

Epitope-resolved Epstein-Barr virus antibody mapping identifies disease-stratifying signatures in nasopharyngeal carcinoma

Corresponding Author: Professor Xu Lin

This manuscript has been previously reviewed at another journal that is not operating a transparent peer review scheme. The manuscript was considered suitable for publication without further review at Nature Communications.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

1. The cohort descriptions require careful revision. The Methods and Results appear to report different NPC/control numbers for the VirScan discovery cohort, e.g., the Methods show that Cohort I contains 36 NPC cases and 47 controls, whereas the Results section says the VirScan discovery cohort as 47 NPC patients and 36 controls. Figure 1A also appears to label NPC n=37 and NC n=46, while Table 1 reports NPC n=36 and NC n=47. It is very confusing. A discrepancy is also seen in Tables 2 and 3. Look at them very carefully. The percentages across TNM stages are identical, even when the patient numbers differ. For all cohort sizes and group labels, tables- each value should be thoroughly rechecked across the manuscript, figures, legends, tables, and supplementary tables.
2. The use of VirScan as a broad discovery platform is appropriate, but the relatively small discovery cohort limits the strength of the conclusion that EBV is the sole viral axis in NPC. The authors should either expand this analysis, validate the viral specificity in an independent subset, or modify the language to reflect that this is an exploratory discovery cohort.
3. The authors should provide more details for the statistical filtering. Like, reporting the minimum read count thresholds, fold-enrichment cutoffs, z-score cutoffs, multiple-testing correction strategy, and how background enrichment was estimated and adjusted?
4. Continuing with this, I agree with the use of EBVScan after Virscan in principle, but I am curious why the authors didn't use the same cohort to analyze both platforms. Using all different cohorts for both analyses weakens the connection between virome-wide discovery and EBV-focused analyses.
5. The EBV serostatus of controls should be clearly reported. Do we know how many healthy samples are EBV seropositive? EBV infection is highly prevalent, so we need to know the status among healthy controls to clearly distinguish between healthy-enriched vs disease-enriched epitopes.
6. The manuscript appropriately emphasizes the challenge of cross-reactivity among human herpesviruses, but it does not clearly demonstrate how EBVScan distinguishes EBV-specific antibody binding from cross-reactive responses to homologous epitopes in other herpesviruses, particularly given the introduction's emphasis on this as a major drawback of existing platforms. I think the paper should discuss how their PhIP-seq platform tackles this question. The authors should provide sequence homology analysis of lead peptides against the human herpesvirus proteome, if possible, or elaborate in the discussion section about how cross-validation needs further exploration.
7. I am not quite convinced by the authors' choice of the precise amino acid or residue-level epitopes. See, the EBVScan library is composed of 56-aa peptides with a 28-aa overlap, correct? However, the manuscript highlights narrow regions such as gp42 144-163, gL 65-84, and GP350 146-165, and interprets them as receptor-interface/protective antibody targets. A 56-aa enriched peptide can show that antibody reactivity occurs somewhere within that region, but it cannot prove that

antibodies bind the exact 20-aa sequence or the specific receptor-contact residues. I think that to claim the 20-AA precise epitope, additional fine-mapping (for example, alanine scanning) must be performed (especially given that most B cell epitopes are actually 8-12 AA). I think authors should rewrite these epitopes as mere structurally overlapping regions within the protein rather than as experimentally resolved epitopes.

8. In addition, I am not entirely convinced by the claim that the identified epitopes are protective, given the lack of functional validation (neutralization or receptor blockade). The authors need to rewrite and significantly understate the claim.

9. I am confused how the authors are calling the serum IgA response “mucosal responses”? How do we know whether the IgA is mucosal or serum? Please elaborate on the use of the term mucosal for IgA, or change it to Serum IgA

10. The internal calibration strategy is interesting, but I believe the manuscript should more clearly define exactly what is happening. The references are incorporated during DNA amplification, but if they are not there in the PhIPseq library itself, how can we compare the responses during the actual immunoprecipitation step? Please clarify.

11. It is good that the authors have deposited the raw sequencing data into the SRA, but these data are useless without the sequences of the peptides in the phipseq libraries because you can't do alignments and generate peptide counts. The normalized peptide counts for each peptide and each sample should be made available as well as the sequences of the peptides that were part of the VirScan and EBVScan libraries. In addition, when I look at the SRA data, it's not clear to me that the sequencing data are available on a per participant basis and that the participant IDs map back to the paper so that someone could recapitulate the analyses done by the authors.

12. PhIP-Seq is a semi-quantitative assay, and the authors need to do more work by presenting more orthogonal quantitative antibody titer data to confirm that their claim of having devised a qPhIP-Seq assay is legitimate.

13. There are numerous claims about diagnostic biomarkers having been identified, but a biomarker is only useful if it differentiates between patients with clinically overlapping conditions that require differentiation. It's not clear to me how NPC patients have meaningful clinical overlap with people who are healthy or who have a runny nose (i.e., nasopharyngitis). In that case, I would not call these diagnostic biomarkers that have been "rigorously tested" for "cancer-specific diagnostic accuracy"

14. Need to add to limitations that phipseq is geared toward discovering linear antigens and frequently missing conformational and/or post-translationally modified epitopes

Reviewer #2

(Remarks to the Author)

This manuscript presents EBVScan for high-resolution mapping of EBV antibody responses in NPC. The authors constructed a comprehensive pan-strain EBV peptide library and identified several diagnostically and prognostically relevant epitopes, including TK139–194, which demonstrated excellent diagnostic performance in a large validation cohort. The work possesses considerable novelty and translational potential. However, several important issues must be addressed before the conclusions can be fully supported.

Major Comments

1. Although the authors describe separate discovery, refinement, and validation cohorts, all samples appear to originate from the same geographic region (Fujian Province). Therefore, the validation cannot be considered truly independent. Validate the diagnostic model in an independent external cohort. If this is difficult, discuss this limitation.

2. The reported diagnostic performance of TK139–194 is impressive. However, the study evaluates only healthy controls and non-NPC outpatient controls. Importantly, no comparison is performed with other EBV-associated diseases such as infectious mononucleosis, Hodgkin lymphoma, and CAEBV. Because these conditions may also induce lytic EBV antibody responses, the specificity of TK139–194 for NPC remains uncertain. The authors had better to include additional disease controls.

3. TK139–194 is identified as the strongest diagnostic epitope, yet little mechanistic explanation is provided regarding why this specific region is preferentially recognized in NPC.

The manuscript would benefit from additional information regarding conservation among EBV strains, structural localization within TK, and functional relevance during viral replication.

4. The manuscript repeatedly refers to gp42, gL, and GP350 epitopes enriched in healthy controls as “protective epitopes.” However, the presented data are entirely observational and cross-sectional. The study demonstrates association with healthy status but does not establish causality or protective function. No experiments demonstrate viral neutralization, inhibition of cell entry, or prevention of transformation. Therefore, the term “protective epitope” appears premature. It is better to replace with more conservative terminology such as “healthy-associated epitopes” unless functional validation is provided.

5. The structural mapping analyses are interesting and suggest that healthy-enriched epitopes overlap receptor-binding interfaces involving HLA-II, EphA2, or CR2. However, these conclusions are based solely on structural proximity. No experimental evidence demonstrates that antibodies targeting these epitopes actually interfere with receptor engagement or viral entry. Functional assays should be considered, such as pseudovirus neutralization assays, receptor-blocking experiments, or cell-entry inhibition studies using Akata-EBV system.

6. Plasma/serum EBV DNA quantification is currently among the most established biomarkers for NPC screening and monitoring. The manuscript compares TK139–194 with EA-IgA and VCA-IgA. Is this possible to compare its performance

with plasma/serum EBV DNA-based screening strategies?

7. The manuscript does not clearly explain how ELISA cutoffs were selected. In addition, whether cutoff determination and validation were performed in separate datasets.

Minor Comments

8. The sample numbers reported in Figure 1 appear inconsistent with those described in the Methods section. Please verify and correct the reported cohort sizes.

9. The manuscript refers to both: 42 EBV strains and 43 EBV strains, in different sections.

Please clarify and ensure consistency throughout the text.

