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Journal Title: Journal of Virology
Volume: Volume 87, Number 24
Publisher: American Society for Microbiology | 2013-12, Pages 13904-13910
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1128/JVI.01947-13
Permanent URL: http://pid.emory.edu/ark:/25593/gj36q

Final published version: http://jvi.asm.org/content/87/24/13904

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Accessed December 27, 2019 6:47 PM EST
Therapeutic Vaccination against the Rhesus Lymphocryptovirus EBNA-1 Homologue, rhEBNA-1, Elicits T Cell Responses to Novel Epitopes in Rhesus Macaques

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Epstein-Barr virus (EBV) is a vaccine/immunotherapy target due to its association with several human malignancies. EBNA-1 is an EBV protein consistently expressed in all EBV-associated cancers. Herein, EBNA-1-specific T cell epitopes were evaluated after AdC–rhEBNA-1 immunizations in chronically lymphocryptovirus-infected rhesus macaques, an EBV infection model. Pre-existing rhEBNA-1-specific responses were augmented in 4/12 animals, and new epitopes were recognized in 5/12 animals after vaccinations. This study demonstrated that EBNA-1-specific T cells can be expanded by vaccination.

Nearly all humans worldwide harbor persistent Epstein-Barr virus (EBV) infection by adulthood (1, 2). This lifelong viral infection is asymptomatic in the vast majority of humans due to control of persistent EBV infection by viral immunity. However, persistent EBV infection is associated with the development of cancers such as Burkitt and Hodgkin lymphomas and nasopharyngeal and gastric carcinomas, which typically arise decades after initial EBV infection (3).

T cell-mediated responses are known to be important for controlling persistent EBV infection since immunosuppression can result in EBV-induced lymphomas in AIDS and transplant patients (4). Furthermore, adoptive transfer of EBV-specific T cells can be preventive or therapeutic against EBV-induced lymphomas in transplant patients (5, 6). Thus, vaccination strategies that enhance EBV immunity may lead to an effective EBV cancer vaccine by reducing the number of virus-infected cells or attacking EBV-positive tumor cells.

EBNA-1 is an EBV nuclear protein expressed in both lytic (7) and latent phases of EBV infection, and it is consistently expressed in all EBV-associated cancers (8–11). The presence of purine-enriched Gly-Ala repeats in the EBV EBNA-1 sequence inhibits RNA translation (12–14) and restricts antigen presentation for CD8+ T cells in vitro (15, 16). Nevertheless, studies have shown that EBNA-1-specific CD4+ and CD8+ T cells are frequently detected in EBV-infected hosts (17–19), and both T cell subsets can be effective in controlling growth of EBV-immortalized B cells in vitro (20, 21).

Rhesus macaques provide the most accurate animal model for EBV infection, because both the human and nonhuman hosts and pathogens share a high degree of genetic and biologic similarities (22–25). They are naturally infected with an EBV-related gammaherpesvirus or lymphocryptovirus (LCV) that shares an identical repertoire of viral genes with EBV (25). The rhesus LCV EBNA-1 homologue (rhEBNA-1) shares approximately 50% amino acid similarity with EBV EBNA-1 (25), has identical molecular functions (26), and, although not a dominant antigen (2), is frequently targeted by both CD4+ and CD8+ T cells in LCV-infected macaques (27, 28). Since only 2 peptides containing rhEBNA-1-specific T cell epitopes have been described in this animal model (27), the goal of our study was to investigate whether the rhEBNA-1-specific T cell response could be expanded in vivo by EBNA-1 vaccination with a specific focus on defining the EBNA-1 T cell epitopes recognized before and after vaccination.

