Novel Cell-Penetrating Peptide-Based Vaccine Induces Robust CD4+ and CD8+ T Cell–Mediated Antitumor Immunity

Madiha Derouazi1, Wilma Di Berardino-Besson1, Elodie Belnoue2, Sabine Hoepner1, Romy Walther5, Mahdia Benkhoucha4, Patrick Teta1, Yannick Dufour1, Céline Yacoub Maroun1, Andres M. Salazar5, Denis Martinvalet6, Pierre-Yves Dietrich1, and Paul R. Walker1

Abstract

Vaccines that can coordinately induce multi-epitope T cell–mediated immunity, T helper functions, and immunologic memory may offer effective tools for cancer immunotherapy. Here, we report the development of a new class of recombinant protein cancer vaccines that deliver different CD8+ and CD4+ T-cell epitopes presented by MHC class I and class II alleles, respectively. In these vaccines, the recombinant protein is fused with Z12, a novel cell-penetrating peptide that promotes efficient protein loading into the antigen-processing machinery of dendritic cells. Z12 elicited an integrated and multi-epitopic immune response with persistent effector T cells. Therapy with Z12-formulated vaccines prolonged survival in three robust tumor models, with the longest survival in an orthotopic model of aggressive brain cancer. Analysis of the tumor sites showed antigen-specific T-cell accumulation with favorable modulation of the balance of the immune infiltrate. Taken together, the results offered a preclinical proof of concept for the use of Z12-formulated vaccines as a versatile platform for the development of effective cancer vaccines.

Introduction

The immune system can recognize and to some extent eliminate tumor cells; however, this antitumor response is often of low amplitude and inefficient. Boosting this weak antitumor response with therapeutic vaccination has been a long sought goal for cancer therapy. Different approaches were evaluated in preclinical and clinical development, including short peptide vaccines (1), long-peptide vaccines (2), and proteins. For the latter, disappointing phase III clinical trial results in melanoma and non–small cell lung cancer indicate that ideal protein vaccines and protocols have yet to be established (3, 4). Nevertheless, in these clinical trials, lack of clinical efficacy was not attributed to the protein nature of the vaccine per se, which if properly formulated, presents many advantages. Indeed, protein can potentially allow multi-epitopic antigen delivery to antigen-presenting cells (APC), such as dendritic cells (DC), without the limitation of restriction to a single MHC allele. Another strength is long-lasting epitope presentation recently described in DCs loaded with proteins (5). Furthermore, proteins require uptake and processing by DCs to achieve MHC restricted presentation with favorable modulation of the balance of the immune infiltrate. Taken together, the results offered a preclinical proof of concept for the use of Z12-formulated vaccines as a versatile platform for the development of effective cancer vaccines. Cancer Res; 75(15); 3020–31. ©2015 AACR.
membranes by both direct translocation and lipid raft-mediated endocytosis (15). We hypothesized that these two mechanisms of entry should promote both MHC class I and II restricted presentation of cargo antigens to CD8+ and CD4+ T cells, respectively. Therefore, we investigated whether this CPP (here called Z12, or its variant called Z13) was able to deliver multi-epitopic peptides to DCs, and subsequently to promote CTL and Th cell activation and antitumor function. To achieve this, we engineered a new generation recombinant vaccine by fusing a CPP to a multi-antigenic protein. This novel chimeric protein was efficiently delivered to APCs and led to multi-epitopic MHC class I and II restricted presentation. Our vaccine generated an integrated immune response with persistent CD8+ effector T cells homing to the tumor site, Th1 polarized CD4+ T cells as well as potent antitumor immunity in different tumor models, including glioma.

Materials and Methods

Cell lines

The DC line 2114 was derived from spleen tumors in CD11c-SV40T-g-transgenic C57BL/6 mice (16); it resembles normal splenic CD8α conventional DCs, but is grown in normal culture medium without addition of cytokines: IMDM-Glutamax supplemented with 10% heat-inactivated FCS, 10 mmol/L HEPES, 50 μmol/L β-mercaptoethanol, 50 μg/mL penicillin, and 50 μg/L streptomycin (all from Life Technologies). All tumor lines are from the EL4 Thymoma cell line. EG7 cells were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (FCS). B16 cells were maintained in DMEM-Glutamax, 10% heat-inactivated FCS, 10 mmol/L HEPES, with 1 μg/mL puromycin and 500 μg/mL of G418 to maintain expression of the transgenes. EG7 is an OVA transfectant from the EL4 Thymoma cell line. EG7 cells were cultured in RPMI-Glutamax, 10% heat-inactivated FCS, 10 mmol/L HEPES, with 400 μg/mL of G418 to maintain expression of the transgenes. B16-LCMV-GP is a lymphocytic choriomeningitis virus (LCMV)-GP33–41 transfectant of B16 melanoma. B16-LCMV-GP cells were cultured in DMEM-Glutamax, 10% heat-inactivated FCS, 10 mmol/L HEPES, with 1 mg/mL of G418 to maintain expression of the transgenes. The cells were treated 48 hours with 50 U/mL IFNγ prior to injection.