10. For the Kaplan–Meier analysis, hazard ratios and 95% confidence intervals should be reported in addition to log-rank P values.

11. The authors should describe the chemotherapy protocol.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

the authors have addressed my concerns

Reviewer #2

(Remarks to the Author)

The authors have adequately addressed my major concerns. In particular, the addition of an independent Sichuan cohort, EBV-associated disease controls, comparison with circulating EBV DNA, and functional infection-blocking experiments substantially strengthens the manuscript. I also appreciate that the authors have appropriately moderated the interpretation of the healthy-enriched regions and acknowledged that peptide-mediated inhibition does not establish antibody-mediated neutralization or receptor blockade. I have no further concerns.

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Response to reviewers

Reviewer #1 (Remarks to the Author):

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Response: We thank the reviewer for identifying these inconsistencies. We re-audited the participant-level records and systematically cross-checked all cohort sizes, group labels, and clinical characteristics throughout the manuscript, including the Methods, Results, figures, figure legends, tables, and Supplementary Information. All discrepancies have been corrected accordingly. The shared VirScan discovery/EBVScan refinement cohort comprises 79 patients with NPC and 94 healthy controls. The validation cohort comprises 100 patients with NPC, 495 healthy controls, and 195 non-NPC outpatients. The treatment-response analysis includes 51 evaluable patients (33 treatment-sensitive and 18 treatment-resistant). For the 79 patients in the VirScan/EBVScan cohort, the corrected stage distribution in Table 1 is 16 patients with stage I, 7 with stage II, 18 with stage III, and 38 with stage IV disease (33 stage IVA and 5 stage IVB). These numbers have now been consistently reported throughout the revised manuscript and associated materials. (Method: line 119-141)

2. The use of VirScan as a broad discovery platform is appropriate, but the relatively small discovery cohort limits the strength of the conclusion that EBV is the sole viral axis in NPC. The authors should either expand this analysis, validate the viral specificity in an independent subset, or modify the language to reflect that this is an exploratory discovery cohort.

Response: We agree with the reviewer's concern and have revised the manuscript to adopt a more

cautious interpretation. VirScan is now presented as an exploratory virome-wide screening approach, and EBV is described as the predominant viral target identified within this dataset, rather than the sole viral axis associated with NPC. The transition from VirScan to EBVScan was based on the marked enrichment of NPC-associated antibody signals against EBV, together with the limited EBV read representation and strain coverage of the broad VirScan library. We have therefore revised the relevant text to avoid implying that the VirScan analysis definitively excludes the involvement of other viruses. (Result: line 371, 390-392, 841-842)

3. The authors should provide more details for the statistical filtering. Like, reporting the minimum read count thresholds, fold-enrichment cutoffs, z-score cutoffs, multiple-testing correction strategy, and how background enrichment was estimated and adjusted?

Response: We have expanded the statistical filtering details in the Methods. Analytical samples required $\geq 4.0 \times 10^5$ peptide-mapped reads. Signals supported by < 3 reads were set to zero, and peptide-sample pairs were considered enriched when the observed count was ≥ 15 , fold enrichment was ≥ 2 , and the upper-tail Poisson P value was ≤ 0.001 ; no z-score cutoff was applied. Background enrichment was estimated from plate-specific serum-free controls and input-library representation. For differential analyses across peptides, multiple testing was controlled using the Benjamini-Hochberg procedure, with $q < 0.05$ considered statistically significant. (Methods: line 193-239)

4. Continuing with this, I agree with the use of EBVScan after Virscan in principle, but I am curious why the authors didn't use the same cohort to analyze both platforms. Using all different cohorts for both analyses weakens the connection between virome-wide discovery and EBV-focused analyses.

Response: We thank the reviewer for raising this point. We agree that the use of non-overlapping cohorts in the original analysis weakened the connection between the virome-wide VirScan discovery and the subsequent EBV-focused analysis. To address this concern, we re-performed the VirScan analysis using the same cohort analyzed by EBVScan (79 NPC cases and 94 healthy controls), thereby linking the two analytical stages. The revised VirScan analysis yielded results consistent with our original findings, with EBV emerging as the predominant NPC-enriched viral

antibody signal (**Fig. 1**). These results provide a more coherent basis for the subsequent EBVScan-based, epitope-resolved characterization. We have revised the corresponding cohort descriptions, Results, and figures accordingly. (Methods: line 132-134; Results: line 373-392)

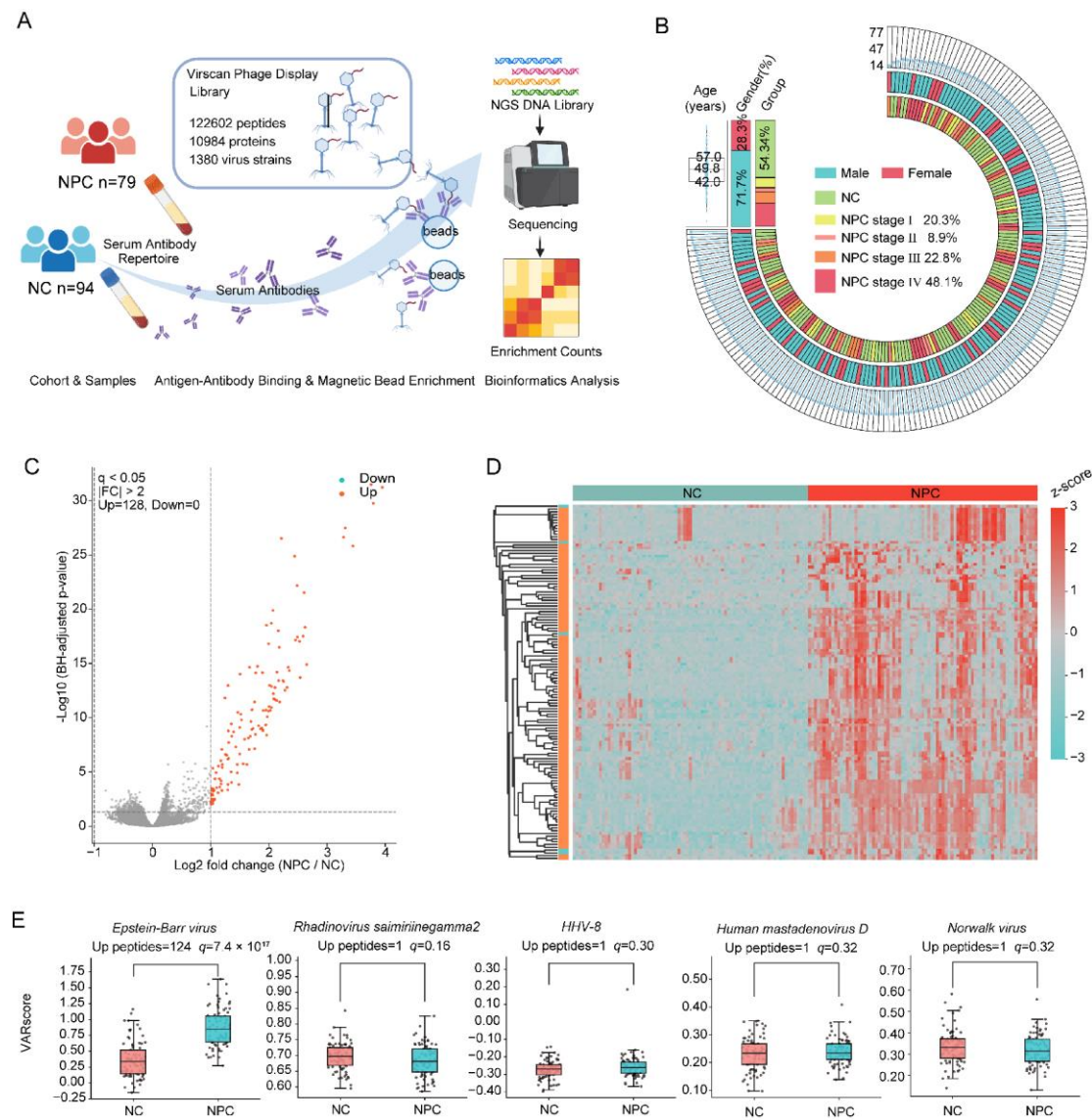
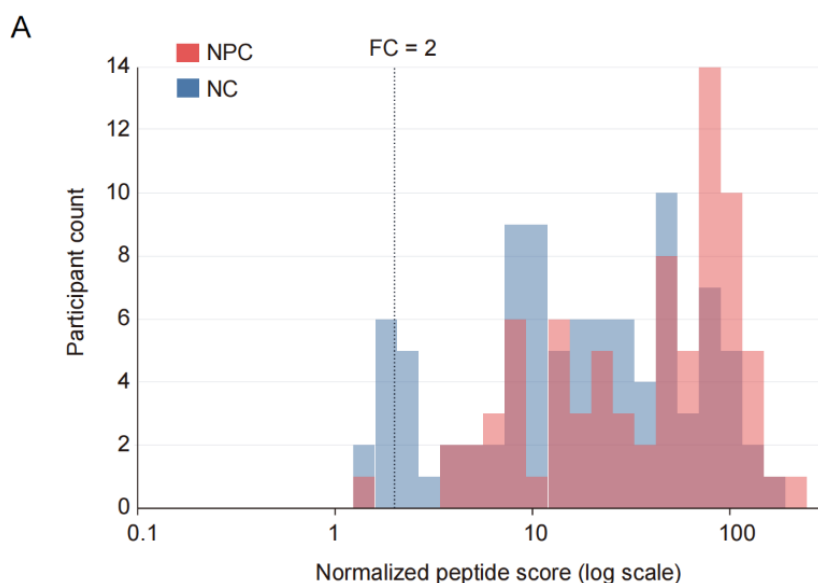