Forty adult rhesus macaques were randomly screened for rhLCV-specific humoral and rhEBNA-1-specific cellular responses, and 15 animals were chosen based on positive rhLCV serology and low positive rhEBNA-1-specific cellular responses (28). The animals were cared for in conformance to the Committee on the Care and Use of Laboratory Animals guidelines (29). All experimental protocols and procedures were approved by the Emory Institutional Animal Care and Use Committee. Assigned animals were divided into 3 groups and immunized twice via the intramuscular route with replication-defective chimpanzee-derived adenovirus vectors (AdC) containing different constructs. Serotype AdC68 was used for priming and AdC6 for boosting immunization. AdC vectors have been shown to stimulate robust transgene product-specific T cell responses in nonhuman primates (30, 31) and do not cross-react with human or rhesus macaque adenovirus serotypes (32), avoiding preexisting immunity against the vaccine constructs. The constructs encoded a herpes simplex virus 1 glycoprotein D (gD) domain fused with an irrelevant antigen (influenza virus nucleoprotein [NP]) or rhEBNA-1 deleted for the Gly-Ala repeats. The rationale for using the gD domain in the rhEBNA-1 vaccine constructs is related to its enhanced binding to the herpesvirus entry mediator (HVEM) compared to that of gD (33), blocking the protein interaction between HVEM and the B and T lymphocyte activator (BTLA)
and consequently inhibiting the negative regulatory pathways of T cell activation (34). Also, antigens fused to gD have shown enhanced immune responses to antigens (35). An illustration representing the different constructs used in this study can be seen in Fig. 1A. A detailed description of these chimpanzee-derived recombinant adenovirus constructs are provided by R. M. Leskowitz, M. H. Fogg, X. Zhou, A. Kaur, E. L. V. Silveira, F. Villinger, P. M. Lieberman, F. Wang, and H. C. Ertl (submitted for publication). Group I animals were vaccinated with the AdCSgD-NP construct (36), group II animals were vaccinated with the AdCSgD–rhEBNA-1 construct, and group III animals were vaccinated with a construct containing an identical rhEBNA-1 but fused to a mutated SgD version incapable of blocking the BTLA-HVEM interaction (AdCNBEFSgD–rhEBNA-1) (35)(Fig. 1) (Leskowitz et al., submitted).

RhEBNA-1-specific T cell epitopes were identified from peripheral blood mononuclear cells (PBMCs) collected pre- and postimmunization by first pulsing cells with a peptide library spanning rhEBNA-1 (GeneMed Synthesis; 15-mer peptides overlapping by 5 amino acids). Excess peptide was removed by washing, and then cells were cultured with rhesus interleukin 7 (rIL-7) (25 ng/ml; Resource for Nonhuman Primate Immune Reagents, Emory University) and human interleukin 2 (hIL-2) (50 IU/ml; Roche). After 12 to 16 days, cultured cells were restimulated with pools, subpools, or single peptides on gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) assay to identify reactivity to specific peptides (17, 27, 37).

A representative epitope-mapping experiment is shown for Rm2-4 (Fig. 2). Initially, T cell responses recognized 1 of 3 peptide pools (pool B) containing 17 overlapping peptides, 1 of 3 peptide subpools (subpool B3) containing 5 of the original 17 peptides (Fig. 2A), and finally peptide 34 (Fig. 2B). CD8+ T cells were responding to peptide 34 based on CD4+ or CD8+ cell depletions performed with specific Dynabeads (Dynal), followed by IFN-γ ELISPOT assay (Fig. 2C). Testing of multiple 9-mer derivatives of peptide 34 showed an optimal response to peptide 34-3 (RGFKK FENM), which had increased responses compared to those of the
original 15-mer peptide 34 (Fig. 2D). Other peptide 34-derived sequences induced lower responses. Of note, peptide 34 is identical to one reported previously as able to stimulate CD8/H11001 T cell cytotoxicity (27).

Using an in silico algorithm (38, 39), the optimal CD8+ T cell epitope (peptide 34-3) for Rm2-4 was predicted as a strong binder to Mamu-B*029 (Fig. 2E; A. Sette, personal communication). Considering those data suggest that Mamu-B*029 allele expression is linked to Mamu-B*017 in rhesus macaques (40) and the particular animal was also Mamu-B*017/H11001, an IFN-γ ELISPOT assay was performed to confirm the epitope binding to these alleles. For this, rhEBNA-1-specific cultured cells were utilized as responder cells and Hmy2.C1r cells transfected with Mamu-B*029 or 721.221 human cells with Mamu-B*017 (APCs) at a ratio of 2:1 and tested by IFN-γ ELISPOT assay in triplicates. *, P < 0.05 (two-sample t test [one tailed] for correlated samples). NS, nonsignificant.

Using this approach, a total of 10 rhEBNA-1 peptide responses were identified among 8 of the 15 animals (Table 1). Five of the rhEBNA-1-specific responses were from CD8+ cell-depleted PBMCs from 3 Mamu-B*029+ animals from another cohort (data not shown). Although both alleles are part of the same inherited haplotype (40), they have different peptide-binding motifs, and only Mamu-B*029 presented the identified epitope.

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These data were confirmed independently using CD4+ cell-depleted PBMCs from 3 Mamu-B*029+ animals from another cohort (data not shown). Although both alleles are part of the same inherited haplotype (40), they have different peptide-binding motifs, and only Mamu-B*029 presented the identified epitope. The epitope was confirmed with peptide-pulsed Mamu-B*029+ APCs, no response was seen with Mamu-B*017+ APCs (Fig. 2F). These data were confirmed independently using CD4+ cell-depleted PBMCs from 3 Mamu-B*029+ animals from another cohort (data not shown). Although both alleles are part of the same inherited haplotype (40), they have different peptide-binding motifs, and only Mamu-B*029 presented the identified epitope.

