Defining the expression hierarchy of latent T-cell epitopes in Epstein-Barr virus infection with TCR-like antibodies

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Epstein-Barr virus (EBV) is a gamma herpesvirus that causes a life-long latent infection in human hosts. The latent gene products LMP1, LMP2A and EBNA1 are expressed by EBV-associated tumors and peptide epitopes derived from these can be targeted by CD8 Cytotoxic T-Lymphocyte (CTL) lines. Whilst CTL-based methodologies can be utilized to infer the presence of specific latent epitopes, they do not allow a direct visualization or quantitation of these epitopes. Here, we describe the characterization of three TCR-like monoclonal antibodies (mAbs) targeting the latent epitopes LMP1125–133, LMP2A426–434 or EBNA1562–570 in association with HLA-A0201. These are employed to map the expression hierarchy of endogenously generated EBV epitopes. The dominance of EBNA1562–570 in association with HLA-A0201 was consistently observed in cell lines and EBV-associated tumor biopsies. These data highlight the discordance between MHC-epitope density and frequencies of associated CTL with implications for cell-based immunotherapies and/or vaccines for EBV-associated disease.

EBV is a persistent herpesvirus acquired as a predominantly asymptomatic infection during childhood in most human communities1. The virus can infect cells of both lymphoid and epithelial origin and its latent infection phase is associated with malignancies that arise from these cell types, including Non-Hodgkin’s lymphoma, Hodgkin’s Lymphoma2 and undifferentiated nasopharyngeal carcinoma (NPC)3. EBV latent gene products found in tumors include Epstein-Barr Virus Nuclear Antigen 1 (EBNA1) and/or Latent Membrane Protein 2A (LMP2A) and/or Latent Membrane Protein 1 (LMP1) depending upon the latency program employed by the virus4,5. Despite the subdominant frequencies of CTLs specific for epitopes derived from these latent gene products (0.05%–1%), they are implicated in the control of EBV infection in vivo5. The presence of these gene products in the majority of EBV-associated tumors suggests that an analysis of their associated CTL epitopes is essential for the design of immuno-targeting approaches including adoptive T-cell therapy.

The expression of EBV-latent epitopes in infected cells has been inferred indirectly from studies employing CTLs, HLA tetramer analysis, and targeted lysis by T-cell lines6. Notably however, T-cell functionality is pivotal for these analyses, and antigen specific CTLs can be rendered dysfunctional by viral immune evasion mechanisms7. A direct measure of HLA-peptide epitopes would circumvent this problem.

The direct analysis of surface EBV latency epitopes presented on MHC class I can be determined using mass spectrometry but this is highly dependent on their hydrophobicity and ionization potential8. An optimal approach is to develop antibodies that recognize viral epitopes in association with MHC9. Termed TCR-like mAbs, these reagents exhibit high affinities and enable direct visualization and quantification of the specific epitope presented7. In this study, antibodies targeting epitopes of EBV latent gene products (LMP1125–133, LMP2A426–434 and EBNA1562–570) were generated and characterized. This allowed an analysis of viral epitope expression using a combination of immunological and biochemical methods.

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including flow cytometry, immunohistochemical staining, and confocal microscopy. We next established the epitope expression hierarchy amongst the three latent epitopes in cell lines and clinically relevant EBV-associated tumor biopsies. Our observations of this hierarchy and its differential binding on strain-associated epitope variants have important implications for diagnosis, immunotargeting and vaccine development.

## Results

### Generation of high affinity TCR-like mAbs with exquisite specificity

In this report, we highlight an adaptation of conventional hybridoma technology that enabled the production of high-affinity TCR-like mAbs targeting three EBV latent epitopes displayed on HLA-A0201. The methodology is illustrated in Supplementary Information (Supplementary Fig. S1). Briefly, membrane-free HLA-A0201 associated with EBV latent peptides (EBNA1562–570; FMVFQLTHL; LMP1125–133; YLLEMLWR1; LMP2A426–434; CLGGLLTMV) were generated to immunize mice using an established protocol. The splenocytes of immunized mice were immunomagnetically selected prior to fusion. It is only with this enrichment that hybridomas producing TCR-like mAbs targeting EBNA1562–570 and LMP2A426–434 in association with HLA-A0201 can be generated (Fig. 1a). For hybridomas producing antibodies targeting LMP1125–133 in association with HLA-A0201, there is an increase in the percentage of such hybridomas isolated following splenocytes enrichment. The optimal representative monoclonal hybridoma for each target was selected for subsequent analyses.