Figure 1. Virome-wide profiling identifies EBV as the predominant NPC-associated viral antibody signal. (A) Schematic of VirScan workflow. Serum samples from patients with nasopharyngeal carcinoma (NPC, n = 79) and healthy controls (NC, n = 94) were subjected to phage immunoprecipitation against a virome-wide library (1,380 species, 122,602 peptides), followed by next-generation sequencing. (B) Clinical characteristics of study cohorts, including age, sex, and TNM stage distributions. (C) Volcano plot showing differentially enriched peptides between NPC and NC groups. Upregulated (orange) hits were defined by $q < 0.05$ (BH-adjusted p-value) and fold change > 2 . (D) Heatmap of 128 differential peptides. Organism labels indicated

that 96.9% of upregulated peptides originated from EBV. (E) Comparison of viral aggregate reactivity score (VARscore) between NPC and NC cohorts for Epstein-Barr virus ($q = 7.4 \times 10^{-17}$), Rhadinovirus saimiriinegamma2 ($q = 0.16$), HHV-8 ($q = 0.30$), Human mastadenovirus D ($q = 0.32$), and Norwalk virus ($q = 0.32$).

5. The EBV serostatus of controls should be clearly reported. Do we know how many healthy samples are EBV seropositive? EBV infection is highly prevalent, so we need to know the status among healthy controls to clearly distinguish between healthy-enriched vs disease-enriched epitopes.

Response: We thank the reviewer for raising this point. EBV infection is highly prevalent in the general population, with seropositivity rates exceeding 90% among adults. To characterize EBV exposure status in the healthy controls, among the 171 IgG samples passing calibration QC, we analyzed IgG reactivity against EBNA1 peptides. EBNA1 IgG positivity was detected in 85/93 healthy controls (91.4%) and 77/78 NPC cases (98.7%) (**Fig. S3**). The seropositivity rate among healthy controls was comparable to previously reported population-based estimates (PMID: 32257152; PMID: 19596032), suggesting that the healthy control cohort represents the expected EBV exposure profile consistent with the expected seroprevalence. (Methods: line 240-248; Results: line 436-442)



Supplementary Figure 3. EBNA1-derived peptide IgG reactivity indicates widespread prior EBV exposure in both healthy controls and patients with NPC.

(A) Distribution of the maximum normalized IgG reactivity score among all EBNA1 peptides for each participant in the NPC (n = 78) and healthy-control (NC n = 93) groups. Scores are displayed on a logarithmic scale; the vertical dotted line denotes the operational cutoff of 2 used to define EBNA1 reactivity, corresponding to a signal-to-background ratio exceeding 2.

6. The manuscript appropriately emphasizes the challenge of cross-reactivity among human herpesviruses, but it does not clearly demonstrate how EBVScan distinguishes EBV-specific antibody binding from cross-reactive responses to homologous epitopes in other herpesviruses, particularly given the introduction's emphasis on this as a major drawback of existing platforms. I think the paper should discuss how their PhIP-seq platform tackles this question. The authors should provide sequence homology analysis of lead peptides against the human herpesvirus proteome, if possible, or elaborate in the discussion section about how cross-validation needs further exploration.

Response: We agree that cross-reactivity among human herpesviruses is an important consideration when interpreting serological signals. To address this issue, we first examined the conservation of the candidate marker TK₁₃₉₋₁₉₄ across EBV strains. TK₁₃₉₋₁₉₄ was conserved among the TK sequences from the EBV strains included in our analysis. We next assessed sequence homology of the full-length TK proteins across EBV, HSV-1, HSV-2, VZV, and KSHV. The pairwise sequence identities were low, ranging from 18.8% to 31.5%, and no significant homologous sequence corresponding to EBV TK₁₃₉₋₁₉₄ was identified in these other herpesviruses (**Fig. S6**). These analyses support the sequence specificity of the TK₁₃₉₋₁₉₄ region for EBV and illustrate how peptide-level profiling can help prioritize antibody-reactive regions with limited sequence homology to other herpesviruses. We have incorporated these analyses and the corresponding discussion into the revised manuscript. (Methods: line 325-334; Results: line 522-527)



Supplementary Figure 6. Conservation, predicted accessibility, and cross-herpesvirus homology of TK₁₃₉₋₁₉₄.

(D) Conservation, normalized quality, and gap frequency across an alignment of canonical human-herpesvirus TK homologs; the region aligned to EBV TK₁₃₉₋₁₉₄ is highlighted.

(E) Residue-level alignment of TK₁₃₉₋₁₉₄ with the corresponding regions of KSHV, HSV-1, HSV-2, and VZV TK homologs. KSHV, Kaposi's sarcoma-associated herpesvirus; HSV, herpes simplex virus; VZV, varicella-zoster virus.

7. I am not quite convinced by the authors' choice of the precise amino acid or residue-level epitopes. See, the EBVScan library is composed of 56-aa peptides with a 28-aa overlap, correct? However, the manuscript highlights narrow regions such as gp42₁₄₄₋₁₆₃, gL₆₅₋₈₄, and GP350₁₄₆₋₁₆₅, and interprets them as receptor-interface/protective antibody targets. A 56-aa enriched peptide can show that antibody reactivity occurs somewhere within that region, but it cannot prove that antibodies bind the exact 20-aa sequence or the specific receptor-contact residues. I think that to claim the 20-AA precise epitope, additional fine-mapping (for example, alanine scanning) must be performed (especially given that most B cell epitopes are actually 8-12 AA). I think authors should rewrite these epitopes as mere structurally overlapping regions within the protein rather than as experimentally resolved epitopes.

Response: We agree with the reviewer that our original description overinterpreted the resolution provided by the EBVScan library. Because EBVScan uses 56-aa peptides with a 28-aa overlap, the enrichment signals identify antibody-reactive regions within these peptides but do not resolve the exact minimal B-cell epitopes or specific antibody-contact residues. The 20-aa sequences highlighted in the original manuscript were selected from the enriched 56-aa peptides based on their overlap with structurally relevant regions and were subsequently synthesized as candidate antibody-reactive regions for proof-of-concept functional testing. They should therefore not be interpreted as experimentally resolved minimal epitopes or as necessarily representing the optimal blocking sequences.

Accordingly, we have revised the relevant terminology throughout the manuscript to describe these sequences more cautiously as candidate antibody-reactive or structurally overlapping regions, where appropriate, rather than as precisely mapped epitopes. We have also clarified in the Discussion that definitive identification of the minimal antibody-binding residues will require additional fine-mapping approaches, such as alanine scanning, systematic truncation, or overlapping peptide competition assays. (Discussion: line 620-622)

8. In addition, I am not entirely convinced by the claim that the identified epitopes are protective, given the lack of functional validation (neutralization or receptor blockade). The authors need to rewrite and significantly understate the claim.

