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Vaccination with human papillomavirus-18 E1 protein plus α-galactosyl-ceramide induces CD8$^+$ cytotoxic response and impairs the growth of E1-expressing tumors

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ABSTRACT

CD8+ T cell-mediated immune response plays a major role in the clearance of virus-infected cells, including human papillomavirus (HPV). The effective treatment of women with normal cytology but persistent high risk-HPV infection or with low-grade intraepithelial lesions could take advantage of novel strategies based on vaccination with viral immunological targets with a wide spectrum of cross-protection. The helicase E1, expressed early during viral replication in HPV infection, is among the most conserved papillomavirus proteins, which makes it a good vaccine candidate. In the present study, we examined E1-specific CD8+ T cell and NK immune responses in a mouse model with α-galactosylceramide (α-GalCer) as an adjuvant. We found that mice immunized with E1 combined with α-GalCer elicited an E1-specific CD8+ T and NK cell cytotoxic responses, which correlated with growth inhibition of grafted melanoma B16-F0 cells expressing E1, both in prophylactic and therapeutic protocols.

1. Introduction

Cervical cancer is the fourth most frequent cancer in women worldwide [1] with high-risk human papillomavirus (HPV) as its etiologic agent [2]. According to their oncogenic potential, HPVs are classified as high- (HR-HPV) or low-risk (LR-HPV), being HPV16 and HPV18 the most common viral types found in cervical cancer patients [4,5]. Persistent infection with high-risk HPV can lead to cervical intraepithelial neoplasia (CIN) that, without treatment, could progress to *in situ* carcinoma [3].

During an HPV infection, viral genes are differentially and sequentially expressed along the various layers of the cervical epithelium [6]. HPV proteins E1 and E2 regulate viral replication [7,8], of which the E1 helicase is necessary for DNA double-strand separation during viral replication [9] and is the most conserved among early expressed HPV proteins [10,11].

Malignant transformation is not a common occurrence of an HPV infection, as only a minor number of cervical lesions infected with HR-HPV types evolve into cervical cancer [12]. However, for unclear reasons the HPV genome, in some cases, integrates randomly into the host DNA leading to cervical transformation, which is mainly due to overexpression of the major HPV viral oncoproteins, E6 and E7 [13,14].

Although the adaptive immune system plays an important role in the elimination of HPV infected cells [2,15], an estimated 15% of women infected with HR-HPV fail to develop an effective response against the virus due to viral immune evasion mechanisms [16], leading to HPV persistent infections [15]. HPV prophylactic vaccines activate the adaptive immune system and induce neutralizing antibodies that prevent infections by viral types covered by the vaccines but cannot eliminate established HPV infections. On the other hand, therapeutic vaccine candidates must be directed at inducing cell-mediated adaptive immune responses through the induction of HPV-specific CD8+ cytotoxic T lymphocytes (CTL) [17,18].

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An ideal therapeutic vaccine candidate should be able to induce cross-reactivity among different HPV types with activation of tumor-specific CD8+ cytotoxic T cells, as well as CD4 + helper T cells [19,20]. A number of lines of evidence have shown that HPV-specific T cells and tumor infiltration by CD8+ T cells are associated with a better outcome in patients [21,22] and tumor regression in experimental models [23]. Although the most common HPV antigens employed for vaccination have been the major oncoproteins E6 and E7, which have shown some benefit against HPV and cervical cancer [24], these oncoproteins differ substantially among HPV types, affecting their potential as wide spectrum HPV therapeutic immunogens. On the other hand, as the HPV-E1 sequence is highly conserved among all known HPV types [10], it could constitute a good target for a universal HPV vaccine.

E1 protein expressed in a plasmid encoding codon-optimized canine oral papillomavirus (COPV) led to therapeutic immunization of COPV-infected Beagle dogs [25,26], which was better than that achieved with plasmids encoding E2 or E7 COPV. Moreover, E1 and E2 from cottontail rabbit papillomavirus (CRPV) induced strong immunity against infection with CRPV particles in rabbits [27,28]. In both models, the adaptive immune system cleared virally-induced papillomas. Recently, it was found that immunization of macaques with E1/E2 sequences in an adenoviral vector induced antigen-specific T cell immunity against established primate papillomavirus (MfPV) infections [29]. Moreover, immunization of mice with HPV-16 E1 in a vaccinia vector induced specific CTL immune responses [30].

Protein antigens contain a variable number of T-cell-epitopes capable of binding to major histocompatibility complex class I and class II molecules (MHC-I and MHC-II) and are recognized by CD8+ CTLs or CD4 + T cells, respectively. However, purified soluble proteins generally fail to induce robust CTL responses [31] unless they are administered together with adjuvants of different chemical natures, which activate innate immunity and inflammation, leading to the induction of costimulatory signals which are critical for antigen-presenting cell (APC) [32] induction of T cell activation. In addition to being an adjuvant, α-galactosylceramide (α-GalCer) that increases the numbers of antigen-specific CD8+ T cells when co-administered with antigens [33,34], stimulates an NKT cell-dependent cell immune cascade involving dendritic cells, NK and T cells [35]. Moreover, α-GalCer appears to promote a better CTL immune response than other adjuvants, such as incomplete Freund's adjuvant, IFN-α, poly (I:C) or TLR-9 agonists [33].

Its high homology among different HPV types makes the E1 protein a good candidate for a therapeutic vaccine against most HPV types in early HR-HPV infections and low-grade cervical intraepithelial lesions, where the viral genome is replicating and E1 expression is at its highest. As yet, it is not known whether antigen-specific CD8+ T cells are capable to eliminate HPV E1-expressing cells *in-vivo*. Here, we used a combination of HPV-E1 with the strong adjuvant α-GalCer to induce specific CD8+ T cell immune responses against HPV18-E1 expressing grafted cells. This combination induced E1-specific CD8+ T cells in mice, which correlated with a decrease in the growth of E1-expressing cells both in prophylactic and therapeutic approaches.

2. Methods

2.1. Mice and cell line

Female C57BL/6 mice (C57BL/6NCrl, originally from Charles River Laboratories International, Inc.) were purchased from Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, México. All animals were kept in a pathogen-free environment and fed ad lib. The Ethics Committees of the Universidad Nacional Autónoma de México and of the Instituto Nacional de Cancerología, México, approved the procedures for care and use of animals. All applicable institutional regulations concerning the ethical use of animals were followed accordingly. The B16-F0 mouse melanoma-derived cell line (ATCC® CRL-6322™) was grown in Dulbecco's modified Eagle's medium F12 supplemented with 10% FBS.

