPgV-1 infection status on PfSPZ vaccine-induced humoral immune response

As IL-2 and IL-17A might contribute to differentiation of naïve B cells into plasma cells and support the survival of activated B cells [46, 47] we examined the potential of HPgV-1 infection to impact on PfSPZ vaccine-induced humoral immunity by comparing antibody titers to PfCSP, the most immunodominant protein recognized amongst the PfSPZ vaccinated volunteers. Anti-PfCSP titres were measured at baseline (n=70) and 14 days past last vaccination (n=54) by ELISA **(Fig. 7 A-B)**. Similar results were observed when PfSPZ vaccine-induced antibody responses were analysed as net (14 days post last vaccination-baseline) (**Fig. 7 C**) and as fold change (14 past last vaccination/ baseline ratios) (**Fig. 7 D**). No significant correlation between HPgV-1 infection status and measured anti-PfCSP antibody titers was found.

Effect of HPgV-1 infection on PfSPZ vaccine efficacy

The high prevalence of HPgV-1 in our cohort allowed us to investigate a potential impact of ongoing viral infection during PfSPZ vaccination on vaccine-induced protection. Protective efficacy of the vaccine was evaluated by presence or absence of asexual blood stage parasitemia following homologous PfSPZ challenge (CHMI) (**Supplementary Fig. 3**). While none of the placebo-receiving participants was protected (0/20), the overall protection in the vaccinated group was 55% (26/47). The HPgV-1 prevalence was comparable in these 2 groups, 35% (7/20) vs 34% (16/47) respectively, suggesting that HPgV-1infection does not facilitate protection against CHMI. To assess the impact of ongoing HPgV-1 during PfSPZ vaccination on vaccine-induced protection, we further compared the protection levels between HPgV-1 positive and negative participant in the vaccinated group **(Fig. 8A)**. Surprisingly, HPgV-1 positive vaccinees

showed higher protection after CHMI (62.5%; 10/16) than HPgV-1 negative individuals (51.6%; 16/31). We also assessed anti-CSP antibodies titres at 14 days past last vaccination. Slightly higher anti-CSP levels were seen in protected than non-protected individuals **(Fig. 8B)**, but without any statistical significance. However, these levels tended to be lower in the HPgV-1 positive individuals than the negative participants

Interaction of HPgV-1 and CHMI induced asexual blood stage

HPgV-1 co-infection has been associated with favourable outcomes in HIV-1 and Ebola infected individuals [23]–[26]. But so far its impact on *P. falciparum* infection and immunity is unknown. We evaluated parasite multiplication rates and pre-patent period in the control volunteers (n=20) undergoing CHMI using PfSPZ challenge. Comparable asexual blood stage multiplication rates and pre-patent periods were observed between HPgV-1 positive and negative individuals (**Fig. 9 A-B)**.

Effect of CHMI on HPgV-1 viremia levels

P. falciparum infection is known to impact viremia levels of some common viruses [49] . We therefore evaluated the effect of an acute *P. falciparum* infection on HPgV-1 viremia by comparing the viral load before and 28 days post CHMI. Detectable levels of HPgV-1 at both time points were quantified in 9 individuals; 5 of those showed an increased HPgV-1 viral load and the other 4 had lower viremia post CHMI with no statistical significance. In addition, 12 trial participants were HPgV-1 positive only for one of the two tested time points; 6 were positive before CHMI and 6 were positive at 28 days post CHMI (**Fig. 9C).**

Discussion

The role of chronic asymptomatic viral infections in modulating immune responses in health and disease is increasingly appreciated[50].The present study sought to better understand the prevalence and genotype distribution of HPgV-1 in East and West-central Africa. We aimed to investigate the potential influence of HPgV-1 infection on experimental malaria vaccine-induced humoral immunity and vaccine-induced protection. By studying a cohort of volunteers undergoing CHMI, we were in a unique position to investigate if an acute malaria episode has an impact on HPgV-1 viremia in chronically infected volunteers.