Response: We thank the reviewer for this important comment. To address this concern, we performed an *in vitro* EBV infection-blocking assay in which target cells were pre-incubated with the candidate peptides before EBV exposure, and infection efficiency was subsequently assessed by flow cytometry. The candidate peptides significantly reduced EBV infection compared with the control peptides, providing functional evidence that these peptide regions possess infection-blocking activity (**Fig. 5G; Fig. S7**).

Nevertheless, we agree that these experiments represent proof-of-concept functional validation and do not establish these sequences as fully defined protective or neutralizing epitopes. We have therefore revised the relevant text to moderate this interpretation and to describe these regions more cautiously in terms of their potential protective relevance and experimentally observed infection-blocking activity. We have also clarified in the Discussion that further studies, including

alanine scanning, systematic truncation, peptide combination screening, and direct receptor-binding or neutralization assays, will be required to define the minimal active sequences, elucidate their mechanisms of action, and optimize their inhibitory efficacy. In addition, peptide stability may influence the observed functional activity and will require further optimization in future studies. (Methods: line 282-302; Results: line 559-572; Discussion: line 620-622)

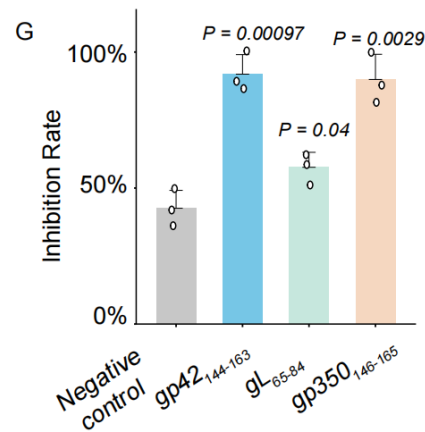
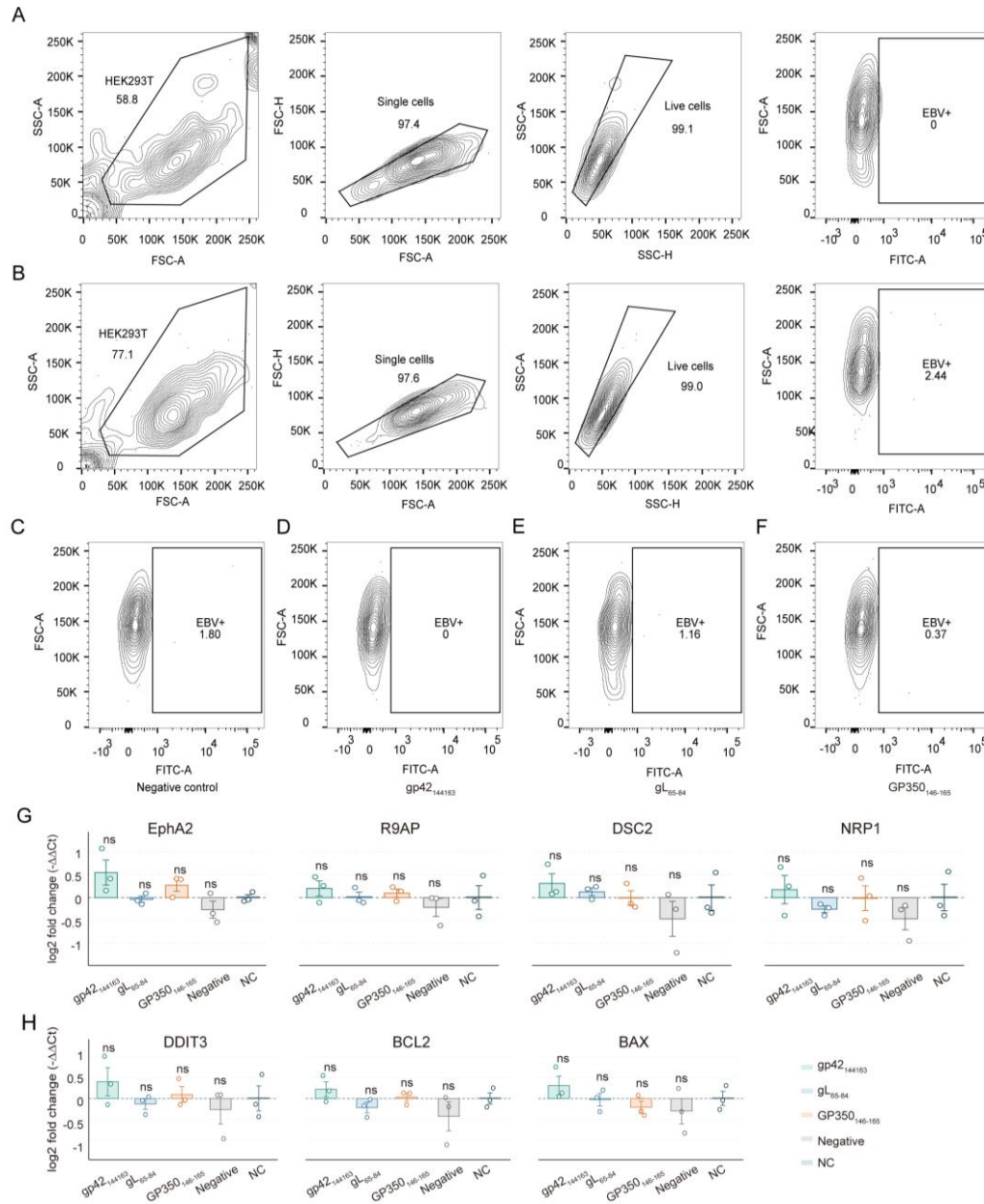


Figure 5. Structural mapping and functional assessment of healthy-enriched epitopes involved in EBV entry.

(G) Inhibition of EBV infection by candidate peptides. Bars indicate the mean, error bars denote SEM. *P* values are relative to the negative control.



Supplementary Figure 7. Effects of peptide blockade on EBV infection and host-cell gene expression.

(A, B) Representative flow-cytometry contour plots illustrating the sequential gating strategy used to identify EBV-positive HEK293T cells in the cells-only control (A) and after EBV exposure (B). The target-cell population, singlets, and live cells were gated sequentially, followed by identification of EBV-positive events according to FITC fluorescence.

F) Representative EBV-positive frequencies after viral exposure in the presence of the negative-control peptide (C), gp42₁₄₄₋₁₆₃ peptide (D), gL₆₅₋₈₄ peptide (E), or GP350₁₄₆₋₁₆₅ peptide (F).

(G, H) RT-qPCR analysis after peptide treatment for candidate cell-surface genes EphA2, R9AP,

DSC2, and NRP1 (G) and cellular stress- and apoptosis-related genes DDIT3, BCL2, and BAX (H). GAPDH was used as the reference gene. Relative expression was calculated by the $2^{-\Delta\Delta C_t}$ method and is presented as log₂ fold change ($-\Delta\Delta C_t$), with the mean of the untreated NC group normalized to zero. Bars show the mean, error bars indicate the SEM. Statistical comparisons were performed on ΔC_t values using two-sided Welch's t-tests against the NC group; all comparisons were not significant ($P \geq 0.05$). NC, untreated control; ns, not significant.

9. I am confused how the authors are calling the serum IgA response “mucosal responses”? How do we know whether the IgA is mucosal or serum? Please elaborate on the use of the term mucosal for IgA, or change it to Serum IgA

Response: We apologize for the confusion. All samples in this study were serum samples, and we have revised the manuscript to replace “mucosal IgA” with “serum IgA.” (line 446, 541)

10. The internal calibration strategy is interesting, but I believe the manuscript should more clearly define exactly what is happening. The references are incorporated during DNA amplification, but if they are not there in the PhIP-seq library itself, how can we compare the responses during the actual immunoprecipitation step? Please clarify.