2.2. E1-codon optimization and plasmid construction

The HPV18-E1 reference gene sequence was codon-optimized to improve its expression in mouse cells. Codon optimization was made by GenScript (New Jersey). The codon-optimized sequence was used to amplify the E1-FL full-length (E1-FL) sequence and three E1 fragments (E1-N-terminal, E1-DNA-binding domain, and $E1_{244-550}$) (Supplementary Fig. 1A). The $E1_{244-550}$ region contains part of the DNA binding domain and C-terminal domain. PCR products were amplified with the primers shown in Supplementary Table 1 and were cloned into pcDNA 3.3 Topo plasmid (Invitrogen) with a Kozak sequence and human influenza hemagglutinin (HA) tag.

2.3. MHCI peptide prediction

To determine whether the E1 protein and the $E1_{202-654}$ region contained enough peptides capable of binding mouse H2-D^b, H2-K^b and human HLA-A2 we took advantage of the RANKPEP software (http://imed.med.ucm.es/Tools/rankpep.html) [36]. Based on this software, with 2% threshold and with a percentile score (% OPT) $\geq$ 15%, we found 120 peptides capable of binding to H2-D^b and H2-K^b in E1 of which 62 were within $E1_{244-550}$ (Supplementary Table 2).

2.4. Cell cultures, transfection, and cell sorting

GFP and pE1-FL or plasmids with truncated E1 sequences (E1-N-Terminal, E1-DNA-binding domain, and $E1_{244-550}$) were co-transfected 24 h after the beginning of culture by means of Lipofectamine 2000 reagent (Invitrogen) into B16-F0 cells grown in supplemented culture medium in 60 mm^2 wells (6 $X10^5$ cells/well). Transfected cells were analyzed by flow cytometry for the expression of GFP and E1. As cells with high expression of GFP (GFP^{hi}) also had high expression of E1 (Supplementary Fig. 2A). these cells were isolated by fluorescence-activated cell sorting in a BD FACSJazz cell sorter to obtain an enriched population of 96% GFP^{hi} cells (Supplementary Fig. 2B). As $pE1_{244-550}$ yielded higher and more consistent expression than the other E1-expressing plasmids, this E1-truncated plasmid was used for the experiments described below.

2.5. Western blot analysis

Extracts from sorted B16-F0 cells expressing $E1_{244-550}$ (B16-F0/$E1_{244-550}$) and from cells transfected with pcDNA3.1/His empty vector were run on 10% SDS-PAGE and transferred onto nitrocellulose membranes, after which they were incubated with rabbit polyclonal anti-HA antibody (Santa Cruz Biotechnology,sc-805), followed by horseradish peroxidase-labeled goat anti-rabbit antibody (Santa Cruz Biotechnology,sc-2030). β-actin was used as loading control and it was detected with mouse monoclonal antibody sc-47778 (Santa Cruz Biotechnology) followed by mouse IgG kappa binding protein conjugated to horseradish peroxidase (Santa Cruz Biotechnology, sc-516102). E1 and β-actin were visualized with enhanced chemiluminescence [37] (Amersham). Western blots were performed three times to assure reproducibility.

2.6. Expression of $E1_{244-550}$ in tumors

To evaluate $E1_{244-550}$ expression *in vivo* (in tumor cells), 7.5×10^5 B16-F0/$E1_{244-550}$ cells with a viability of $\geq$ 97% (trypan blue) were injected subcutaneously (s.c.) in the left flank of untreated C57BL/6 mice. Tumors were excised at different times from euthanized mice in accordance with the Animal Research Advisory Committee (ARAC)-NIH regulations. $E1_{244-550}$ was analyzed in Western blots of tumor lysates as described above.

2.7. Recombinant $E1_{202-654}$ protein

A bacterial protein expression system was used to produce and purify the E1 protein 202 to 654 ($E1_{202-654}$) that corresponds to the DNA-binding (DBD) and carboxyl-terminal domains (Supplementary Fig. 1A) plus a 6x histidine tag. This fragment was obtained and purified by GenScript and dissolved in PBS and 0.5 M L-arginine, pH 7.4, and loaded onto SDS-PAGE to corroborate its molecular weight and integrity (Supplementary Fig. 1B).

2.8. Prophylactic and therapeutic immunization protocols

For the prophylactic protocol, seven to eight-week-old female C57BL/6 mice were distributed into four groups of six mice each. Group P1 ($E1_{202-654}$): immunized subcutaneously (s.c.) with 4 μg recombinant $E1_{202-654}$; Group P2 ($E1_{202-654}$ + α-GalCer) immunized s.c. with 4 μg recombinant $E1_{202-654}$ plus 3 μg α-GalCer; Group P3 (α-GalCer): inoculated s.c. with 3 μg α-GalCer; and Group P4 (control): inoculated s.c with 70 μl PBS. Fourteen days later, a second identical dose was administered s.c. to mice of each group. The dose for $E1_{202-654}$ immunization was chosen because it elicited the highest number of CTL as determined by a dose-response curve, with the optimal timing for boosting also determined by measuring cytotoxic CTLs at different times after immunization (not shown). The dose of α-GalCer administered s.c. has been reported by others [33]. To examine the effect of the different treatments on the growth of B16-F0/$E1_{244-550}$ cells, immunized C57BL/6 mice were injected s.c. five days later in the left flank with 1.8×10^6B16-F0/$E1_{244-550}$ cells (≥97% viability by trypan blue). In non-immunized mice, tumor masses were detectable 3 to 5 days after inoculation. Tumor growth was measured every three days with a digital caliper and the estimated volume was determined with the equation; Volume = (width^2 × length)/2 as described [38–41]. Mice were euthanized when the tumor mass size reached 20 mm in any one dimension, or up to a maximum of 21 days after inoculation.

For the therapeutic protocol, 24 seven to eight-week-old female C57BL/6 mice were inoculated with 1.8×10^6 B16-F0/$E1_{244-550}$ cells (viability of $\geq$ 97% by trypan blue). Five days after cell inoculation, when all mice had palpable tumors, they were distributed into four groups of six mice each, and inoculated with the same antigen and adjuvant doses as those of the prophylactic protocol: groups were T1 ($E1_{202-654}$), T2 ($E1_{202-654}$ + αGalCer), T3 (αGalCer) and T4 (PBS). Fourteen days later, a second identical dose was administered s.c. to each group. Tumor growth was measured every three days and the estimated volume was determined as described above. Again, mice were euthanized when the tumor mass size reached 20 mm in any one dimension, or up to a maximum of 23 days after inoculation.