Mice

Female C57BL/6 mice (CD45.2) were purchased from Charles River Laboratories. T-cell receptor (TCR) transgenic mice were all on a C57BL/6 background, but in some cases expressed congenic markers used for their identification after adoptive transfer. P14 transgenic mice that express a TCR directed against MHC class I restricted epitope (LCMV)-GP33–41 were kindly provided by H. Pircher (University of Freiburg, Freiburg, Germany). SMARTA TCR transgenic mice, expressing a TCR for the MHC II restricted epitope LCMV-GP61–80 were kindly provided by P. Ohashi (University of Toronto, Toronto, Canada). OT1, OT2, and D011.10 mice express TCR specific for OVA epitopes (OT1: MHC class I-restricted OVA257–264; OT2 and D011.10 MHC class II-restricted OVA323–335). They were kindly provided by F. Romero (University of Lausanne, Lausanne, Switzerland); T. Schüler (Institute of Immunology, Charité Berlin, Berlin, Germany), or B. Huard (University of Grenoble, Grenoble, France). All animals used in this study were between 6 and 12 weeks of age at the time of experiments. These studies have been reviewed and approved by the institutional and cantonal veterinary authorities in accordance with Swiss Federal law on animal protection.

Proliferation tests

Bone marrow–derived DCs (BMDCs) were prepared from C57BL/6 mice as previously described (18), and used at day 9 to 10 of culture as APCs for proliferation tests. Briefly, BMDCs were loaded with 0.3 μmol/L of the indicated protein or peptide for 4 hours at 37°C, washed three times, then matured overnight at 37°C with 100 ng/mL LPS (from Salmonella abortus, equi S-form; Enzo Life Sciences). Antigen-loaded mature BMDC were then mixed at a ratio of 1:10 with the indicated TCR transgenic mouse splenocytes that had been stained with 10 μmol/L 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Life Technologies). After 5 days coincubation of spleen cells and BMDCs, antigen-specific proliferation was assessed by flow cytometry, measuring CFSE dilution on CD8 and live-gated cells.

Figure 1. Z12 promotes antigen cross-presentation in vitro and in vivo. A, Z12 promotes antigen cross-presentation in vitro. Processing of Z12-OVALP by BMDCs and antigen presentation to OVA257–264-specific, MHC class I-restricted (H-2Kb) OT1 T cells in vitro. CFSE-labeled splenocytes from OT1 mice were cocultured for 5 days alone, or with mature BMDCs that had been loaded with Z12-OVALP or pulsed with OVA257–264 peptide. Proliferation was assessed by CFSE dilution analyzed by flow cytometry, gating on live CD8+ T cells (n = 4). B, processing of OVALP by endogenous APCs in vivo and stimulation of polyvalent OVA-specific CD8+ T cells. Mice were vaccinated twice at 14 days of interval with Z12-OVALP and the indicated adjuvants (± anti-CD40 for Hiltonol). B16MC were isolated 7 days after the second vaccination and OVA-specific CD8+ T cells were quantified by H-2Kb-OVA257–264 tetramer staining, analyzed by flow cytometry (n = 4).
Cell preparation

Peripheral blood and spleen mononuclear cell suspensions for flow cytometry were isolated on lymphocyte separation medium 1077 (GE Healthcare). To obtain brain-infiltrating leukocytes (BIL), mice were transcardially perfused with Ringer’s solution prior to cell isolation, as previously described (19).