Response: We thank the reviewer for recognizing the potential value of this calibration strategy and for raising this important point. We agree that the timing at which the internal reference is introduced determines which sources of technical variation it can control. In PhIP-seq, two commonly used immunoprecipitation strategies are protein G/A-based capture and human IgG- or IgA-specific capture. In our unpublished work using protein G/A-based PhIP-seq, reference peptides were displayed on T7 phage and spiked into the library before immunoprecipitation, together with their corresponding positive-control antibodies. Because these reference phage undergo the complete enrichment workflow, they can provide quality control for both the immunoprecipitation/enrichment step and the subsequent amplification and sequencing steps.

In the present study, however, we used human IgG- and IgA-specific immunoprecipitation. Implementing the same pre-IP reference strategy would require suitable human IgG- or IgA-format antibodies directed against the reference epitopes or tags (*e.g.*, FLAG or AviTag) so that the reference phage could be efficiently captured by the same isotype-specific system. We

were unable to identify suitable reagents for this purpose. Therefore, the internal-reference DNA used in this study was introduced at the PCR amplification stage rather than before immunoprecipitation.

We would like to clarify that, because these DNA references do not pass through the immunoprecipitation step, they are not intended to monitor or correct variation in antibody-phage enrichment itself. Rather, they serve as internal calibrators for downstream technical variation arising from PCR amplification and sequencing, improving the comparability of calibrated relative signals across samples. We have revised the Methods and corresponding description in the manuscript to make the scope and limitation of this internal calibration strategy explicit. (Methods: line 206-208)

11. It is good that the authors have deposited the raw sequencing data into the SRA, but these data are useless without the sequences of the peptides in the phipseq libraries because you can't do alignments and generate peptide counts. The normalized peptide counts for each peptide and each sample should be made available as well as the sequences of the peptides that were part of the VirScan and EBVScan libraries. In addition, when I look at the SRA data, it's not clear to me that the sequencing data are available on a per participant basis and that the participant IDs map back to the paper so that someone could recapitulate the analyses done by the authors.

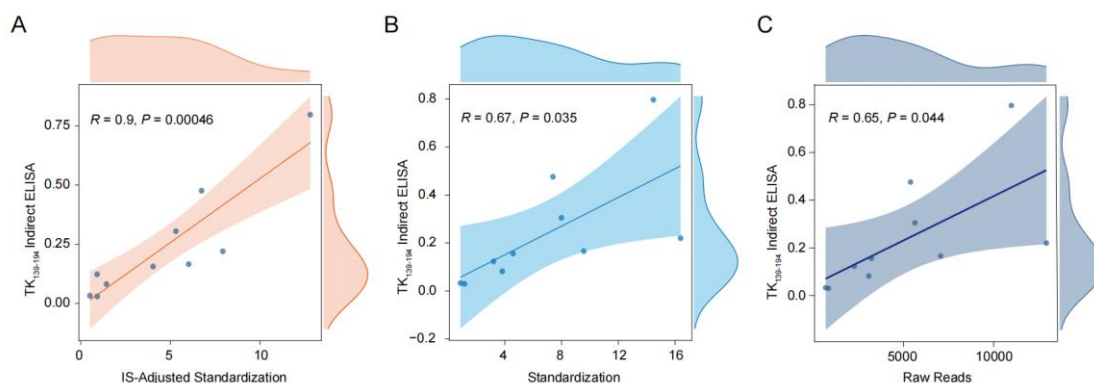
Response: We thank the reviewer for this suggestion. The deposited materials now include (i) the complete peptide sequences of the VirScan and EBVScan libraries, (ii) preprocessed peptide-by-sample read count matrices following next-generation sequencing, and (iii) participant-level metadata including mapping between sequencing sample identifiers and manuscript participant identifiers. All relevant files have been deposited in Zenodo and are publicly ccessible at <https://doi.org/10.5281/zenodo.21944590>. (Methods: line 355-359)

12. PhIP-Seq is a semi-quantitative assay, and the authors need to do more work by presenting more orthogonal quantitative antibody titer data to confirm that their claim of having devised a qPhIP-Seq assay is legitimate.

Response: We thank the reviewer for this important suggestion and agree that orthogonal

validation is necessary to assess the quantitative performance of our calibration strategy. Because EBV encodes a large number of proteins and currently available serological assays generally quantify antibody responses to individual antigens rather than the total EBV-specific antibody repertoire, repertoire-wide absolute quantification is technically challenging.

We therefore selected TK₁₃₉₋₁₉₄, one of the representative antibody-reactive peptides identified in our study, for orthogonal validation by ELISA. The ELISA measurements showed a high correlation with the internal-reference-adjusted qPhIP-seq values ($r = 0.90$, $P = 0.00046$), which was stronger than that observed with conventionally standardized PhIP-seq values ($r = 0.67$, $P = 0.035$) and raw sequencing reads ($r = 0.65$, $P = 0.044$) (**Fig. S2**). These results provide evidence that the internal-reference calibration improves the relative quantitative concordance of PhIP-seq measurements with an independent antibody assay. We agree, however, that this validation does not establish absolute quantitative accuracy across the entire EBV antibody repertoire. We have therefore revised the manuscript to clarify that qPhIP-seq provides improved relative semi-quantitative measurements, rather than absolute antibody titers, and have moderated the corresponding claims accordingly. (Results: line 425-433)



Supplementary Figure 2. Orthogonal ELISA validation of the relative semi-quantitative performance of qPhIP-Seq for TK₁₃₉₋₁₉₄.

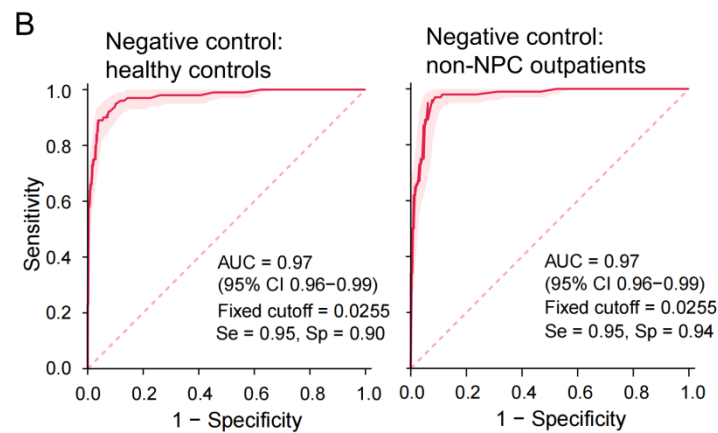
Correlation of indirect ELISA reactivity against TK₁₃₉₋₁₉₄ with (A) internal-reference-adjusted qPhIP-Seq values, (B) conventionally standardized qPhIP-Seq values, and (C) raw sequencing reads in NPC serum samples. Pearson correlation coefficients and two-sided P values are shown in each panel. The solid line represents the fitted linear regression and the shaded band represents its 95% confidence interval; marginal density plots show the distributions of the paired measurements. The internal-reference-adjusted values showed the strongest agreement with indirect ELISA ($r =$

0.90, $P = 0.00046$), compared with conventional standardization ($r = 0.67$, $P = 0.035$) and raw reads ($r = 0.65$, $P = 0.044$).

13. There are numerous claims about diagnostic biomarkers having been identified, but a biomarker is only useful if it differentiates between patients with clinically overlapping conditions that require differentiation. It's not clear to me how NPC patients have meaningful clinical overlap with people who are healthy or who have a runny nose (i.e., nasopharyngitis). In that case, I would not call these diagnostic biomarkers that have been "rigorously tested" for "cancer-specific diagnostic accuracy"

Response: We agree with the reviewer that a diagnostic biomarker requires rigorous clinical evaluation, particularly in patient populations with symptoms or clinical presentations that overlap with the target disease. In our original validation, in addition to healthy controls, we included an independent cohort of 195 non-NPC patients recruited from nasopharyngeal outpatient clinics, including patients with nasopharyngitis and other nasopharyngeal conditions that may present with symptoms overlapping with NPC. In this cohort, TK₁₃₉₋₁₉₄ distinguished NPC from non-NPC outpatients with an AUC of 0.97 (95% CI, 0.96-0.99), comparable to its performance against healthy controls (AUC = 0.97; 95% CI, 0.96-0.99), with no statistically significant difference between the two comparisons (**Fig. S5**).