2.9. Preparation of splenocytes, NK and CD8+ T cell isolation

Spleens were excised, placed in PBS, sliced into small fragments, settled onto 70 μm mesh nylon cell strainers (Corning Life Sciences) and pressed through with a syringe plunger onto 50 mL conical tubes. Cells were washed with PBS and centrifuged at 500 × *g* for 5 min. Cell pellets were incubated with lysing solution (Becton Dickinson PharmLyse™) for 2 min at 37 °C. The cell preparation was washed twice and centrifuged (500 × *g* /5 min). CD8+ T cells were isolated with CD8α$^+$ T Cell Isolation Kit II (Miltenyi Biotec), and NK cells were isolated with NK Cell Isolation Kit (Miltenyi Biotec), according to the manufacturers' protocols from viable splenocytes counted in a TC20 automated cell counter (BIORAD). Purity of CD8+ T cells was assessed by staining with CD8+/PE, CD3+/Alexa Fluor488 and with CD3+/Alexa Fluor488, NK 1.1/Alexa Fluor647 for NK cells (purity of 94% and 87%, respectively)

2.10. Ex-vivo cytotoxic activity assay for CD8+ T-cell or NK cell cytotoxicity

Purified CD8+ T and NK cells with a viability ≥ 90% were used for the cytotoxic activity assays. Thus, $4x10^5$ CD8+ T cells or $1x10^5$ NK cells from each individual mouse of the four groups were co-cultured with B16-F0/$E1_{244-550}$ target cells ($4x10^4$ and $1x10^5$, respectively). In both systems, fluorophore-labelled antibodies AF488-anti-CD107a (1D4B) and AF488-anti-CD107b (M3/84) directed at the granular membrane proteins CD107a and CD107b (0.25 μg/10^6 cells) or isotype controls were added to the cultures at the beginning of the assay. As it is a time-course curve, the exposure of CD107a/b molecules on cell membrane is indicative of living cells with cytotoxic activity. NK and CD8+ T cells from unimmunized mice stimulated with phorbol-12-myristate-13-acetate (PMA, 300 ng/ml, Sigma Chemical Company, St. Lous MO) and ionomycin (1,500 ng/ml, Sigma) were used as positive effector controls. In all cases, cell cultures were incubated for up to 5 h, and monensin 5 mg/ml (BD Biosciences) was added for the final 3 h of culture [42]. Cells were fixed with 1% paraformaldehyde and

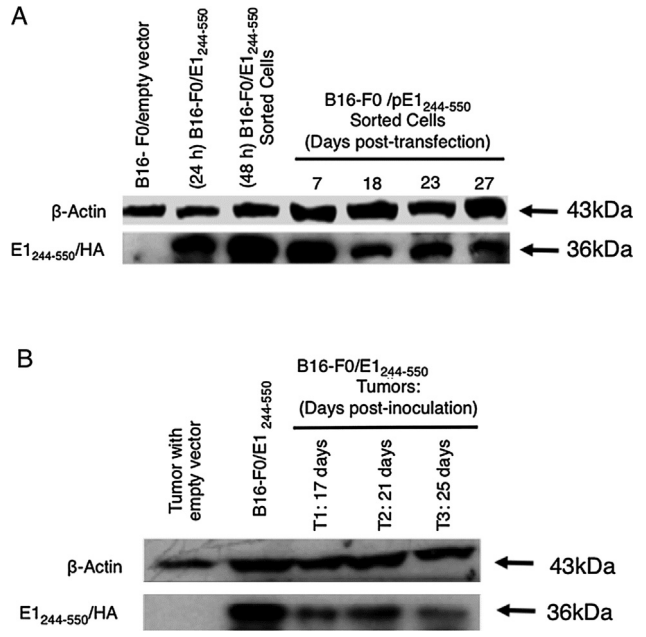

Fig. 1. Expression of codon-optimized $E1_{244-550}$. (A) Sorted B16-F0 cells expressing $E1_{244-550}$ were analyzed at different times post-transfection (7, 18, 23 and 27 days) by western blot using an anti-HA antibody. An extract from B16-F0 cells transfected with the empty vector was used as negative control. Extracts from un-sorted B16-F0 cells transfected with p$E1_{244-550}$ (24 h post-transfection) and sorted B16-F0 cells transfected with p$E1_{244-550}$ (48 h post-transfection) were used as positive controls. (B) Detection of $E1_{244-550}$ in tumors originated from sorted B16-F0/$E1_{244-550}$ cells. Untreated mice were inoculated with sorted B16-F0/$E1_{244-550}$ cells and the expression of $E1_{244-550}$ was analyzed in tumor extracts collected at different times post-inoculation (17, 21 and 25 days). B16-F0-p$E1_{244-550}$ cell extracts collected at 24 h post-transfection were used as positive control. Extracts from a tumor originated from B16-F0/empty vector was used as negative control.

CD107a/b expression on NK1.1 + and CD8+ T cells was analyzed by flow cytometry (see below).

2.11. Flow cytometry

Flow cytometry was achieved with a FACSAria II flow cytometer (Becton Dickinson, Mountain View, CA) using FACS Diva software 8.0.1 and further analyzed with FlowJo software (version 10.2). Typically, 50,000 events were acquired for CD8+ T cell cytotoxic activity assays and 10,000 events for NK cytotoxic activity assays. Monoclonal antibodies used for the flow cytometry and lineage-specific panels were: PerCP-labelled anti-CD8a (53-6.7), Alexa Fluor 488®-labelled anti-CD3 (17A2), Alexa Fluor 488® (AF488)-labelled anti-CD107a (1D4B), Alexa Fluor 488® (AF488)-labelled anti-CD107b (M3/84), PerCP-labelled anti-CD62L (MEL-14), brilliant violet 421™-labelled anti-CD127 (A7R34) and Alexa Fluor® 647 (AF647)-labelled anti-NK1.1 (all from Biolegend). To test E1-transfection efficiency in B16F0 melanoma cells, Alexa Fluor 647® (AF647)-labelled anti-HA.11 (16B12) antibody (Biolegend) was used.

2.12. Statistical analysis

Statistical analysis was performed by one-way ANOVA and Tukey's multiple comparison tests (GraphPad Prism Software, Inc.). Error bars represent standard error of the mean (SEM) and *P* values of < 0.05 were considered as statistically significant.