In vivo cytotoxicity assay

For target cell preparation for the in vivo cytotoxicity assay, spleen cells from C57BL/6 CD45.1 congenic mice were pulsed with 10 μmol/L OVA257–264 peptide (or vehicle control), then labeled with either CellTrace Violet Proliferation Kit (Life Technologies), as described in the manufacturer’s protocol, or with CFSE, as described for proliferation tests. They were injected i.v. and in parallel analyzed by flow cytometry to determine the input ratio. After 16 hours, splenocytes were isolated, stained with CD45.1- and CD45.2-specific antibodies to distinguish transferred cells, and the proportions of CFSE and Violet-stained target cells were determined by flow cytometry. Percentage vaccine-induced peptide-specific in vivo cytotoxicity was calculated according to the following formula:

\[
1 - \frac{(\text{target} + \text{peptide/target} - \text{peptide}) \text{vaccinated/input ratio} - \text{peptide/target} + \text{peptide} \times (\text{target} + \text{peptide/target} - \text{peptide}) \text{naive}}{100}.
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Antibodies and flow cytometry

For surface staining, after FcR blocking, the following mAb were used: CD4 (RMA4-4), CD8 (53-6.7), CD11b (M1/70), CD19 (6D5), CD45.1 (A20), CD45.2 (104), CD62L (Mel-14), Ly6C (AL21), and Ly6G (1A8) all from BD Biosciences. KLRG1 (2F1/ KLRG1) was from BioLegend. Dead cells were identified with LIVE/DEAD yellow fluorescent reactive dye (L34959) from Life Technologies and were excluded from analyses. MHC-peptide multimers were from ProImmune, Immudex or P. Guillaume (University of Lausanne, Lausanne, Switzerland). Multimer gating strategy used a dump gate (CD4, CD11b, and CD19) and excluded dead cells. Intracellular cytokines were stained after restimulation with the indicated peptides for 6 hours in the presence of Brefeldin A (GolgiPlug; BD Bioscience) with mAb

Figure 2.

Z12 vaccination induces a persistent CD8+ T-cell response with potent antigen-specific cytotoxicity. A, C57BL/6 mice were vaccinated (Vac) four times at the indicated week number (W0-W19) with adjuvanted Z12-OVALP. AT1 W24 they were adoptively transferred with target cells (ATT) for in vivo cytotoxicity testing and sacrificed 16 hours later. B, PBMC were isolated 7 days after vaccinations 2, 3, and 4, and OVA-specific CD8+ T cells were quantified by H-2Kb-OVA257–254 multimer staining, analyzed by flow cytometry. *P < 0.05, Mann–Whitney (n = 4 for each adjuvant combination listed). C, an in vivo killing assay performed 7 weeks postvaccination to assess cytotoxicity of the persistent T cells. Target cells were 4 × 10^6 OVA257–254-pulsed splenocytes (CFSE stained) mixed with 4 × 10^6 nonpulsed splenocytes (violet dye stained), both from CD451 C57BL/6 congenic mice. They were injected i.v. into vaccinated or control mice, which were sacrificed after 16 hours. Splenocytes were isolated, stained for CD45.1 and CD45.2, and then proportions of CFSE and violet stained adoptively transferred target cells were analyzed by flow cytometry, with in vivo vaccine-induced peptide-specific cytotoxicity calculated as described in Materials and Methods. Error bars, mean ± SD (n = 4 for each adjuvant combination and n = 4 for nonvaccinated mice).
to IFN-γ (XMG1.2), tumor necrosis factor (MP6-XT22), interleukin-4 (BVD4-1D11), and corresponding isotype controls (BD Biosciences). Anti-Granzyme B (GB11) and control isotype mAb were from Caltag. Anti-Foxp3 (FJK-16S) and control isotype were from eBioscience. Fixation and permeabilization was carried out using BD Biosciences (for cytokines) and eBioscience (for Granzyme B and Foxp3) kits according to the manufacturer’s instructions. Cells were analyzed using a Gallios flow cytometer (Beckman Coulter) and results were processed with FlowJo (TreeStar, Inc.) or Kaluza (Beckman Coulter) software.