Nevertheless, we agree that TK₁₃₉₋₁₉₄ has not yet been evaluated in a prospective, large-scale clinical study specifically designed for diagnostic validation. We therefore consider the term “diagnostic biomarker” to be stronger than warranted by the current evidence. Accordingly, we have revised the terminology throughout the manuscript and now refer to these markers as “NPC-associated serological markers” (line 463, 477-480, 529, line 604, line877)



Supplementary Figure 5. Structural mapping and functional assessment of healthy-enriched epitopes involved in EBV entry.

(B) ROC analyses of TK₁₃₉₋₁₉₄ in patients with NPC (n = 100) using healthy controls (n = 495) or non-NPC outpatients (n = 195) as separate negative-control groups.

14. Need to add to limitations that phipseq is geared toward discovering linear antigens and frequently missing conformational and/or post-translationally modified epitopes

Response: We have added this limitation explicitly in the revised Discussion. While highlighting the advantage of our approach in resolving antibody responses at the epitope level, we also acknowledge that PhIP-seq is limited in detecting conformational and/or post-translationally modified epitopes. (Discussion: line 660-662)

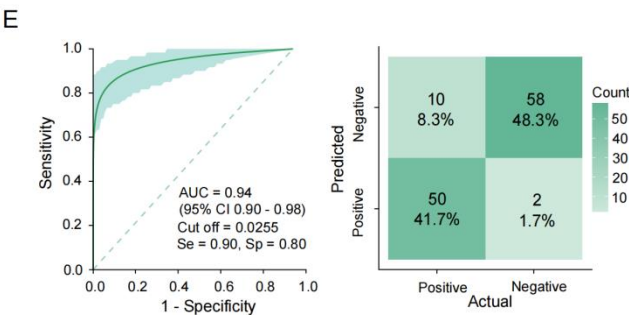
Reviewer #2 (Remarks to the Author):

This manuscript presents EBVScan for high-resolution mapping of EBV antibody responses in NPC. The authors constructed a comprehensive pan-strain EBV peptide library and identified several diagnostically and prognostically relevant epitopes, including TK₁₃₉₋₁₉₄, which demonstrated excellent diagnostic performance in a large validation cohort. The work possesses considerable novelty and translational potential. However, several important issues must be addressed before the conclusions can be fully supported.

Major Comments

1. Although the authors describe separate discovery, refinement, and validation cohorts, all samples appear to originate from the same geographic region (Fujian Province). Therefore, the validation cannot be considered truly independent. Validate the diagnostic model in an independent external cohort. If this is difficult, discuss this limitation.

Response: We thank the reviewer for this important suggestion. We agree that all cohorts included in the original study were recruited from Fujian Province, which limited the geographic independence of the original validation. To address this concern, we performed external validation in an independent cohort from Sichuan Province comprising 60 patients with NPC and 60 healthy controls. In this cohort, the TK₁₃₉₋₁₉₄-based panel achieved an AUC of 0.94 (95% CI, 0.90-0.98) (Fig. S5), indicating that its discriminatory performance was generally maintained in an independent cohort from a different geographic region. Nevertheless, we recognize that both the Fujian and Sichuan cohorts were recruited within China. Further validation in larger and more ethnically and geographically diverse populations, including international cohorts, will therefore be required to establish the broader generalizability of these findings. We have added this limitation to the revised Discussion. (Methods: line 137-138; Results: line 503-505; Discussion: line 662-664)



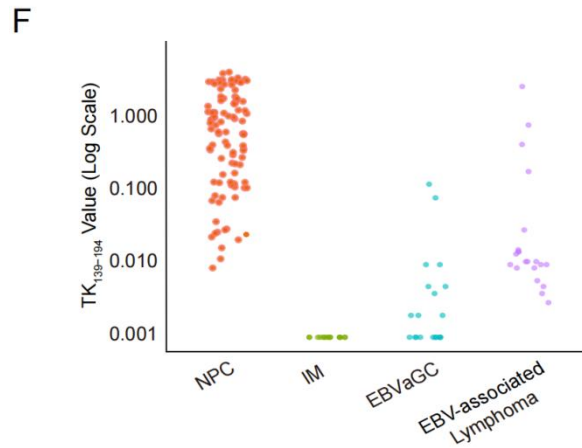
Supplementary Figure 5. Structural mapping and functional assessment of healthy-enriched epitopes involved in EBV entry.

(E) ROC curve and corresponding confusion matrix for TK₁₃₉₋₁₉₄ in an independent external cohort from Sichuan comprising 60 patients with NPC and 60 healthy controls.

2. The reported diagnostic performance of TK₁₃₉₋₁₉₄ is impressive. However, the study evaluates only healthy controls and non-NPC outpatient controls. Importantly, no comparison is performed with other EBV-associated diseases such as infectious mononucleosis, Hodgkin lymphoma, and CAEBV. Because these conditions may also induce lytic EBV antibody responses, the specificity of TK₁₃₉₋₁₉₄ for NPC remains uncertain. The authors had better to include additional disease controls.

Response: We thank the reviewer for this valuable suggestion. To further evaluate TK₁₃₉₋₁₉₄ in the context of other EBV-associated diseases, we included additional disease-control groups comprising patients with infectious mononucleosis, EBV-associated gastric cancer, and EBV-associated lymphoma. TK₁₃₉₋₁₉₄ was negative in all patients with infectious mononucleosis (0/15), whereas positivity was detected in a subset of patients with EBV-associated gastric cancer (2/20) and EBV-associated lymphoma (5/21) (**Fig. S5**). Samples from patients with CAEBV were not available in the current study, and we therefore acknowledge that evaluation in CAEBV will be important in future studies.

These findings indicate that larger-scale clinical studies, including a broader spectrum of EBV-associated diseases, will be required to further establish the diagnostic and screening performance of TK₁₃₉₋₁₉₄. At the same time, the observed reactivity in subsets of EBV-associated NPC, lymphoma, and gastric cancer may reflect shared features of EBV activation across these EBV-associated malignancies. We have added the corresponding results and discussion to the revised manuscript. (Methods: line 139-141; Results: line 506-511; Discussion: line 632-635)



Supplementary Figure 5. Structural mapping and functional assessment of healthy-enriched epitopes involved in EBV entry.

(F) Distribution of TK₁₃₉₋₁₉₄ values in patients with NPC, infectious mononucleosis (IM; n = 15), EBV-associated gastric carcinoma (EBVaGC; n = 20), and EBV-associated lymphoma (n = 21). TK₁₃₉₋₁₉₄ values are displayed on a logarithmic scale.

3.TK₁₃₉₋₁₉₄ is identified as the strongest diagnostic epitope, yet little mechanistic explanation is provided regarding why this specific region is preferentially recognized in NPC. The manuscript would benefit from additional information regarding conservation among EBV strains, structural localization within TK, and functional relevance during viral replication.

Response: We thank the reviewer for this insightful suggestion, which helped us better characterize the specificity of TK₁₃₉₋₁₉₄. We have added analyses of sequence conservation, structural localization, surface accessibility, and cross-reactivity with other herpesviruses. TK₁₃₉₋₁₉₄ was fully conserved across the 40 EBV TK sequences analyzed and showed limited sequence homology to canonical TK homologs of HSV-1, HSV-2, VZV, and KSHV (**Fig. S6**). The region lies within the N-terminal non-catalytic portion of EBV TK and was predicted to be relatively surface accessible. Previous studies suggest that the broader N-terminal region may influence the intracellular distribution of TK; however, the specific functional role of TK₁₃₉₋₁₉₄ during EBV replication has not been established. We have therefore revised the manuscript to distinguish these structural and evolutionary observations from mechanistic inference. We have added these discussions to the revised manuscript. (Methods: line 325-334; Results: line 512-529; Discussion: line 635-636)

and consensus percentages. The TK₁₃₉₋₁₉₄ region is highlighted.

(B) Solvent-accessible surface area (SASA) along the EBV TK sequence, shown as raw residue-level values and rolling means with windows of 5 and 15 residues.