The overall prevalence of HPgV-1 in our cohort was 29.2 %, roughly the same for Tanzania and Equatorial Guinea. The prevalence reported here is likely underestimated as we observed fluctuations of HPgV-1 viral loads and detection rates studied at different time points, with some volunteers showing HPgV-1 positivity in only one, two or all three measured time points**.** These detection variations might indicate either viral clearance or continuously ongoing viral replication with viremia fluctuations sometimes below the detection limit of the RT-qPCR assay. The drawbacks of RT-qPCR based methods over deep sequencing methods in virus identification are well known and to overcome these limitations the combination of qPCR and sequencing is recommended [50, 51]. The variation in HPgV-1 vireamia levels between serum and cellular have been previously reported, with as high as 10 $X10^7$copies found in serum alone [22, 27]. We did not detect HPgV-2 RNA in any of our volunteers but we cannot completely exclude the possibility of the presence of HPgV-2 infections as antibody titers against the HPgV-1 and HPgV-2 E2 envelope proteins were not measured.

Similar to a study in Mexico, we observed two broad groups, low and high, of HPgV-1 viremic individuals, categorized by a cut off value of 600,000 RNA copies/ml. This observation likely reflects the different viral replication states within infected volunteers [24]. We observed

similar numbers of high and low viremic individuals, who are infected with HPgV-1 genotype 2. The potential role of a distinct viral genotype on this pattern remains unclear, given the small number of volunteers in this study and limited heterogeneity of the detected HPgV-1 genotypes.

Currently, 7 HPgV-1 genotypes are described globally [52, 53] and some of these genotypes have been implicated in varied clinical outcomes [25, 28, 54]. HIV/HPgV-1 co-infection studies showed lower CD4 T cell counts in individuals infected with HPgV-1 genotype 2a than genotype 2b [55, 56] and higher HPgV-1 viral loads in individuals with genotype 1 compared to genotypes 2a and 2b [58]. Higher serum levels of IFN-ɣ were described in HIV-1 positive women co-infected with genotype 2 compared to genotype 1 [23].

Phylogenetic analyses in our cohort revealed presence of genotype 1 (n=2, 7.1%) and 2 (n=24, 92.3%). Most of our genotype 2 strains clustered with group 2a, originally described from Venezuela. Genotype 1 and 2 have been previously reported in Tanzania but there is no published data for Equatorial Guinea [59, [60]. The predominance of genotype 2 in our study is somewhat surprising. Given the diverse geographic origin of our volunteers recruited from East and West-central Africa, we had expected to find extensive HPgV-1 genetic diversity. Studies in neighbouring countries including Cameroon, the Democratic Republic of Congo and Gabon documented a high prevalence of genotype 1[61]–[65]. Lower numbers of genotypes 2 and 5 were also seen, when phylogenetic studies included molecular markers other than 5` UTR region like envelope protein 1 (E1), non-structural protein 3 (NS3) and non-structural protein 5A (NS5A) [62, 66].

The limitation of using only the amplification of the 5` UTR, a highly conserved region, to discriminate closely related isolates is known [67, 68]. We therefore amplified the E2 region in addition to a successfully amplified 5` UTR. We were able to detect and sequence the E2 coding RNA only from subjects with high viremia (n=9). Based on the E2 sequences, these nine

isolates clustered with strains described elsewhere in Africa. It is possible that the failure to amplify E2 from all volunteers positive for 5` UTR detection is due the low sensitivity and high diversity of the E2 region [44]. While it is known that the detection of HPgV-1 based on amplification of the E2 region is highly specific, it requires very high amount of RNA input [44] and individuals with low HPgV-1 viremia are likely missed. Alternatively, it is possible that E2 genetic variants could not be amplified with the primers used in these studies due to sequence mismatch. The E2 region is highly variable and this diversity contributes to structural, functional and immunogenic properties of the virus [69].

Cytokines, chemokines and growth factors are important for inter-cellular communication and regulation of immune processes [70]. Any changes in levels of these immune mediators can act as markers of inflammation, immunity or vaccine uptake [28, 71, 72]. We therefore investigated if altered levels of cytokines and chemokines unique to ongoing HPgV-1 infection could be identified. We analysed serum samples taken at baseline for 45 cytokines in a Luminex platform. Volunteers with chemokine and cytokine levels above the lower limit of detection were stratified according to the HPgV-1 infection status. Of all 23 differentially detected cytokines and chemokines, IL-2 and IL-17A were significantly higher in HPgV-1 positive compared to HPgV-1 negative individuals.