The binding specificities of these antibodies were examined using flow cytometric analysis of T2 cells pulsed with 12 different HLA-A0201 restricted peptides. The mAbs exhibited exquisite specificity for their respective target peptide and not other HLA-A0201 restricted epitopes from a variety of human pathogens (Fig. 1b).

To determine the binding affinities of the three antibodies, surface plasmon resonance (SPR) was employed. All three mAbs exhibited strong binding affinities for their respective ligands (anti-HLA-A02/EBNA1562–570 K_D = 6.02 nM; anti-HLA-A02/LMP1125–133 K_D = 1.85 nM; anti-HLA-A02/LMP2A426–434 K_D = 6.98 nM) (Fig. 1c).

To further examine the specificity of each TCR-like mAb in comparison to specific CTLs, we determined their ability to inhibit CTL lysis. The three mAbs inhibited the activity of their respective CTLs in a specific dose-dependent manner, as shown by the inhibition of CTL-infected 51Cr release from target cells (Fig. 1d). Thus, we can infer a degree of overlap in the targeting of TCRs and TCR-like mAbs for the same viral epitopes. With these mAbs endowed with TCR specificity, we can visualize and quantitate the expression profile of latent EBV epitopes in infected cells.

### Epitope variants are differentially recognized by respective TCR-like mAbs

A factor that impacts upon epitope presentation is strain differences in the encoding sequence of the latent antigens that translates to CTL epitope variants. The classical methodology of EBV typing does not adequately distinguish the pathogenic/tumorigenic nature of various virus strains. This typing does not give due consideration to the clinical diversity of EBNA1, LMP1, and LMP2A, which are the only three latent genes observed in associated malignancies. Using alternative classifications that were adopted follows the presence or absence of the latent proteins from which the epitopes were derived, as indicated by RT-PCR and immunoblot (Figure 2c). The absence of an LMP1 immunoblot for C666-1A2 was due to the mutations in the LMP1 (China1) variant that altered the binding site for the anti-LMP1 antibody used.

### Mapping the expression hierarchy of three latent epitopes on EBV infected cells

We next analyzed the endogenous expression of EBNA1562–570, LMP1125–133, and LMP2A426–434 epitopes on three HLA-A0201 EBV-infected tumor cell lines of lymphoid and epithelial origins: CCRF-SB, RPMI-6666, and C666-1A2. The epitope variant of each cell line was determined by sequencing their associated EBV virus (Fig. 2a). A flow cytometry based bead-calibration kit, QIFIKIT® (Dako), allowed the quantitation of the three EBV latent peptides presented by HLA-A0201 (Supplementary Fig. S2). This enabled us to map the ligand density and expression hierarchy amongst the three latent EBV epitopes in the cell lines (Fig. 2b). HLA-A0201/EBNA1562–570 complexes were strongly expressed by the RPMI-6666 and CCRF-SB cell lines (1500 ± 61 and 2050 ± 112 complexes respectively). An intermediate level of expression was observed on the C666-1A2 cell line (590 ± 144.3 complexes). In contrast, HLA-A0201/LMP1125–133 and/LMP2A426–434 complexes exhibited relatively intermediate or weak expression on CCRF-SB (437 ± 127 and 668 ± 66 complexes respectively) and RPMI-6666 (208 ± 22 and 280 ± 59 complexes respectively) and negligible complexes on C666-1A2. This represents the first analysis of an expression hierarchy of HLA-A0201/peptide complexes of latent EBV antigens. Surprisingly, the high expression and dominance of HLA-A0201/EBNA1562–570 complexes was consistent for all cell lines tested. With the exception for the LMP1 (China1) variant of C666-1A2, our mAbs could bind the variants of each epitope. This was consistent with our previous data on binding of epitope variants (Fig. 1e).

The mAbs were also utilized to determine the cellular localization and relative expression of the 3 latent EBV HLA-A0201/peptide complexes by confocal microscopy. A high degree of surface localization of the HLA-A0201/EBNA1562–570 complexes was observed in all three cell lines (Supplementary Fig. S4) and the staining intensities of the three latent antigens correlated with the quantitation data shown in Figure 2b. In all cases, expression of HLA-A0201/EBNA1562–570 complexes on the surface of the tumor cells was stronger than/LMP1125–133 or/LMP2A426–434. Our observations cannot be ascribed to the presence or absence of the latent proteins from which the epitopes were derived, as indicated by RT-PCR and immunoblot (Figure 2c). The absence of an LMP1 immunoblot for C666-1A2 was due to the mutations in the LMP1 (China1) variant that altered the binding site for the anti-LMP1 antibody used.