(C) Predicted three-dimensional structure of EBV TK showing the peripheral location of the TK₁₃₉₋₁₉₄ region.

(D) Conservation, normalized quality, and gap frequency across an alignment of canonical human-herpesvirus TK homologs; the region aligned to EBV TK₁₃₉₋₁₉₄ is highlighted.

(E) Residue-level alignment of TK₁₃₉₋₁₉₄ with the corresponding regions of KSHV, HSV-1, HSV-2, and VZV TK homologs. SASA, solvent-accessible surface area; KSHV, Kaposi's sarcoma-associated herpesvirus; HSV, herpes simplex virus; VZV, varicella-zoster virus.

4. The manuscript repeatedly refers to gp42, gL, and GP350 epitopes enriched in healthy controls as “protective epitopes.” However, the presented data are entirely observational and cross-sectional. The study demonstrates association with healthy status but does not establish causality or protective function. No experiments demonstrate viral neutralization, inhibition of cell entry, or prevention of transformation. Therefore, the term “protective epitope” appears premature. It is better to replace with more conservative terminology such as “healthy-associated epitopes” unless functional validation is provided.

Response: We thank the reviewer for raising this important point regarding the interpretation of these regions as protective. To address this concern, we performed an *in vitro* EBV infection-blocking assay in which target cells were pre-incubated with the candidate peptides before EBV exposure, and infection efficiency was subsequently assessed by flow cytometry. The candidate peptides reduced EBV infection compared with the control peptides, providing proof-of-concept functional evidence that these peptide regions possess infection-blocking activity (Fig. 5G; Fig. S7).

We agree, however, that these experiments do not establish the identified regions as fully defined neutralizing or protective epitopes. Accordingly, we have moderated the terminology in the revised manuscript and now describe these regions more cautiously as candidate protective regions or peptide regions with infection-blocking activity, as appropriate. Further studies, including fine-mapping, antibody-based neutralization, and receptor-blocking assays, will be

required to establish the precise antibody-contact residues and determine whether antibody responses against these regions mediate protection. We have clarified these points in the revised Discussion.. (Methods: line 282-302; Results: line 559-572; Discussion: line 620-622)

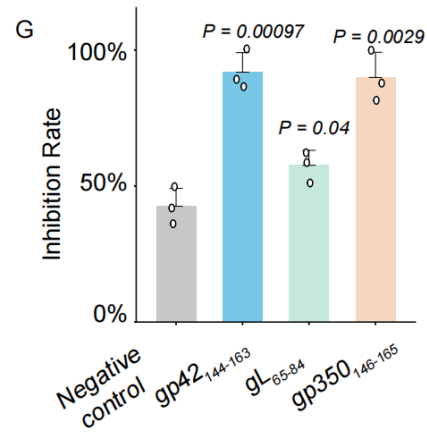
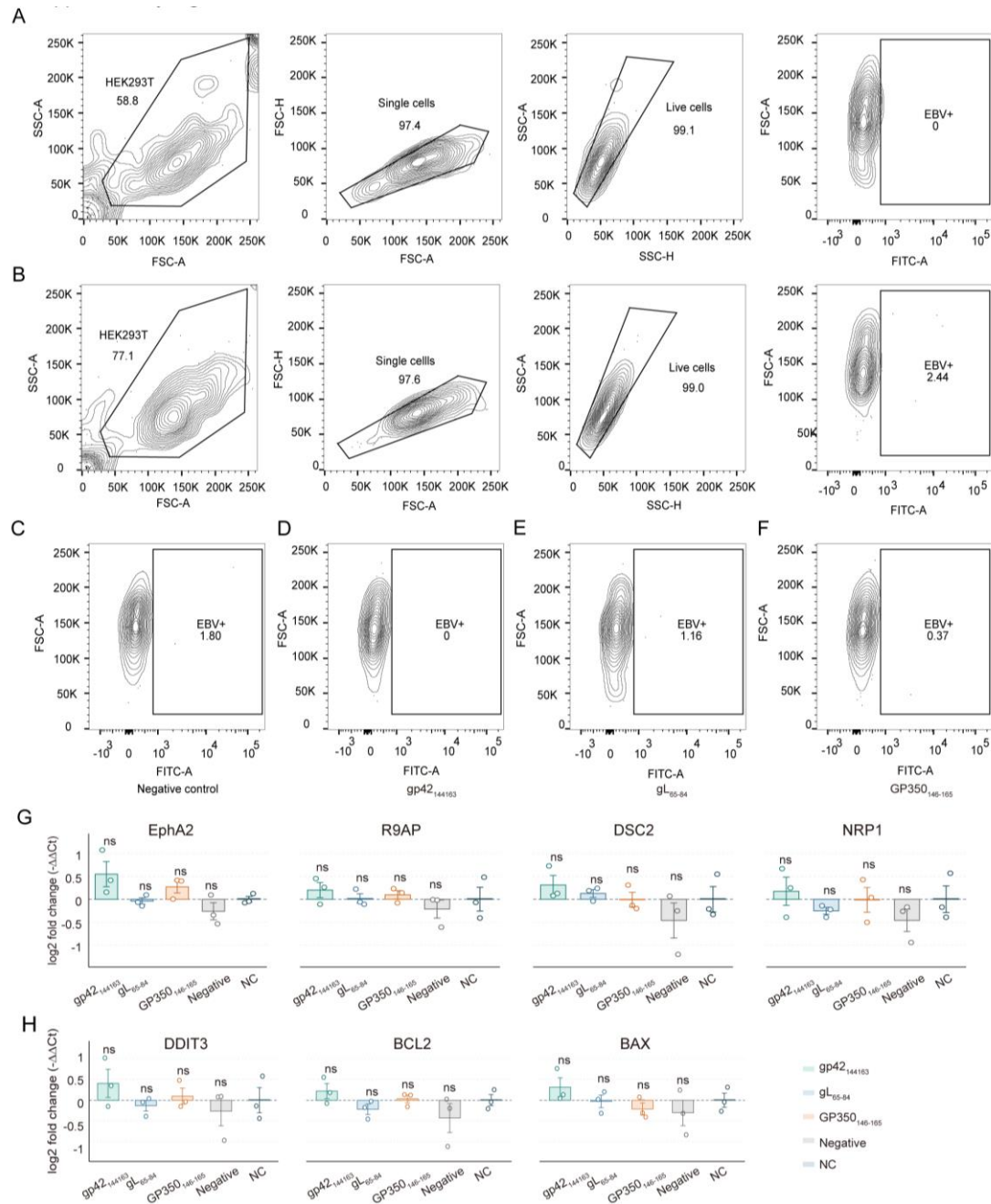


Figure 5. Structural mapping and functional assessment of healthy-enriched epitopes involved in EBV entry.

(G) Inhibition of EBV infection by candidate peptides. Bars indicate the mean, error bars denote SEM. *P* values are relative to the negative control.



Supplementary Figure 7. Effects of peptide blockade on EBV infection and host-cell gene expression.

(A, B) Representative flow-cytometry contour plots illustrating the sequential gating strategy used to identify EBV-positive HEK293T cells in the cells-only control (A) and after EBV exposure (B). The target-cell population, singlets, and live cells were gated sequentially, followed by identification of EBV-positive events according to FITC fluorescence.

F) Representative EBV-positive frequencies after viral exposure in the presence of the negative-control peptide (C), gp42₁₄₄₋₁₆₃ peptide (D), gL₆₅₋₈₄ peptide (E), or GP350₁₄₆₋₁₆₅ peptide

(F).

(G, H) RT-qPCR analysis after peptide treatment for candidate cell-surface genes EphA2, R9AP, DSC2, and NRP1 (G) and cellular stress- and apoptosis-related genes DDIT3, BCL2, and BAX (H). GAPDH was used as the reference gene. Relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method and is presented as log2 fold change ($-\Delta\Delta Ct$), with the mean of the untreated NC group normalized to zero. Bars show the mean, error bars indicate the SEM. Statistical comparisons were performed on ΔCt values using two-sided Welch's t-tests against the NC group; all comparisons were not significant ($P \geq 0.05$). NC, untreated control; ns, not significant.