IL- 2 is an essential survival factor for T and B lymphocytes [48, 73] and induces development and survival of regulatory CD4 T cells critical for maintenance of immune tolerance [74]. Fama et al., showed increased levels of circulating soluble IL-2 receptor (sIL-2R) in HPgV positive volunteers but the authors did not quantify IL-2 levels [75] . The increased concentrations of IL-2 seen amongst the HPgV-1 positive individuals could be linked to either on-going antiviral immunity [76] or serves as a survival mechanism used by the virus to establish persistence in immune cells. A similar mechanism has been described in the apicomplexan pathogen *Theileria*

parva that infects T and B lymphocytes in cattle [77]. Contrary to our observations are results from HPgV/HIV coinfection studies which have shown reduced T-cell activation and IL-2 release in individuals positive for both viruses [78, 79]. The HPgV-1 envelope protein 2 (HPgV1-E2) has been implicated in these outcomes, due to its ability to inhibit T cell-receptor mediated signalling and IL-2 signalling pathways [78, 79].

IL-17A induction has been associated with bacterial, fungal, autoimmune and inflammatory diseases [80]. IL-17A stimulates production of chemokines such as monocyte chemoattractant protein-1 which mediates tissue infiltration of monocytes. The role of IL-17A in the context of HPgV infection is unknown. However, in other viral infections like HIV and Hepatitis C, IL-17A has been shown to promote T-cell mediated anti-viral responses through activation and recruitment of dendritic cells, monocytes and neutrophils [81, 82]. Other cytokines and chemokines which could be detected, albeit not significantly different in in HPgV-1 positive individuals included SCF (lower) and IL-1beta, IL-12p70, MCP-1, LIF, VEGF.A, HGF, and TNF-α (higher). BDNF, EGF, Eotaxin, GRO-alpha, IFN-ɣ, IL-7, IP-10, MIP1-a, Mip-1b, PDGF.BB, PIGF.1, RANTES, SDF-1a, and VEGF.D were comparable between the two groups. The levels of these cytokines and chemokines are within a comparable range as previously reported [28, 29]. While most of the previous HPgV-1 studies had focused on at risk populations, particularly on HIV-1 positive persons, our investigations are in healthy individuals [28, 75], therefor some of the observed differences could be due to health status.

Here, we observed lower, albeit not statistically significant, median anti-CSP titres in the HPgV-1 positive versus the HPgV-1 negative group at baseline and 14 days past last vaccination. These observations mirror findings by Avelino-Silva et al., who found no association between HPgV infection status/viremia with yellow fever specific neutralizing antibody titers in HIV-1 positive individuals immunized with yellow fever vaccine [83]. While

studies have extensively tried to understand potential inhibition mechanisms induced by HPgV-1 (and other Flaviviruses) on T cell activation [78, 84] activation pathways that might be affected in B cells are less explored. It is also possible that the effect of HPgV viruses on immune responses against vaccines is negligible when studied singly, but this impact is significantly synergized in the presence of other, co-infecting viruses like EBV, CMV and HSV [85, 86]. Hence, the potential role played by the combined human virome in shaping vaccine-induced responses in different populations needs to be further explored in larger cohorts.

Clinically silent, chronic viral infections are known to modulate host immunity [17] and in turn, acute co-infections are known to drive the re-activation of asymptomatic viral infections [87]. Several viruses, like HIV, Ebola and HCV have been implicated in the pathogenesis and clinical outcome of ongoing malaria infections through a range of different mechanisms [88]–[90]. It has been suggested that HIV infections worsen *P. falciparum* presentations by depleting the CD4 T-cell compartment essential for driving malaria-specific antibody responses and for clearance of malaria infected red blood cells [88]. In contrast, better survival outcomes have been reported in Ebola infected individuals with *P. falciparum* co-infections [89]. Reports have also suggested delayed emergence of *P. falciparum* asexual blood stages in Gabonese individuals chronically infected with HCV [90]. Thus, we studied the impact of HPgV-1 positivity on asexual *P. falciparum* parasitaemia and multiplication rates during CHMI. Vice versa, we also looked at the impact of PfSPZ vaccination and PfSPZ challenge on HPgV-1 viremia. We could not find evidence of an association between HPgV-1 infection status and asexual blood stage parasite multiplication rates after CHMI. Notably, a slight trend towards longer pre-patent period was seen in HPgV-1 positive individuals. HPgV-1 positivity appears to increase malaria vaccine-induced protection, since slightly higher proportion of CHMI protected individuals were seen in HPgV-1 positive trial participants (62.5% vs 51.6%).This outcome can be partially explained by immune modulation properties of HPgV-1 on immune