3. Results

3.1. Expression of codon-optimized $E1_{244-550}$ in B16-F0 cell line and mouse tumors

To generate cell lines expressing HPV-E1, four different plasmids encoding codon-optimized E1-full-length and three E1 fragments (E1-N-Terminal, E1-DNA-binding domain, and $E1_{244-550}$) were transfected into B16-F0 cells. All of the plasmids yielded transient expression of E1 sequences and although a stable expression could not be achieved with any of them, the $E1_{244-550}$ sequence remained expressed *in vitro* up to 27 days post-transfection (Fig. 1A). As high GFP-expressing cells were also $E1_{244-550}$ high expressers, with a mean 96% GFP + cells were used as targets in our experiments (Supplementary Fig. 2B).

$E1_{244-550}$ protein expression was clearly identified in western blots of *in vivo* grown tumor lysates up to 25 days after inoculation of mice with $7.5x10^5$ B16-F0/$E1_{244-550}$ cells (Fig. 1B).

Therefore, the presence of a number of potential peptide binders to mouse H2-D^b, H2-K^b (Supplementary Table 2) and the high expression of $E1_{244-550}$ in these cells make them potential immunological targets for antigen-specific CTLs.

3.2. Immunization with $E1_{202-654}$ + α-GalCer protects against B16-F0/$E1_{244-550}$ tumor challenge (prophylactic approach)

To determine whether antigen-specific immune response impairs tumor formation and growth, we examined the protective efficacy of immunization with $E1_{202-654}$ + α-GalCer in a mouse tumor model (Fig. 2A). Tumor growth was monitored daily in the four different groups of mice implanted with syngeneic B16-F0/$E1_{244-550}$ cells at day 17th (Fig. 3A, B, C, and D). As seen, mean tumor size was significantly smaller in the group immunized with $E1_{202-654}$ + α-GalCer (Fig. 3E), when compared with mice receiving PBS (control), α-GalCer alone or $E1_{202-654}$ alone (n = 6 each). Importantly, four out of six mice in the $E1_{202-654}$ + α-GalCer group did not show any tumor growth after 21 days. Of interest, mean tumor size in mice inoculated with α-GalCer alone was also significantly smaller than those inoculated with PBS (Fig. 3E).

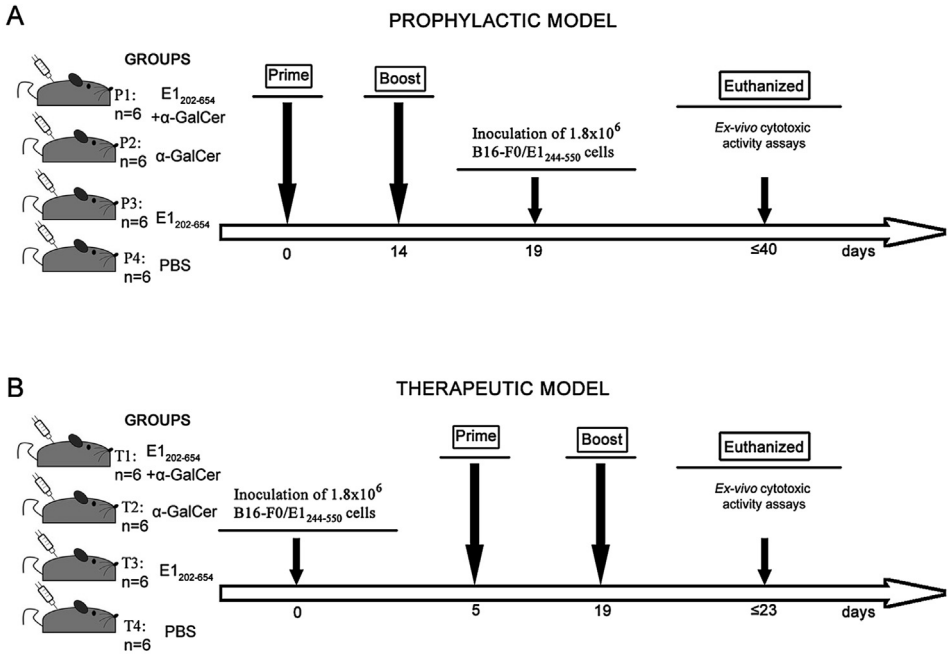

Fig. 2. Immunization protocol. (A) For the prophylactic immunization, six mice per group were used. They were inoculated and boosted at two-week after prime inoculation. Five days later, all mice were challenged with B16-F0/$E1_{244-550}$ cells. Mice were euthanized up to a maximum of 21 days after inoculation and spleens were removed to perform direct *ex-vivo* cytotoxic activity assays. (B) For the therapeutic immunization, twenty-four female C57BL/6 mice were inoculated with B16-F0/$E1_{244-550}$ cells. Five days after cell inoculation when all mice developed detectable tumors, they were distributed randomly into four groups of six mice each and inoculated as specified. Mice were euthanized up to a maximum of 23 days after inoculation and spleens were removed to perform direct *ex-vivo* cytotoxic activity assays.

3.3. $E1_{202-654}$ + α-GalCer inhibits the growth of established B16-F0/ $E1_{244-550}$ tumors (therapeutic approach)

To examine whether $E1_{202-654}$ + α-GalCer inoculation confers protection to animals with established tumors derived from cells expressing E1, four groups of mice were initially inoculated with syngeneic B16-F0/$E1_{244-550}$ cells, and after five days, when, all mice had developed detectable tumors, were immunized as described in Methods (Fig. 2B). Tumor growth was monitored daily until day 23 after implantation of B16-F0/$E1_{244-550}$ cells. As shown in Fig. 4B, vaccination with $E1_{202-654}$ + α-GalCer impairs tumor growth, when compared with the other treatments (Fig. 4A, C, and D). As before,