CPP preparation and DC transduction
Recombinant Z12-MultiE was prepared as previously described (20). The constructions used in this study are represented in Supplementary Fig. S1. Coomassie staining and anti-His Western blot analyses were used to assess the protein purity (85%, Supplementary Fig. S2). After endotoxin removal, the level was between 0.3 and <0.01 EU/μg. Synthetic Z12 constructs were synthesized in-house. Endotoxin levels of the peptides were always <0.01 EU/μg. A variant of Z12 called Z13 was also synthesized, with a serine residue replacing the

Figure 3.
Z13 vaccination induces polyclonal immune response against gp100. A, C57BL/6 mice were vaccinated three times at the indicated weeks (W) with 10 nmol of the indicated CPP or short peptides, with Hiltonol and anti-CD40 as adjuvant. After 1 week restimulation with cognate peptide, gp100-specific CD8$^+$ T cells were quantified by H-2D$^b$-gp10025–33 multimer staining in the splenocytes and analyzed by flow cytometry (n = 4). B, the same experiment was performed with MPLA as adjuvant.
cysteine residue at position 186. Transduction of either BMDC or DC line 2114 was at 37°C for 4 hours. For removal of membrane-bound peptide prior to BMDC transduction, an acid wash (0.2 mol/L Glycine, 0.15 mol/L NaCl pH 3) was performed, essential to remove membrane-bound CPPs; omitting this step resulted in higher fluorescence (data not shown), similar to previous observations using trypsin (21). For the β-lactamase reporter system, after loading DC line 2114 cells with Z12-β-lactamase coumarincephalosporin fluorescein acetoxyethyl ester (CCF2-AM) substrate was added according to the manufacturer’s instruction (Life Technologies) and cells were analyzed by flow cytometry.

Vaccinations

Vaccines were prepared in PBS, with the peptide/protein component (10 nmol peptide/1–1.5 nmol protein) injected subcutaneously, with the addition of 100 μg anti-CD40 (FGK4.5; Bioxcell) where indicated. The TLR agonist adjuvants were administered intramuscularly, close to the peptide/protein injection site. Hiltonol (50 μg. Poly-ICLC) was provided by Oncovir, Inc.; MPL

![Image of Figure 4]

**Figure 4.** Z12 vaccination modulates the immune infiltrate of GL261-QUAD tumors and promotes accumulation of tumor-specific CD8⁺ T cells. A, C57BL/6 mice were intracranially implanted with 5 × 10⁶ GL261-QUAD cells, then vaccinated (Vac) twice at the indicated week (W) number with Hiltonol and anti-CD40 adjuvanted Z12-MultiE, and sacrificed at W4. PBMC and BILs were isolated and analyzed by flow cytometry. B, OVA-specific CD8⁺ T cells were quantified by H-2Kb-OVA257-254 multimer staining in PBMC and BILs (mice accumulated from two experiments, in total n = 6 for blood monitoring, n = 10 for BIL monitoring). C, Tregs, defined as Foxp3⁺ CD4⁺ T cells, were quantified in the CD4⁺ BILs (n = 4 control mice, n = 6 vaccinated mice). Error bars, mean ± SD. **, P < 0.01, unpaired t test. D, CD11b⁺ cells in the CD45⁺ BILs (n = 10 control mice, n = 10 vaccinated mice). Error bars, mean ± SD. **, P < 0.01, unpaired t test. E, CD11b⁺ cells in the CD45⁺ BILs (n = 5 control mice, n = 5 vaccinated mice) were stained for Ly6C and Ly6G expression (n = 6). Monocytic MDSCs are defined as CD45⁺CD11b⁺ Ly6ChighLy6Glow. Error bars, mean ± SD. *, P < 0.05, unpaired t test. F, correlation between proportions of antigen-specific CD8⁺ T cells (H-2Kb-OVA257-254 multimer⁺ CD8⁺) and CD11b⁺CD45⁺ cells in BILs (n = 10 control mice, n = 10 vaccinated mice). Spearman correlation coefficient r = −0.8632. Solid line and dashed lines represent regression line and 95% confidence bands, respectively. Regression analysis confirms that percentage of CD45⁺CD11b⁺ decreases when percentage of multimer⁺ CD8⁺ T cells increases (P = 0.0001).
Results and Discussion

Z12 efficiently transports a cargo into the cytosolic compartment of DCs

Induction of tumor-antigen specific CD8+ T cells is an essential requirement of a therapeutic cancer vaccine. To achieve this, the vaccine vector must deliver antigens into the cytosol, to access the processing pathway that leads to presentation of immunogenic peptides on MHC class I molecules. Because CPPs have the potential to transport cargoes across cellular membranes (12, 13), we tested whether the 42 residue Z12 fragment of the ZEBRA CPP (Supplementary Fig. S1) was able to deliver its cargo to the cytosol of DCs, and subsequently to induce CD8+ T cells.