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Figure 2: Representative sequential steps to identify a Mamu-B*029-restricted CD8+ T cell epitope sequence for monkey Rm2-4. Values represent net spot-forming cell (SFC) averages (per 10⁶ PBMCs) ± standard deviations (SD) adjusted by subtraction of SFCs observed with cells restimulated without peptide (negative control) or with a simian immunodeficiency virus Gag peptide pool. The positive control consisted of total PBMC-derived T cells restimulated with PHA-16 (0.5 μg/ml) or ConA (5 μg/ml). Samples were collected at 2 (A and B), 4 (C), 6 (D), and 16 (F) weeks post-boost immunization. (A to D) Unsorted rhEBNA-1-restimulated PBMCs were tested against peptide subpools (A) or individual peptides by IFN-γ ELISPOT assay in duplicates. (E) In silico prediction of rhEBNA-1-specific CD8+ T cell epitope binding to Mamu-B*029. (F) Restimulated PBMCs were mixed with peptide-pulsed cells transfected with Mamu-B*029 or with Mamu-B*017 (APCs) at a ratio of 2:1 and tested by IFN-γ ELISPOT assay in triplicates. *, P < 0.05 (two-sample t test [one tailed] for correlated samples). NS, nonsignificant.
Identification of rhEBNA-1-Specific T Cell Epitopes

TABLE 1 rhEBNA-1-elicited T cell response in immunized rhesus macaques

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccine</th>
<th>Animal</th>
<th>rhEBNA-1-specific peptide response</th>
<th>T cell response</th>
<th>Preimmunization</th>
<th>Post-boost immunization</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AdCSgD-NP</td>
<td>Rm1-1</td>
<td>29.GTDGDGEQPPGAVEQ</td>
<td>CD8⁺</td>
<td>50 ± 36</td>
<td>1,523 ± 5251ᵇᵉ</td>
<td>30.5</td>
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<td></td>
<td></td>
<td>Rm1-2</td>
<td>40.RLTLGLRPGFYAEP</td>
<td>CD4⁺</td>
<td>1,380 ± 481</td>
<td>6,165 ± 933ᵇᵉ</td>
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<tr>
<td></td>
<td></td>
<td>Rm1-3</td>
<td>UND</td>
<td>UND</td>
<td>UND</td>
<td>UND</td>
<td></td>
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<tr>
<td>2</td>
<td>AdCSgD–rhEBNA-1</td>
<td>Rm2-1</td>
<td>44.AECVKDALRDYIMTK</td>
<td>CD8⁺</td>
<td>87 ± 96</td>
<td>820 ± 322ᵇ</td>
<td>9.4</td>
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<tr>
<td></td>
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<td>Rm2-2</td>
<td>34.GGRFKEFENMAKLN</td>
<td>CD8⁺</td>
<td>205 ± 290</td>
<td>3,565 ± 219ᵇ</td>
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<tr>
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<td>Rm2-3</td>
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<td>UND</td>
<td>UND</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>Rm2-4</td>
<td>40.RLTLGLRPGFYAEP</td>
<td>CD8⁺</td>
<td>0 ± 0</td>
<td>138 ± 67</td>
<td></td>
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<tr>
<td>3</td>
<td>AdCNBEFSgD–rhEBNA-1</td>
<td>Rm3-1</td>
<td>40.RLTLGLRPGFYAEP</td>
<td>CD8⁺</td>
<td>0 ± 0</td>
<td>2,175 ± 113ᵇ</td>
<td>28.8</td>
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<tr>
<td></td>
<td></td>
<td>Rm3-2</td>
<td>B pool (18–34)</td>
<td>NT</td>
<td>70 ± 121</td>
<td>2,015 ± 205ᵇᵉ</td>
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<tr>
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<td>Rm3-3</td>
<td>C pool (35–51)</td>
<td>NT</td>
<td>0 ± 0</td>
<td>1,180 ± 14ᵇ</td>
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<tr>
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<td></td>
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<td>Rm3-6</td>
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<td>385 ± 184ᵇ</td>
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<td></td>
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<td>Rm3-6</td>
<td>46.SQVTQTVTFEDPVML</td>
<td>CD4⁺</td>
<td>0 ± 0</td>
<td>1,0205 ± 533ᵇ</td>
<td></td>
</tr>
</tbody>
</table>