cells activation and systemic cytokines like the higher IL-2 and IL-17A observed in HPgV individuals in this cohort. However, the current study is limited by the low sample sizes and further investigations with larger cohorts are required to corroborate these findings. Importantly, PfSPZ vaccination and PfSPZ challenge did not impact HPgV-1 viremia levels in our cohort suggesting that the conduct of CHMI is safe in HPgV-1 infected volunteers.

Conclusions

Notable effects have been reported in HPgV co-infections with other RNA viruses such as HIV-1 and Ebola. Although our study is constrained with limited sample size, we have highlighted the epidemiology and genetic distribution of HPgV-1 in areas endemic for malaria. We have reported for the first time genotype distribution of HPgV in Equatorial Guinea. We examined the potential influence of HPgV infection status on PfSPZ vaccine-induced CSP-antibody titers and CHMI outcome without finding any striking correlation. Our study provides first time evidence that intravenous vaccination using large numbers of attenuated *P. falciparum* sporozoites and CHMI does not increase HPgV viremia in already infected volunteers.

List of abbreviations

CHMI: Controlled human malaria infection

CSP: Circumsporozoite protein

E1/2: Envelope glycoproteins (1 and 2)

HPgV: Human pegivirus

IVT: In vitro transcription

LEfSe: Linear discriminant analysis effect size

NK: Natural killer cells

NS5A: Non-structural protein 5A

PfCSP: Plasmodium falciparum circumsporozoite protein

PfSPZ: Plasmodium falciparum sporozite

PMR: Parasite Multiplication Rate

RNase P: Ribonuclease P

SSA: Sub-Saharan Africa

TBS: Thick blood smear

UTRs: Untranslated regions

DECLARATIONS

Ethics approval and consent to participate

The studies were registered at Clinicaltrial.gov. under the registration numbers NCT02132299 (BSPZV1), NCT02613520 (BSPZV2), NCT03420053 (BSPZV3a) and NCT02859350 (EGSPZV2). All clinical trials were approved by the Institutional Review Board for the Ifakara Health Institute (IHI-IRB), Tanzanian Food and Drug Administration (TFDA), Tanzanian National Institute for Medical Research (NIMR) and the Ethical Committee of Northern and Central Switzerland (EKNZ). Written informed consent was obtained from all participants prior enrolment. All trial procedures were conducted in accordance to good clinical practice (GCP) and under the Declaration of Helsinki.

Consent for publication

Not applicable

Availability of data and materials

Data are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was funded by Equatorial Guinea Malaria Vaccine initiative. AT is supported by Swiss government, through ESKAS scheme scholarship grant no 2016.0056. KS was supported by NIH grant AI128194.

Authors' contributions

Study concept and design: AT, TS, CD, Investigation: AT, TS, JP, MP, KS, Technical support and resources: SM, DM, Analyses and interpretation of data: AT, TS, NOF, CD, Drafting the manuscript and reviewing: AT, TS , NOF, CD, and all other authors reviewed the manuscript, Study supervision: CD; AO, SJ, Funding acquisition: CD, MT.

Acknowledgements

The authors would like to thank all participants of the clinical trials for their willingness to participate. We extend our appreciation to the clinical and laboratory staff involved in these trials (BSPZV1, BSPZV2, BSPZV3a and EGSPZV2), both at the Ifakara Health Institute in Tanzania and the Malaria Vaccine Initiative in Malabo, Equatorial Guinea. We would also like to thank: Prof. Jack Stapleton (University of Iowa), Prof Laurent Kaiser and Dr. Samuel Cordey (University of Geneva) for providing the plasmids which were used as positive controls and for generating standards for HPgV strains; Ashery Mbilinyi for technical assistance; Tanzania commission of science and technology (COSTECH) for providing us with high performance computing facility.