### Detection of latent viral epitope expression in a humanized EBV mouse model

To analyze the expression of the three antigens in the context of a natural EBV infection, we employed an EBV-infected humanized NOD/SCID/IL2Rγnull (Hu-NSG) mouse model based on reported protocols. The latency program exhibited by these mice was type III and the same as that observed in lymphoproliferative disorders in immuno-compromised patients. A schematic of our experimental approach is provided (Fig. 3a). Following hematopoietic stem cells (HSC) reconstitution in NSG mice and infection with EBV, the spleens were harvested for staining. Immunofluorescence staining was carried out using R-Phycerothrin (PE) conjugated TCR-like mAbs (Fig. 3b). Due to the haplotype specific nature of our mAbs, only mice engrafted with HLA-A0201 HSC exhibited staining for all three complexes (Fig. 3b). Thus, these staining validated the employment of our TCR-like mAbs for epitope presentation studies in EBV-infected Hu-NSG mouse model.
Dominance of EBNA1<sub>562–570</sub> epitope expression in EBV-tumor biopsies. To determine the clinical relevance of our observations, cryo-sections of HLA-A0201 EBV-positive NPC biopsies were stained with the respective TCR-like mAbs. EBV infected cells in these biopsies stained strongly for HLA-A0201/EBNA1<sub>562–570</sub> complexes and weakly for HLA-A0201/LMP2A<sub>426–434</sub> complexes. Sections were also stained with BB7.2 to determine their expression of HLA-A02 molecules. A representative data set (from 5 patients) is shown (Fig. 3c). The staining intensity correlated with that observed in the NPC cell line C666-1A2 (Supplementary Fig. S3). The LMP1<sub>1125–133</sub> complex staining was not observed due to epitope mutation in the EBV NPC-linked China1 strain, correlating with the data in Figure 1e.

Discussion

TCR-like mAbs are valuable reagents for direct epitope visualization and detection. Several studies had illustrated its usefulness in epitope discovery<sup>15</sup>, epitope processing<sup>16</sup>, and immuno-targeting<sup>17</sup>. However, the numbers of documented antibodies are limited.

Previous reports documenting the generation of these mAbs used either classical hybridoma fusion following murine immunization or phage display methodologies. These resulted in antibodies that are relatively low in affinity<sup>18</sup> and stain poorly for endogenously generated epitopes<sup>19</sup>. By enriching for immunized splenocytes prior to fusion, we increased the number of hybridomas producing antibodies targeting LMP1 compared to non-enrichment. Moreover, it is only with this...
enrichment approach that hybridomas producing two of our TCR-like mAbs targeting EBNA1 and LMP2A could be isolated. Furthermore, our antibodies exhibit high specificity for their respective epitopes and binding affinity in the nanomolar range. Our data validates this approach for isolating such rare hybridomas\(^5\) that are technically challenging to generate\(^20,21\).

The high affinity and exquisite specificity of these reagents enabled us to examine epitope variants presented on HLA-A0201 BLCLs. These epitope mutations are due to viral sequence variation observed in each of the latent EBV genes (LMP1, LMP2A and EBNA1). Our antibodies bind to the epitope variants with differential activity suggesting that the associated amino acid substitutions impact upon presentation. Given that B95-8 is the only GMP-grade EBV strain employed for the expansion of specific CTL for adoptive immunotherapy, our data indicate that more attention must be paid to the EBV strain infecting the patient to ensure in vivo efficacy of the adoptively transferred T-cells.

Staining for endogenously produced epitopes/peptides is a major challenge for TCR-like mAbs\(^19\). Therefore, it was necessary to investigate the binding ability of our mAbs for its respective endogenously generated cognate peptide/MHC. As observed, our reagents enabled the visualization of intracellular and extracellular localization of endogenously generated epitopes. These reagents were also used to visualize latent EBV epitope expressing cells in an EBV-infected humanized mouse model. Coupled with QIFIKIT\(^\text{©}\), we also quantitated the density of endogenously generated epitopes in these three EBV infected cell lines. A distinct epitope expression hierarchy was consistently observed for all three cell lines with the dominance of EBNA1\(^{562–570}\) over the other two epitopes analyzed. This was similarly observed in clinically relevant EBV-associated NPC biopsies.

The dominance of EBNA1\(^{562–570}\) epitope did not correlate with the reported frequency of the respective specific CTLs\(^22\) where CTLs targeting LMP2A\(^{426–434}\) are dominant over the other two epitopes (EBNA1\(^{562–570}\) and LMP1\(^{125–133}\)).