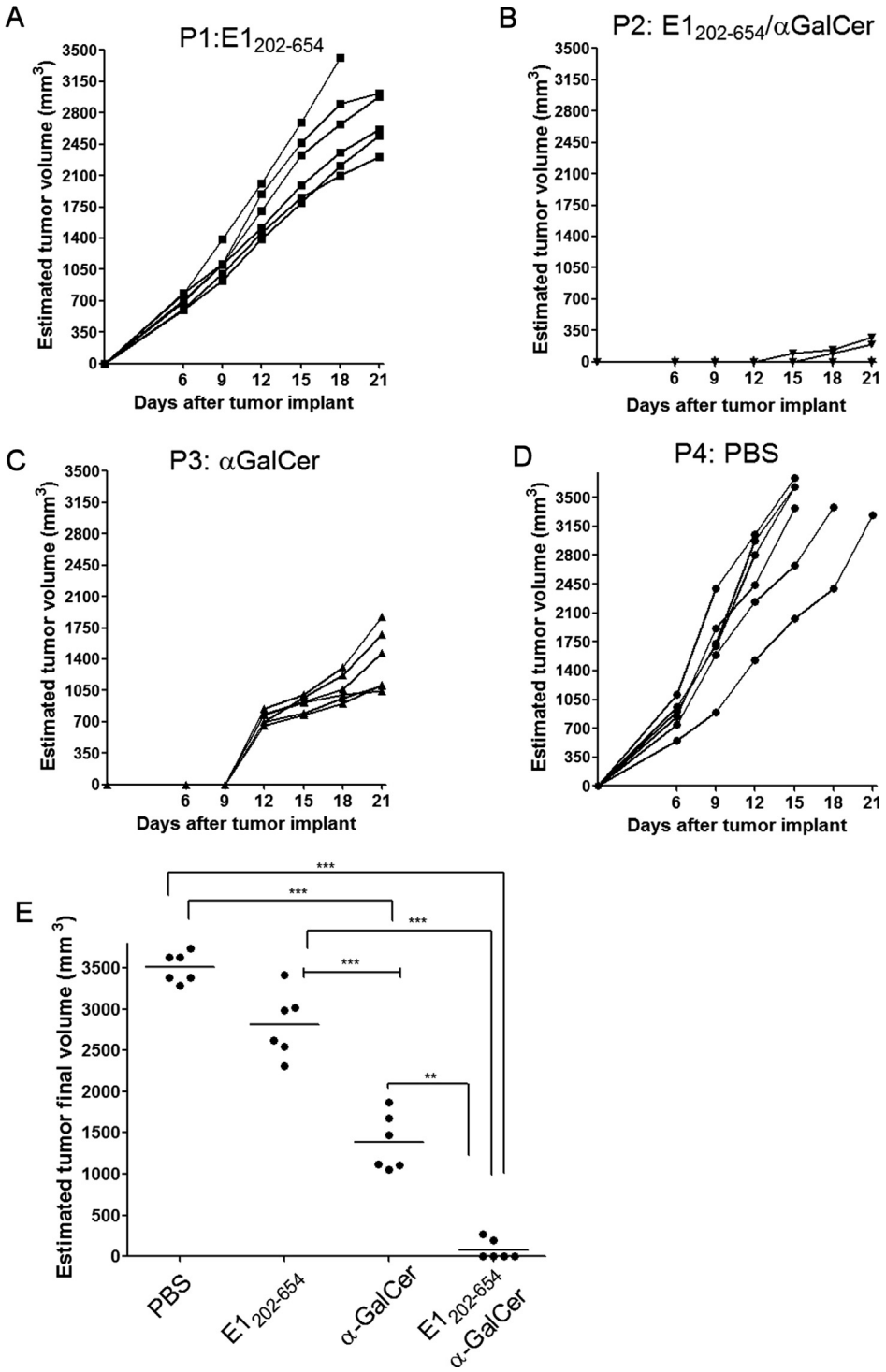

Fig. 3. Effect of prophylactic immunizations on tumor growth *in-vivo*. Each mouse group was inoculated with B16-F0/ $E1_{244-550}$ cells and tumor growth was monitored over time for (A) $E1_{202-654}$ (n = 6), (B) $E1_{202-654}$ + α-Galcer (n = 6), (C) α-GalCer (n = 6) and (D) PBS (n = 6) treatments. Tumor size was measured as described in "Methods" and its estimated volume was plotted over time. (E) Effect of immunizations on tumor growth. Once the mice were euthanized, the estimated final tumor size was determined as described in "Methods" and reported in mm^3. Tumor size was plotted for each mouse and the mean is depicted for each group. The statistical significance with $^{**}p < 0.01$ and $^{***}p < 0.0001$ (multiparametric one-way ANOVA and Tukey's Multiple Comparison Test).

the mean tumor size in mice inoculated with α-GalCer alone was significantly smaller (1937.6 mm^3) than that of PBS inoculated mice (3624 mm^3) (Fig. 4E). These results, indicate that immunization with *$E1_{202-654}$ + α-GalCer* is capable of reducing tumor mass when tumor growth has already started, and a role for α-GalCer alone can be seen, as mice of this group also had a reduced tumor size.

3.4. Immunization with $E1_{202-654}$ plus α-GalCer promotes antigen-specific CD8+ T cell cytotoxic response in both prophylactic and therapeutic protocols

As cytotoxic granule exocytosis leads to the expression of CD107a and CD107b on the surface of CD8+ T cells [42] their newly appearance on CD8+ T cells and NK cells are widely accepted as