ᵃ PBMCs were collected preimmunization (at 4 months) and post-boost immunization (2, 4, or 6 weeks) and tested in duplicates or triplicates by IFN-γ ELISPOT assay. Peptide pools: B, 17 15-mer peptides spanning residues 171 to 345; C, 17 15-mer peptides spanning residues 341 to 511. Values are averages of IFN-γ-producing cells ± SD. NT, not tested; UND, undetermined. Positive response, >50 IFN-γ-producing cells per million of PBMC-derived T cells; fold change, average of IFN-γ-producing cells post-boost immunization/preimmunization.
ᵇ PBMCs collected at 2 weeks post-boost immunization.
ᶜ PBMCs collected at 4 weeks post-boost immunization.
ᵈ PBMCs collected at 6 weeks post-boost immunization.
ᵉ Significant difference between preimmunization and postimmunization (P < 0.05).

(2). Three additional CD8⁺ epitopes plus one CD4⁺ epitope have also been mapped to 15-mer peptides (Table 1). Finally, we defined the exact sequences of rhEBNA-1 CD8⁺ (RGFKKFENM) and CD4⁺ (RLTLGLRPGFYAP) T cell epitopes in distinct animals (data not shown).

The rhEBNA-1 therapeutic immunizations either markedly expanded existing responses or appeared to elicit novel ones when comparing preimmunization and post-boost immunization time points in responder animals (Table 1). rhEBNA-1-specific responses were not enhanced after AdCSgD-NP vaccinations in the control group 1 animals. But in group 2 and 3 animals, rhEBNA-1 peptide-specific responses were detected in 4/12 animals before immunization and in 8/12 animals after rhEBNA-1 vaccination. In the 4 animals for which rhEBNA-1-specific responses were detected before immunization, the preexisting responses were augmented in a range of 4.5- to 28.8-fold after boost immunization (Table 1). In 5 animals, 6 rhEBNA-1 epitopes were detected only after boost vaccination. There was no convincing evidence that an HVE-binding SgD domain enhanced the response among the small numbers of animals in group 2 versus group 3. However, the combined results of animals in groups 2 and 3 suggest that rhEBNA-1-specific responses were enhanced by rhEBNA-1 vaccination.

To confirm these findings, a Mamu-B*029-RGFKKFENM tetramer was synthesized (NIH Tetramer Core Facility) and tested for staining of rhEBNA-1-specific CD8⁺ T cells in expanded cells from Rm2-4 (Mamu-B*029⁺) collected preimmunization and post-boost immunizations. The gating strategy for the quantification of the tetramer-positive cells is described for Fig. 3A. While Mamu-B*029⁺ cells collected 2 weeks after boost immunization and Mamu-B*029⁺ collected before immunization stained negative, cells from the same Mamu-B*029⁺ animal collected 2 weeks after the primary but mainly after the boost immunization showed clear staining with the tetramer (Fig. 3B). Cultured cells from another Mamu-B*029⁺ animal (Rm2-5) failed to identify peptide 34-3 by IFN-γ ELISPOT assay, but a small population reacted with the tetramer after the boost immunization (Fig. 3B and C).

A concern for the development of vaccine/immunotherapy against EBV is the potential to elicit autoimmune responses (41–44). All animal plasma samples collected post-booster immunization were, however, negative for autoantibodies to Sm, ribonucleoprotein, Scl-70, SSA/Ro, SSB/La, histidil tRNA-synthetase, and CENP-B (data not shown), suggesting our approach to be safe.

Enhancing immune responses to subdominant antigens can confer some degree of protection against several infections (45–49). Pathogens like EBV restrict host T cell responses during infection, facilitating pathogen replication and/or persistence by avoiding broader immune responses. Recent studies also showed that EBNA-1-specific responses are reduced in nasopharyngeal carcinoma patients compared to those in healthy EBV⁺ donors.
Therefore, our results suggest that the mapping of new rhEBNA-1 epitopes provides important tools for studying vaccination strategies in the rhesus macaque animal model, and EBNA-1 responses can be increased by vaccination in persistently infected hosts.

ACKNOWLEDGMENTS

We are indebted to Chris Ibegbu (Emory Vaccine Center), the veterinary and Research Resource personnel from Yerkes National Primate Research Center for providing excellent technical assistance, the NIH Tetramer Core Facility (Emory University), and the Resource for Nonhuman Primate Immune Reagents (Emory University) for the provision of reagents. These studies were supported by ARRA grant 1RC2CA148325 and NIH ORIP grant 51POD1113 in support of Yerkes. Services at the New England Primate Research Center were supported by a base grant (P51OD011103).

There were no competing financial interests among all authors.

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