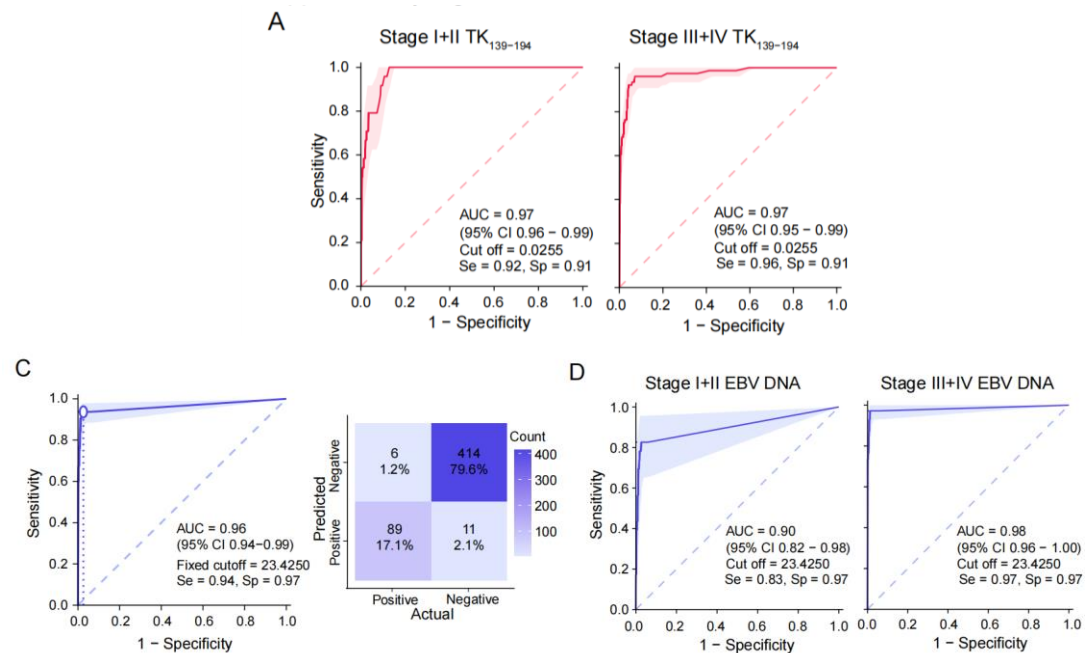
5. The structural mapping analyses are interesting and suggest that healthy-enriched epitopes overlap receptor-binding interfaces involving HLA-II, EphA2, or CR2. However, these conclusions are based solely on structural proximity. No experimental evidence demonstrates that antibodies targeting these epitopes actually interfere with receptor engagement or viral entry. Functional assays should be considered, such as pseudovirus neutralization assays, receptor-blocking experiments, or cell-entry inhibition studies using Akata-EBV system.

Response: We thank the reviewer for this suggestion. To further validate the functional relevance of the identified peptide regions, we performed an *in vitro* EBV infection-blocking assay by pre-incubating target cells with candidate peptides prior to EBV exposure, and assessing infection efficiency using flow cytometry. The candidate peptides showed inhibitory effects on EBV infection compared with control peptides, supporting their potential role in blocking viral entry (**Fig. 5G; Fig. S7**). We acknowledge, however, that this experiment does not establish that antibodies recognizing these regions interfere with specific receptor interactions involving HLA-II, EphA2, or CR2, nor does it define the mechanism underlying the observed inhibitory effects. Further studies, including antibody-based neutralization, receptor-binding or receptor-blocking assays, and fine-mapping of the relevant antibody-contact residues, will be required to clarify the underlying mechanism. These points have been incorporated into the revised Discussion. (Methods: line 282-302; Results: line 559-572; Discussion: line 620-622)

6. Plasma/serum EBV DNA quantification is currently among the most established

biomarkers for NPC screening and monitoring. The manuscript compares TK₁₃₉₋₁₉₄ with EA-IgA and VCA-IgA. Is this possible to compare its performance with plasma/serum EBV DNA-based screening strategies?

Response: We analyzed the subset of participants for whom circulating EBV DNA measurements were available (95 patients with NPC and 425 controls). To enable a comparable assessment, the performance of TK₁₃₉₋₁₉₄ was evaluated in the same subset of participants. Circulating EBV DNA achieved an AUC of 0.96 overall, with AUCs of 0.90 for early-stage NPC and 0.98 for advanced-stage NPC. In the corresponding stage-stratified analyses, TK₁₃₉₋₁₉₄ achieved an AUC of 0.97 for both early- and advanced-stage NPC (**Fig. S5A, S5C-D**). These results indicate that both circulating EBV DNA and TK₁₃₉₋₁₉₄ showed good discriminatory performance in this cohort. Notably, TK₁₃₉₋₁₉₄ showed a numerically higher AUC than EBV DNA in the early-stage subgroup (0.97 versus 0.90), whereas their performance was comparable in advanced-stage disease. We have added these analyses and the corresponding interpretation to the revised manuscript. (Methods: line 279-281; Results: line 474-477, line 496-502)



Supplementary Figure 5. Structural mapping and functional assessment of healthy-enriched epitopes involved in EBV entry.

(A) Receiver operating characteristic (ROC) analyses of TK₁₃₉₋₁₉₄ for early-stage NPC (stages I-II; n = 24) and advanced-stage NPC (stages III-IV; n = 76), each compared with the combined non-NPC control group (n = 690; 495 healthy controls and 195 non-NPC outpatients).

(C) ROC curve and corresponding confusion matrix for circulating EBV DNA in participants with available EBV DNA results (95 patients with NPC and 425 controls).

(D) Stage-stratified ROC analyses of circulating EBV DNA for early-stage NPC (stages I-II; n = 23) and advanced-stage NPC (stages III-IV; n = 72).

7. The manuscript does not clearly explain how ELISA cutoffs were selected. In addition, whether cutoff determination and validation were performed in separate datasets.

Response: We thank the reviewer for this important comment. For the ELISA analysis, NPC patients were defined as the positive group and non-NPC participants as the negative group. The optimal cutoff was determined in the primary ELISA cohort by maximizing the Youden index, with priority given to higher sensitivity and then higher specificity in the event of a tie. Once established, this cutoff was fixed and applied without further adjustment to subsequent subgroup analyses and to the independent external cohort.

To ensure comparability across experimental batches, predefined quality-control samples were included in each ELISA batch. The same cutoff was used for data analysis when the corresponding quality-control criteria were met. We have clarified the cutoff determination, quality-control procedures, and application of the fixed cutoff in the revised Methods. (Method: line 309-324)

Minor Comments

8. The sample numbers reported in Figure 1 appear inconsistent with those described in the Methods section. Please verify and correct the reported cohort sizes.

Response: We thank the reviewer for identifying this inconsistency. We re-audited the participant-level records and confirmed that the shared VirScan discovery/EBVScan refinement cohort comprised 79 patients with NPC and 94 healthy controls. We have corrected the sample numbers in Figure 1 and systematically cross-checked the corresponding cohort sizes throughout the Methods, Results, figure legends, tables, and Supplementary Information to ensure consistency. (Method: line 131-134)

9. The manuscript refers to both: 42 EBV strains and 43 EBV strains, in different sections. Please clarify and ensure consistency throughout the text.

Response: We thank the reviewer for this correction. The number of EBV strains has been corrected to 42 throughout the manuscript. (Method: line 163, 327; Result: line 403)

10. For the Kaplan-Meier analysis, hazard ratios and 95% confidence intervals should be reported in addition to log-rank *P* values.

Response: We thank the reviewer for this suggestion. We have added the hazard ratio, 95% confidence interval, and log-rank *P* value to the revised Results and corresponding figure (HR for high versus low signal, 3.16; 95% CI, 1.02-9.77; Cox *P*=0.046; log-rank *P*=0.035). (Result: line 594-595)

11. The authors should describe the chemotherapy protocol.

Response: We have added a detailed description of the neoadjuvant chemotherapy regimens, treatment schedules, treatment cycles, and RECIST 1.1-based response assessment in a new Methods subsection entitled “Neoadjuvant chemotherapy and treatment response assessment”. (Method: line 147-161)