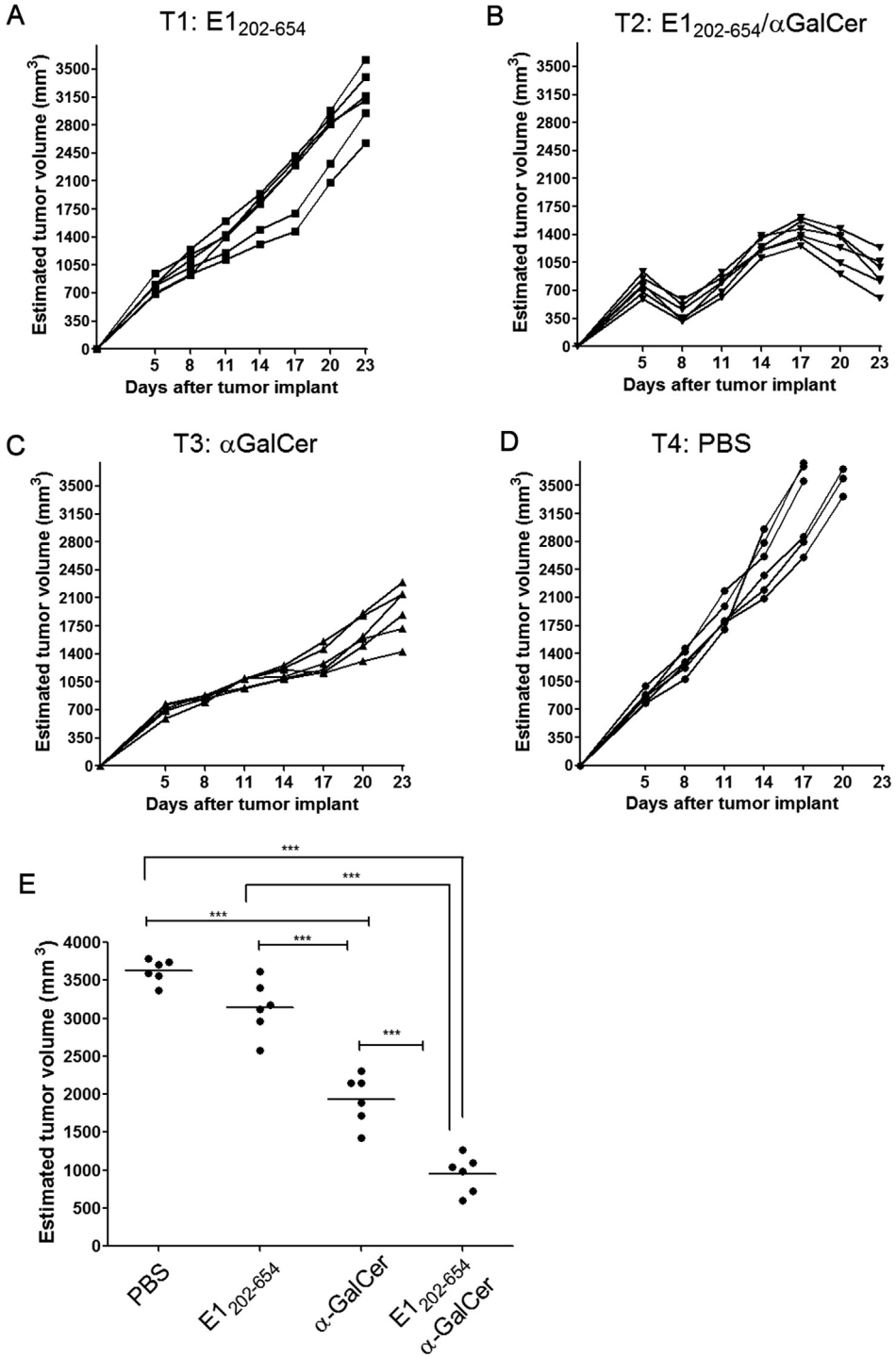

Fig. 4. Effect of therapeutic immunizations on tumor growth *in-vivo*. Each group was inoculated with B16-F0/ $E1_{244-550}$ cells and tumor growth was monitored over time for (A) $E1_{202-654}$ (n = 6), (B) $E1_{202-654}$ + α-Galcer (n = 6), (C) α-GalCer (n = 6) and (D) PBS (n = 6) treatments. Tumor size was measured as described in "Methods" and its estimated volume was plotted over time. (E) Effect of immunizations on established tumors. Once the mice were euthanized, the estimated final tumor size was determined as described in "Methods" and reported in mm^3. Tumor size was plotted for each mouse and the mean is depicted for each group. *** $p < 0.0001$ (multiparametric one-way ANOVA and Tukey's Multiple Comparison Test).

markers of cytotoxicity [42–44]. Therefore, we examined first the time course of appearance of CD107a/b cytotoxic activity markers in splenic CD8+ T cells (Supplementary Fig. 3).

To examine E1 antigen-specific cytotoxic activity for the prophylactic and therapeutic protocols, CD8+ T cells from each individual mouse were co-cultured with B16-F0 and B16-F0/$E1_{244-550}$ cells as targets. We observed that in CD8+ T cells from E1 + α-GalCer immunized mice co-cultured with B16-F0/$E1_{244-550}$ cells, there was a striking increase in surface expression of CD107a/b, indicating the presence of an E1 antigen-specific CD8+ T cell cytotoxic activity (Fig. 5A and B), which correlated with the inhibition of B16-F0/$E1_{244-550}$ growth in both protocols. In the prophylactic (Fig. 5A) and therapeutic protocols (Fig. 5B), the PBS, E1 and α-GalCer groups did not show an E1 antigen-specific response, as measured through CD107a/b detection on CD8+ T cell surface.

We found that in the prophylactic as well as in therapeutic protocols, B16-F0/$E1_{244-550}$ growth inhibition seen in the α-GalCer only-treated mice was not due to CD8+ T cell activity, because CD8+ T cells fail to express CD107a/b in response to B16-F0 and B16-F0/$E1_{244-550}$ cells.

3.5. α-GalCer also promotes NK cell responses in the prophylactic and therapeutic protocols

As NK cell response has been associated with antitumor effects [34,45,46], and α-GalCer is a well-known NK cell activator, we examined the role of NK cells in tumor growth inhibition. First, we determined the time course of appearance of CD107a/b cytotoxic markers in splenic NK cells (Supplementary Fig. 4). To examine NK cytotoxic activity for the prophylactic and therapeutic protocols, NK cells from each individual mouse were co-cultured with B16-F0/$E1_{244-550}$ as targets. In both protocols, mice inoculated with α-GalCer alone showed the strongest NK cytotoxic activity (Fig. 6A for prophylactic and 6B for therapeutic) compared with all groups. Nevertheless, the inoculation of E1 + α-GalCer in both protocols also induced significant NK cytotoxic activity compared with PBS and $E1_{202-654}$ mice groups (Fig. 6A and B). $E1_{202-654}$ immunized mice from the prophylactic and therapeutic protocols showed a basal NK cytotoxic activity, similar to that observed in the PBS group (Fig. 6A and B). These results indicate that in addition to tumor-specific CD8+ cytotoxic responses, NK cells can