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Figures, tables and additional files:

Figure 1. Unbiased search for RNA molecules encoding human viruses in RNA-seq transcriptomics data.

A) Overall prevalence of 9 human viruses detected in 172 whole blood samples B) Number of viral RNA-seq reads detected for each of the identified viruses. Virus names are plotted on the y-axis and prevalence (A), number of reads (B) on the x-axis. (C) Distribution of the 9 different viruses across the 28 individuals included. Virus names are plotted on the y-axis and volunteer IDs on the x-axis. Each bar indicates viral reads for an individual. The log viral RNA-seq reads are plotted, in increasing order ranging from 0-3; green indicating low number and red high number of reads.

Figure 2: Proportion of individuals with (purple) and without (grey) HPgV- 1 infection. A) Total cohort of 96 vacinees, B) separated by gender, C) Country of origin, D) HIV-1 infection status. All individuals are between 18-35 years of age. Chi square with Yates correction for group comparisons (*, $P<0.05$).

Figure 3: Comparisons of HPgV-1 viral loads.

No differences in HPgV-1 viral loads between Equatorial Guinea (green, n=16**)** and Tanzania (blue, n=12) volunteers **(A)**. Two distinct groups with low (blue) and high (grey) viremia levels in plasma are found in HPgV-1 infected individuals **(B).** The two groups were divided based on a cut off value of 600,000 RNA copies/ml plasma.

Figure 4. Phylogenetic inferences of the HPgV-1 isolates based on 5´ UTR.

Phylogenetic tree was constructed using Neighbour joining method and Kimura two-parameter model of the 5´ UTR. The 5´ UTR sequences from Tanzania and Equatorial Guinea (n=26) were compared to selected references spanning genotype 1 to 7 from different countries available in the NCBI database. The accession numbers for the reference sequences were: AF488786, AF488789, KC618399, KP710602, U36388, JX494177, Y16436, and MF398547 (Genotype 1, Pink); AB003289, AF104403, D90600, JX494179, MG229668, JX494180, U4402, U59518 (Genotype 2; 2a light brown), MH000566, U59529, U63715, MH053130 (Genotype 2; 2b Brown); AB008335, KR108695, JX494176, D87714 (Genotype 3, Green); AB0188667, AB021287, HQ3311721 (Genotype 4, Maroon); DQ117844, AY949771, AF488796, AF488797 (Genotype 5, Light blue), AB003292, AF177619 (Genotype 6, Bright green); HQ331235, HQ 3312233 (Genotype 7, Golden) and Hepatitis C (AJ132997, Black) was used as outgroup.

Figure 5. Phylogenetic inferences of the HPgV-1 isolates based on E2 region.

Phylogenetic tree was constructed using Neighbour joining method and Kimura two-parameter model of the E2 region of HPgV-1. The E2 sequences from Tanzania and Equatorial Guinea (n=9) were compared to selected references spanning genotype 1 to 6 from different countries available in the NCBI database including; KP701602.1, KM670109, U36380, KP710600, KC618399, AB003291 (Genotype 1, Pink); AF121950, MK686596, D90600 (Genotype 2; 2a Brown), U63715 (Genotype 2; 2b Brown) D87714 (Genotype 3, Green); AB0188667 (Genotype 4, Brown); AY94977, KC618401, AY951979 (Genotype 5, Light blue) and AB003292 (Genotype 6, Green). Equatorial Guinean and Tanzanian strains identified in this study are denoted by strain number followed with letters EG or TZ, respectively (Red). Chimpanzee HPgV-1 strain (AF70476, Black) was used as outgroup and U4402 (Genotype 2,

Golden) was used for mapping of our sequences to identify regions of similarity. The scale bar under the tree indicates nucleotide substitution per site.

Figure 6: HPgV-1 infection is associated with increased systemic levels of IL-2 and IL-17A.

Cytokine, chemokine and growth factors levels were analysed by Luminex and levels compared between HPgV-1 negative (5` UTR- **,** Grey, n=35**)** and HPgV-1 positive (5´ UTR+, Purple, n=9) volunteers. Absolute serum concentrations levels (pg/mL) of Interluekin-2 (IL-2) and Interluekin-17A (IL-17A) at samples taken before vaccination are shown. Significantly higher IL-2 and IL-17A are seen in the HPgV-1^{+} compared to the HPgV-1^{-}. Wilcoxon rank sum test was used to determine significance (p-value * < 0.05) which are indicated on top of top for each group comparison.