Previous reports have intimated that EBNA1 inhibits its own processing and presentation\(^23\). However, epitope expression can be inferred from the presence of EBNA1 specific CTLs-this has been proposed as evidence for dendritic cells cross-presentation of EBNA1 peptides\(^24\). Despite such indications, we are the first to directly visualize and detect HLA-A0201/EBNA1\(^{562–570}\) complex expression in infected cells of both hematopoietic and non-hematopoietic origin. This suggests that EBNA1 can be processed and presented through

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**Figure 2** Expression hierarchy of latent EBV epitopes in infected HLA-A0201 cell lines. (a) Epitope comparison amongst the variants and those observed in the 3 cell lines studied. Red letters denote amino acid mutation. (b) Quantitation of HLA-A0201/LMP1/LMP2A and EBNA1 on CCRF-SB, RPMI-6666 and C666-1A2 using TCR-like mAbs based on flow cytometry. Error bars are standard deviation from an average of three independent experiments. **p < 0.01. n.d. denotes not detectable. (c) LMP1, LMP2A and EBNA1 in each of the cell lines was determined using RT-PCR and Western blot. β-actin was used as control. The blots and gels were cropped from full-length blots/gels presented in Supplementary Figure S3.
the classical MHC class I pathway. Although the presentation of HLA-A0201/EBNA1562–570 was reported to be low or non-existent in previous reports employing CTL lines, we were able to **directly** detect this “rare” epitope in relatively high numbers. This discordance could be due to dysfunctional CTLs or suggest that the density of peptide–MHC complexes is not necessarily the key factor in CTL selection and/or dominance. The immunodominance of CTLs can be affected by an interplay of cellular and molecular factors such as CTL precursor frequencies, quality of TCR-peptide-MHC complexes and the relative binding affinity of the peptide epitope for its associated MHC molecule. These components and the microenvironment can influence both the scale and form of the CTL response but were not addressed as part of this study.

In summary, by employing TCR-like mAbs, we have determined the expression hierarchy of EBV latent antigens. Our data indicates that EBNA1562–570 and LMP2A426–434 are possible biomarkers of EBV-associated tumors in HLA-A0201 patients. The dominance of EBNA1562–570 amongst the three epitopes was validated in cell lines and ex vivo staining of clinical NPC biopsies. This observation has important implications for both immunotherapy and EBV vaccine development.

**Methods**

**Cell lines.** The cell lines RPMI-6666 (CCL-113, Hodgkin’s lymphoma), CCRF-SB (CCL-120, acute lymphoblastic leukemia) and T2 (CRL-1992, TAP negative lymphoblast) were obtained from the American Type Culture Collection. C666-1A2 is a NPC cell line transduced with HLA-A201 haplotype. The B cell lymphoblastoid cell lines were generated from donors.

**Antibody and peptide.** The HLA-A02-restricted peptides Epstein-Barr virus EBNA1562–570 (FMVFLQTHI), EBNA1 Raji variant (FIVFLQTHI), LMP1 (B95-8)125–133 (FMVFLQTHI), LMP1 China1 variant (CLGGLLTMV), LMP2A NPC variant (SLGGGLTMV), Mycobacterium tuberculosis Ag85B143–152 (FYIAGSLSAL) and Hepatitis B virus sAg183–191 (FLLTRILTI) were synthesized by Mimotopes to 95% purity and was analyzed by electrospray mass spectrometry. The murine IgG1 isotype control antibody MOPC 21 was purchased from Sigma-Aldrich. The rabbit anti-beta-2 microglobulin polyclonal antibody and horseradish-peroxidase-conjugated goat anti-mouse IgG were purchased from Dako. Alexa Fluor cocktail (anti-EBV LMP1 antibody) and PE-conjugated goat anti-mouse IgG were purchased from Thermo Fisher Scientific. E1-2.5 (anti-EBV EBNA1 antibody) were purchased from Abd Serotec. Anti-human CD20 antibody was purchased from Becton Dickinson. Anti-CD45 antibodies were purchased from Invitrogen. 14B7 (anti-EBV LMP2 antibody) and CS1-4 (anti-CD45 antibody) were purchased from Abcam. 14B7 (anti-EBV LMP2 antibody) and CS1-4 (anti-CD45 antibody) were purchased from Abcam.