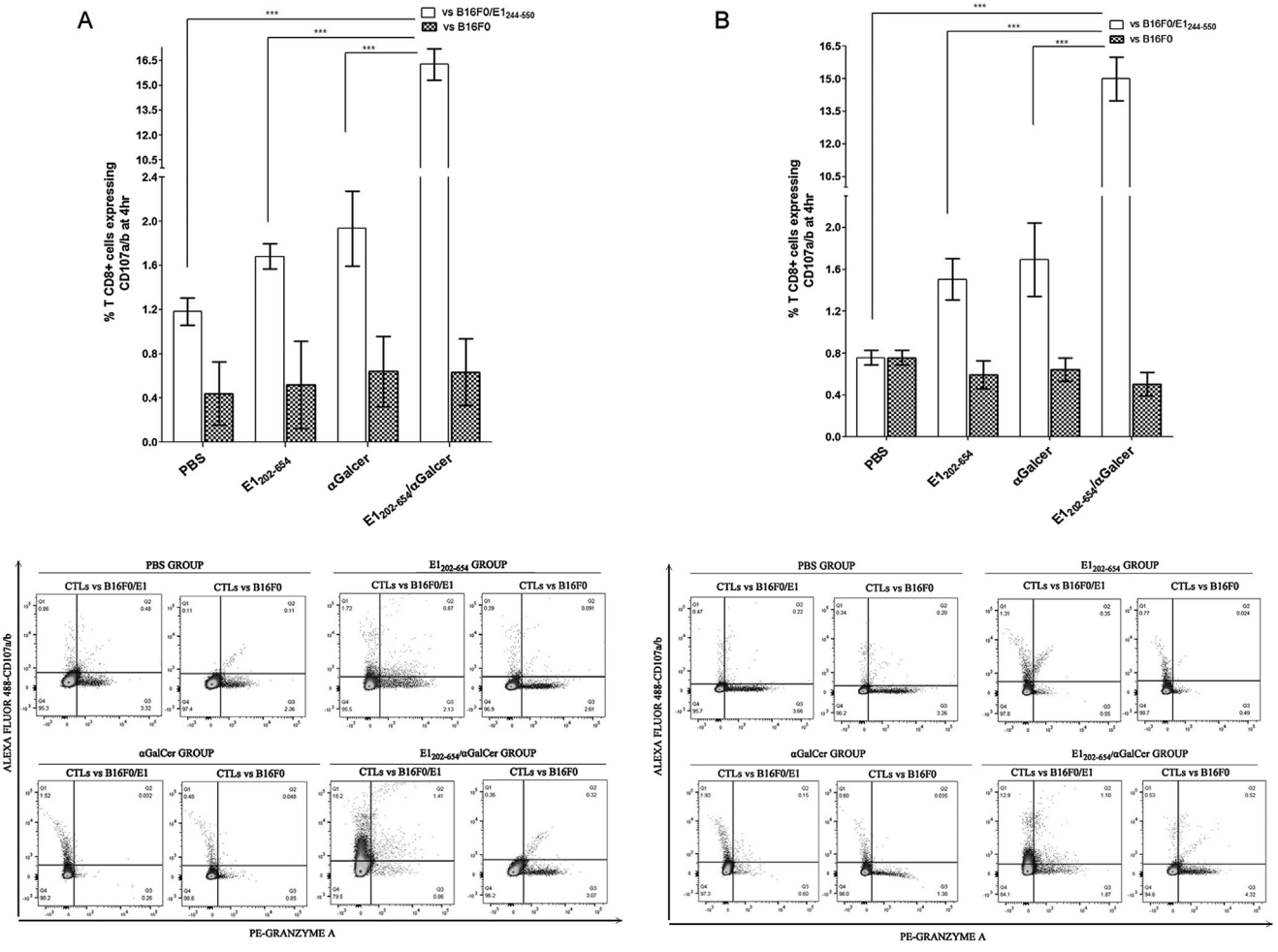

Fig. 5. E1-specific CD8+ T cell cytotoxic response by immunization with $E1_{202-654}$ + α-Galcer. CD107a/b expression on cell surface was measured in splenic CD8+ T cells from mice inoculated with $E1_{202-654}$ + α-Galcer (n = 6), PBS (n = 6), $E1_{202-654}$ (n = 6) and α-Galcer (n = 6) co-cultured with B16-F0 cells (empty bars) or B16-F0/$E1_{244-550}$ transfected cells (checkered bars) for up to 4 h in the presence of anti-CD107a/b-Alexa Fluor 488 to compare if the T CD8+ cells have an antigen-specific cytotoxic activity both in (A) prophylactic and (B) therapeutic protocols. The lower panels in A and B show representative flow cytometry images of cytotoxic assays where the CD107a/b molecules are detected at the cell surface. $^{***}p < 0.0001$ (multiparametric one-way ANOVA and Tukey's Multiple Comparison Test).