Figure 7: HPgV-1 infection status does not impact on anti-PfCSP antibody titres.

Total IgG antibodies recognizing full length PfCSP were measured by ELISA in HPgV-1 negative (5 UTR- **,** Grey), HPgV-1 positive (5 UTR+, Purple) volunteers. **A)** Shows baseline (pre-vaccination) anti-PfCSP IgG levels of HPgV-1 negative (n=47) compared to HPgV-1 positive (n=23) volunteers. **B)** Anti-PfCSP IgG levels at 14 days past last vaccination in HPgV-1 positive individuals (n=17) versus the HPgV-1 negative (n=37) group. (**C-D)** Comparison of vaccine-induced changes in anti-PfCSP IgG titres as net responses (14 days post last immunization - baseline) as well as fold (14 days post last vaccination/baseline)**.** Only vaccinated individuals were included for 14 days post last immunization, net and fold change responses**.** One HPgV-1^{+} individual was not included in these subsequent analyses due to missing antibody data. Log anti-PfCSP titres expressed in arbitrary units are shown**.** Each point represent an individual, box plot with horizontal bar show median values for each group.

Statistical significance was calculated by using Wilcoxon rank sum test (p-value * < 0.05). P values are indicated on top for each group comparison.

Figure 8: HPgV-1 infection does not influence *P. falciparum* pre-patent periods and parasite multiplication rates during CHMI.

Parasitaemia was determined in whole blood by qPCR and thick blood smear microscopy (TBS). The analysis included only placebo participants, positive and negative for HPgV-1. **(A)** Shows Log fold change of parasitaemia in 48 hours between HPgV-1 negative (5 UTR-, Grey, n=13) and HPgV-1 positive (5 UTR+, Purple, n=7) volunteers. **B)** Comparison of days post CHMI to malaria positivity by microscopy in HPgV-1 negative (5 UTR-**,** Grey, n= 11) and HPgV-1 positive (5 UTR+, Purple, n=7). **C)** HPgV-1 viral loads before (red) and 28 days post CHMI (green) in HPgV-1 infected individuals. Each point represents an individual, box plots show data distribution with horizontal bar denoting viral load at each visit. Lines connect viremia levels in individuals found positive for HPgV-1 on both time points. Geometric means were compared between groups and unpaired t-test was used to calculate significance. Horizontal bars represent mean with standard deviation Wilcoxon rank sum test was used to compare viremia levels before and after CHMI. P-values are indicated on top of each comparison.

Figure 9: Association of HPgV-1 infection status with PfSPZ CHMI outcome and anti-CSP titers in immunized volunteers.

Individuals were treated with either normal saline (placebo) or PfSPZ Vaccine (vaccinees). Presence or absence of malaria parasites was determined in whole blood by thick blood smear microscopy (TBS) and confirmed by qPCR. Total IgG antibodies recognizing full length PfCSP were measured by ELISA. **A)** Proportion of non-protected (cream) and protected (blue) in vaccinated volunteers with and without HPgV infection. Proportions are indicated inside the bar and volunteer numbers on top. **C)** Total anti-CSP IgG levels at 14 days past last vaccination in the protected (malaria negative) and non-protected (malaria positive) groups, with and without HPgV-1 infection. Log anti-PfCSP titres expressed in arbitrary units are shown**.** Each point represent an individual, and box plot with horizontal bar show median values for each group. Chi square with Yates correction was for group comparisons of categorical values (*, $P<0.05$). Wilcoxon rank sum test was used to compare anti-CSP titres in the two groups. P values are indicated on top of each comparison

Supplementary Materials

Sup. Figure 1: Flow chart of volunteers included in virome pilot study and analyses pipeline.