**Surface plasmon resonance (SPR).** Kinetic studies were evaluated by surface plasmon resonance using a BIAcore 3000TM (GE Healthcare). The monoclonal antibodies were immobilized covalently onto the surface of microbeads CM5 by amine coupling. To determine the dissociation constant (Kd) of the antibodies, the antibodies were immobilized covalently onto the surface of sensor chip CM5 by amine coupling. To determine the dissociation constant (Kd) of the antibodies, the antibodies were immobilized covalently onto the surface of sensor chip CM5 by amine coupling. To determine the dissociation constant (Kd) of the antibodies, the antibodies were immobilized covalently onto the surface of sensor chip CM5 by amine coupling. To determine the dissociation constant (Kd) of the antibodies, the antibodies were immobilized covalently onto the surface of sensor chip CM5 by amine coupling.
Western blot. CCRF-SB, RPMI6666, and C666-1A2 cells were cultured in 100 mm dishes and lysed with 1× RIPA buffer (Thermo Fisher Scientific) supplemented with protease inhibitors (Roche Applied Science). The lysates were boiled for 15 min and separated by 10% SDS-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membrane (GE Healthcare) overnight. The membranes were probed with CS1-4 antibody cocktail (anti-EBV LMP1) antibody (1:2000 dilution) (Dako), 1:487 (anti-EBV LMP2A antibody) (1:5000 dilution) (AbD Serotec) or E1-2.5 (anti-EBV EBNA1 antibody) (1:5000 dilution) (AbD Serotec). The membranes were subsequently incubated with horseradish-peroxidase-conjugated goat anti-mouse IgG or goat anti-rat IgG (Thermo Fisher Scientific). The proteins were detected with enhanced chemiluminescence reagent (Western Lightning ECL kit, Perkin Elmer).

Detection of LMP1, LMP2A and EBNA1 transcripts by RT-PCR. RNA from 1 × 10^6 cells was extracted using High Pure RNA Isolation kit (Roche Diagnostics) and incubated for 3.5 h in 200 μl) for 1 h at 37°C in a CO2 incubator. On day 2 after incubation, the media of the wells were supplemented with 20 μg/ml IL2 to expand specific peptide stimulated CD8+ T cells. On day 10, CTLs were cloned by limiting dilution assay and expanded in AIM V media supplemented with 2% AB serum, 20 U/ml IL2, 5 mg/ml IL7 and 100 μg/ml IL12 (R&D Systems) at a density of 500,000 cells/well in 96-well plates seeded with irradiated PBMC feeder cells pulsed with 1 μM of respective specific peptides.

Confocal microscopy. RPMI-6666 and CCRF-SB were seeded onto poly-L-lysine coated glass coverslips while C666-1A2 was seeded overnight on glass coverslips coated with 1% gelatin. The sections were fixed and permeabilized with 4% paraformaldehyde, followed by blocking with 5% goat serum. The tissues were stained with PE-conjugated IgG (H+L) (Dako) or Alexa Fluor 488-mAb staining of Hu-NSG mouse tissues

Quantitation assays. Epstein-Barr virus infected HLA-A02 cells were stained with the various TCR-like mAb followed by PE-conjugated goat-anti-mouse IgG (Dako). The level of fluorescence intensity was compared with that of calibrated beads containing pre-determined quantity of mouse monoclonal antibody per bead (QIIFIT® calibration beads). By means of a standard curve based around the calibration beads, the number of complexes on the cell surface was determined from the fluorescence intensity of each TCR-like monoclonal antibody.

Raising of specific CTL lines. The HLA-A0201 restricted CTL specific for peptides of Epstein-Barr associated proteins – LMP1125–133 (YLL EMLW RRL), LMP2A426–434 (RFSWAT) and EBNA1202–210 (PMIQWREIAA) were prepared on poly-L-lysine coated slides. The sections were fixed and permeabilized with 4% paraformaldehyde, followed by blocking with 5% goat serum. The tissues were stained with PE-conjugated antibody.

Biopsy and blood collection. Informed consent from healthy volunteers and patients were taken before biopsy or blood extraction. NPC patients at the National University Hospital were enrolled with informed consent. The Institutional Review Board of the hospital approved this study (IRB no: 07-043E).

Statistical analysis. Empirical data were analyzed using GraphPad Prism, version 5.0. 1-way ANOVA with Bonferroni post-test.

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### Author contributions
A.C.N.S. performed most of the experiments. C.T.T., M.Y.E., and N.S. generated the hybridomas. D.A.L.T. and S.U.G. generated the C666-1A2 cell line. S.W.P. sequenced and analyzed the epitopes. Z.S. stained the biopsies. M.Z.O. assisted with the mouse experiments. J.L. performed the *in vitro* experiments. K.O.L., T.K.S.L., J.C. and S.H.C. shared expertise and materials. A.C.N.S. and P.A.M. designed all experiments, analyzed the results, and wrote the manuscript.

### Additional information
Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: S.H.C. and P.A.M. hold the patent for the antibody generation (US Patent: PCT/SG2010/000438).

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