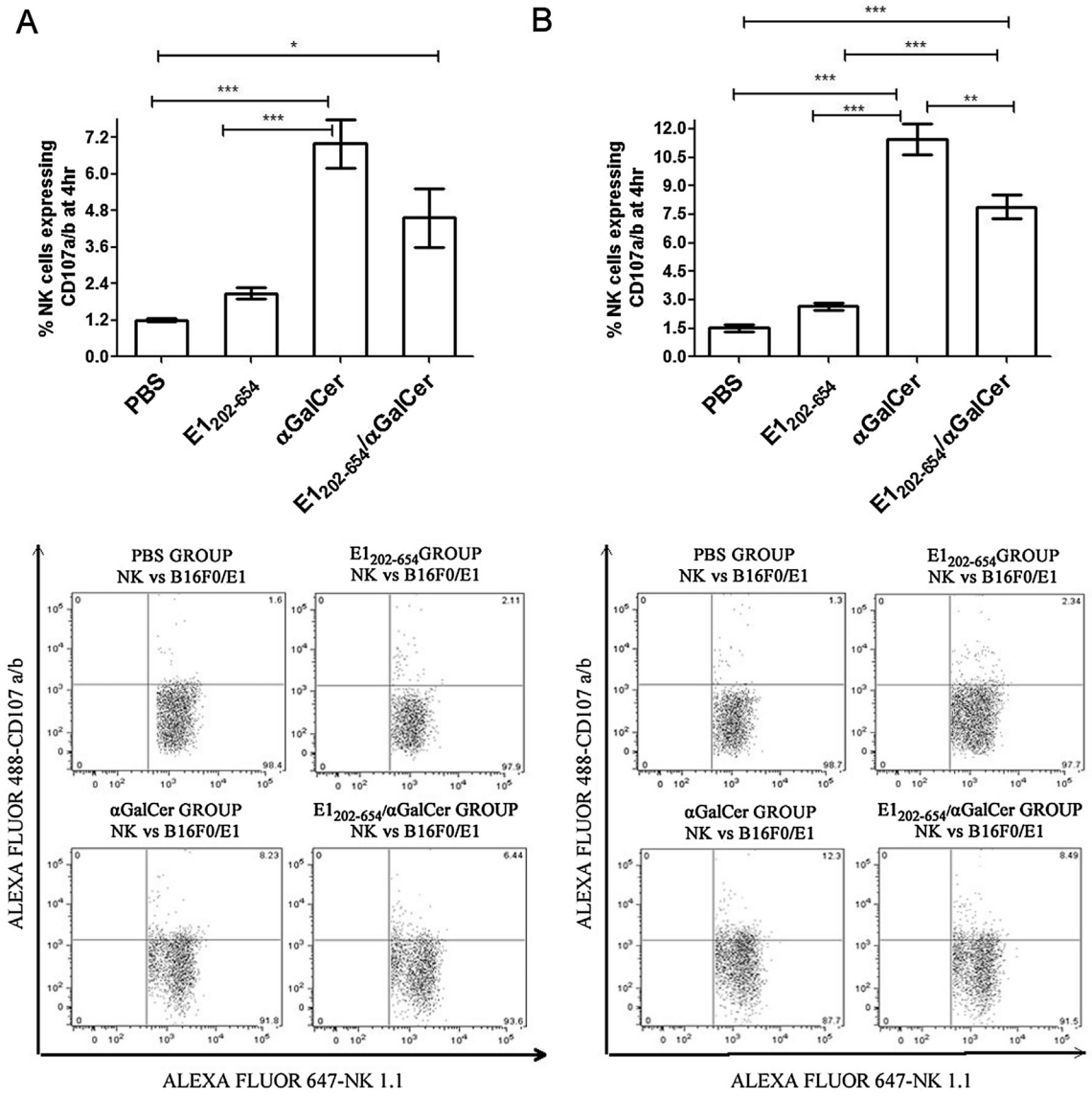

Fig. 6. Expression of CD107a/b on the cell surface of NK as a cytotoxic marker. Splenic NK cells from mice inoculated with $E1_{202-654}$ + α-Galcer (n = 6), PBS (n = 6), $E1_{202-654}$ (n = 6) and α-Galcer (n = 6) were co-cultured with B16-F0/$E1_{244-550}$ transfected cells for up to 4 h in the presence of anti-CD107a/b-Alexa Fluor 488 to evaluate NK cell cytotoxic activity against tumor cells, both in (A) prophylactic and (B) therapeutic protocols. The lower panels in A and B show representative flow cytometry images of cytotoxic assays where CD107a/b molecules are detected on the cell surface. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.0001$ (multiparametric one-way ANOVA and Tukey's Multiple Comparison Test).

provide an additional antitumor activity that contributes to the therapeutic and prophylactic potential of E1 + α-GalCer immunization.

4. Discussion

Induction of cytotoxic T cell responses to viral antigens constitutes the main goal of therapeutic vaccination in chronic viral diseases, including those related to cancer, such as cervical cancer. In the current studies, we examined the role of an HPV-E1 protein fragment as an immunogen with therapeutic and/or prophylactic potential and capable of inducing HPV-E1-specific CD8+ cytotoxic T cells in a mouse model. For this, B16F0 murine melanoma cells were transfected with a codon-optimized $E1_{244-550}$ plasmid, which, in contrast to the full-length HPV18-E1 reference sequence, was efficiently expressed, as reported for COPV E1 [26].

We found that mice inoculated with $E1_{202-654}$ in combination with the potent adjuvant α-GalCer [33,34], impaired the growth of B16-F0/$E1_{244-550}$ bearing target cells in a therapeutic as well as in a prophylactic protocol. The resulting tumors in mice of the therapeutic protocol were smaller than in control mice, whereas in the prophylactic model four out of six mice did not develop any tumor at all. This indicates that vaccination with E1 + α-GalCer can induce a strong antitumor and potentially therapeutic response.

As E1- bearing tumor growth inhibition was observed in the α-GalCer and $E1_{202-654}$ + α-GalCer immunized groups, although inhibition was stronger in the $E1_{202-654}$ + α-GalCer group, it was important to examine separately the ability of NK and CD8+ T cells from either mouse group *ex-vivo*. As expected, only mice immunized with $E1_{202-654}$ + α-GalCer showed specific cytotoxic activity against B16-F0/$E1_{244-550}$ cells, which is consistent with previous findings in mice immunized with Ankara vaccinia virus encoding E1 from HPV-16 [30]. We next measured an NK cell response in the α-GalCer treated mice from both protocols, which showed an NK cytotoxic activity, correlating with the unspecific antitumor effect observed in these groups. Moreover, NK cytotoxic activity was also detected in mice treated with $E1_{202-654}$ + α-GalCer,

indicating that the protective and therapeutic effects of $E1_{202-654}$ + α-GalCer against HPV involve an adaptive branch represented by CD8+ CTLs plus an increase in NK cell activity, which acting together could contribute to a stronger anti-tumor response.

The fact that the mean tumor size of the $E1_{202-654}$ + α-GalCer group was much smaller than that of the α-GalCer inoculated group in both protocols indicates that immunization with $E1_{202-654}$ plus α-GalCer, which induces a strong CD8+ T cell E1 antigen-specific immune response, is involved in the elimination of B16-F0/$E1_{244-550}$ cells. It should be noted that the percentage of CD8+ T cells actively cytotoxic is greater than the percentage of NK cells actively cytotoxic.

The current results could be the basis to propose that E1-specific CD8+ adaptive immune response could play a role in the clearance of HPV infected cells in patients with early HPV-infection, including those with normal cytology or with low-grade cervical intraepithelial lesions, where E1 is necessary for viral replication. Moreover, as the most conserved of early HPV proteins, E1 could potentially induce immunity against various HPV types. This remains to be tested in models inoculated with tumors bearing E1 from other HPV types. It is important to note that the finding that tumor cells transiently expressing $E1_{244-550}$ antigen were eliminated, strengthens the potential role of E1 as an immunizing antigen for broad-spectrum therapeutic vaccines against HPV. Additionally, α-GalCer served as a potent adjuvant to elicit $E1_{244-550}$ antigen-specific CD8+ T cell response also inducing a strong innate immune response through NK cells.

With these results, it is now important to predict whether a similar response could be obtained in humans immunized with the same E1 sequences. Thus, we used the same analysis criteria to predict peptides for HLA-A2, which is the largest and most diverse allele family in humans [47]. This revealed that the E1 full-length protein contains 303 highest-scoring predicted peptides and $E1_{244-550}$ sequence contains 243 (Supplementary Table 2).

Finally, although we did not address CD4 + T cell help in these studies, the $E1_{202-654}$ fragment is long enough and contains potential epitopes that, together with the adjuvant effect of α-GalCer, should have induced CD4 help to enhance CD8 T cell responses. This will be the subject of further studies. This is an important issue because, depending on the phenotype of the CD4 + T cells induced, they would lead to distinct types of responses, including help or suppression, which could affect the outcome of tumor-infiltrating CTLs.

5. Authors' contributions

AAM participated in the study design, cytotoxic and flow cytometry assays, analysis, interpretation of data and writing of the manuscript. CTM was involved in the cytotoxic and flow cytometry assays. DRR and ISO participated in the analysis and interpretation of flow cytometry assays. EPC coordinated the management of the mouse model and analysis of the data. EL and JM participated in the study design, data analysis and writing of the manuscript. ML designed the study and was involved in the analysis and interpretation of the data and writing of the manuscript. All authors reviewed and approved the final manuscript for submission.

Ethical statement

The Ethics Committees of the Universidad Nacional Autónoma de México (UNAM, Mexico City) and of the Instituto Nacional de Cancerología, México (017/001/IBI), approved the procedures for care and use of animals. All applicable institutional regulations concerning the ethical use of animals were followed accordingly.

Conflicts of interest

The authors declare to have no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2018.12.036.

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