A) Flow chart of volunteers included in virome pilot study and analyses. Samples for transcriptomic studies were selected from a subset of volunteers of BSPZV-1 (n=28). RNA sequencing was performed and, differential gene expression and blood transcriptome modules were analysed. Non –human reads data was used for virome analyses. B) **Virus identification**: Pilot virome study analysis pipeline**-"Bagamoyo viromescan"** i) Non-human (un-mapped reads) were searched for "suspected" viral hits in NCBI database containing more than 7424 viral genomes using bowtie 2. ii) Removal of low quality and complexity reads as well as reads

mapping to human genome, transcriptome and repeat regions by bowtie 2, knead data and tandem repeat finder algorithms respectively. iii) Search for viral hits in the "clean" viral reads using virome scan and Taxonomer and for viral proteins using Diamond tool. iv) The non-human unmapped reads were also analysed by Fast virome explorer, without filtering host reads to allow the identification of endogenous retroviral elements and other viruses that may have been missed by Taxonomer and viromescan. C) **Viral confirmation**: i) Pre-selection criteria for suspected viral hits by each tool ii) In-silico confirmation of suspected viral hits through blasting in NCBI and mapping against specific viral whole genomes in geneous tool; and removal of viral contaminants. iii) Laboratory confirmation of viruses by reverse transcription polymerase chain reaction

Sup. Figure 2: Impact of HPgV-1 infection on systemic cytokines and chemokines. Absolute cytokines, chemokines and growth factor levels at baseline are shown based on HPgV status: HPgV negative (-), grey (**n=35)** and HPgV positive (+), Purple **(n=9).** Comparable median levels of Brain derived neutrophin factor (BDNF), Epidermal growth factor (EGF), Eosinophil chemoattractant cytokine (Eotaxin/ CCL11),Growth regulated oncogene-alpha (GRO-alpha),Interferon gamma (IFN-ɣ), Interluekin-7 (IL-7), Interferon gamma induced protein- 10 (IP-10), Macrophage inflammatory protein 1-alpha(MIP1-a), MIP1-b (Macrophage Inflammatory protein 1-beta), Platelet derived growth factor BB (PDGF.BB), Placental growth factor (PIGF.1), Regulated on activation normal T cells and excreted (RANTES), Stromal derived factor 1 alpha (SDF-1a) , and Vascular endothelial growth factor D (VEGF.D); Lower median levels of Stem cell factor (SCF); and higher median levels of Monocyte chemoattractant protein 1 (MCP-1), Leukemia inhibitory factor (LIF), Vascular endothelial growth factor A (VEGF.A), Hepatocyte growth factor (HGF) and Tumor Necrosis Factor-alpha (TNF-α) in the HPgV-1 positive individuals. Cytokines, chemokines and growth factors with values above their predefined lower detection limit were considered substantial. Wilcoxon rank

sum test was used to compare the two groups and P-values are indicated on top for each comparison.

Sup. Figure 3: Vaccine trial design and procedures .

Volunteers are enrolled and randomized into placebo (Black icons) and vaccine groups (Green icons). Immunized with specified dose of irradiated-attenuated whole sporozoites or whole sporozoites with antimalarial drug (V1, V2; V3 etc) and subsequently challenged with homologous PfSPZ parasites used for vaccination (CHMI). Volunteers are monitored in a controlled setting up to 21 days with venous blood drawn daily to monitor presence (malaria positive, not protected) or absence (malaria negative, protected) of asexual blood stage parasitaemia. All volunteers were treated with an anti-malarial drug either once turning TBS positive or at day 28 after start of CHMI. Further monitoring of volunteers occurred at 56 days post CHMI. HPgV-1 infection was evaluated in plasma samples from the time points highlighted in blue

Sup. Figure 4: HPgV RNA positivity and viremia across study visits (Baseline, CHMI and CHMI+28) in Tanzania and Equatorial Guinea:

HPgV-1 viral plasma RNA was measured by RT-qPCR at baseline(Pre-vaccination), before (CHMI) and 28 days post immunization (CHMI+28 days) in Tanzanian (n=45) and Equatorial Guinean (n=51) volunteers. Here four volunteers from the whole cohort are displayed as a representation. The figure depicts inter-individual variability in HPgV RNA detection with some individuals negative or positive at one, two or all three measured time points. Log 10 viral loads are plotted on the y-axis and the time points in the horizontal axis. Each square plot represents an individual with volunteer identification numbers indicated on top. Each dot corresponds to a single time point connected to the next by a solid line. The horizontal dashed line indicates the threshold value of zero viremia