Z12 efficiently transduced through the cell membrane of BMDCs (Supplementary Fig. S3A). Around 80% of the cells were transduced within 4 hours of incubation with 900 nmol/L fluorescein-conjugated Z12 (FAM-Z12-OVA), with 60% of DCs transduced with 300 nmol/L of the CPP. The retro-isomer of the well-described CPP derived from the HIV-Tat (Tat57-49) has been described to be a better transporter than Tat49-57 (22). We compared the transduction efficacy in BMDCs of Z13 and Tat49-57 at concentrations as low as 300 nmol/L (Supplementary Fig. S3B) and observed a faster and higher transduction efficacy of Z13 in this experimental setting. Although the mechanism of CPP-mediated transduction remains controversial, it is generally accepted that the dominant pathway is endocytosis (23). Endosomal escape is often essential for biologic activity of the compound transported and can be considered as the limitation of CPPs in drug development (23). To determine whether Z12 transported its cargo into the cytosol (rather than uniquely the endosomal compartment), we used the β-lactamase reporter system and its membrane-permeable substrate (CCF2AM) that allows monitoring of free cytosolic protein. This reporter system uses live cells and thereby avoids cell fixation artifacts that could wrongly indicate cytoplasmic uptake unrelated to CPP function (21). A direct correlation between protein concentration and transduction efficiency of the 36-kDa cargo was observed (data not shown), with β-lactamase activity detected with Z12 concentrations as low as 100 nmol/L. At 300 nmol/L of β-lactamase alone, no cleavage of CCF2AM was observed (data not shown).
Figure 7. Z13 and Z12 vaccination in a therapeutic setting results in prolonged survival in EG7, B16, and GL261 models. A, Kaplan-Meier survival curve of C57BL/6 mice implanted s.c. with $3 \times 10^5$ EG7-OVA tumor cells in the left flank and vaccinated in the right flank three times (d5, d13, and d24) with 10 nmoles of Z13-OVACD8CD4, the long peptide OVACD8CD4, or the two minimal short peptides OVACD8 and OVACD4 epitopes, with MPLA as adjuvant. Tumor size was measured with a caliper. Median survivals are indicated on the graph (m.s.). $P < 0.01$ (log-rank test, $n = 7$ per group). B, individual tumor growth curves for each group. C, the Kaplan–Meier survival curve of C57BL/6 mice implanted s.c. with $1 \times 10^5$ B16-LCMV-GP tumor cells in the left flank and vaccinated twice (d0 and d11) with 10 nmoles Z13-LCMVgp33 or a long peptide carrying the LCMV-GP33-41 epitope (LCMVgp33). Hiltonol and anti-CD40 were used as adjuvant. (Continued on the following page.)
These data contrast with previous studies reporting inefficient cargo transduction to the cytosol at low CPP concentrations or with large molecular cargoes (24). Thus, Z12 as a CPP offers the advantage of cytosolic antigen delivery without the necessity of complex modifications to improve endosomal escape (24).

In order to test whether cytosolic Z12 and its cargo efficiently entered the MHC class I–restricted processing pathway, we loaded BMDCs with a long peptide composed of 16 residues incorporating the OVA257–264 epitope fused to the CPP (Z12-OVALP; Supplementary Fig. S1). Processing and presentation of this MHC I–restricted epitope was monitored by measuring the proliferation of naive, monoclonal OVA257–264–specific CD8+ T cells from OTI TCR transgenic mice in vitro. Z12-OVALP–loaded BMDCs cross-presented the (OVA)257–264 epitope to OTI T cells, inducing proliferation that was comparable with either CPP–loaded BMDCs, or with BMDCs loaded with a saturating concentration of OVA257–264 peptide (Fig. 1A). This result confirms that Z12 is a CPP allowing the cytosolic delivery of an antigen that can then be processed and presented by MHC class I molecules. Although Z12 is highly efficient at transducing DCs delivering cargo antigens for MHC class I presentation, this has also been shown for other CPPs, including penetratin (25), LAH4 (26) and TAT (27). Indeed, based on such observations, some groups have used CPP-transduced DCs as vaccine vectors (28). However, for clinical development, protein vaccination rather than a DC-based vaccine is a more straightforward option.

To determine whether Z12 could promote cross-presentation in vivo, by transducing endogenous DCs, we used a stringent ex vivo assay detecting expansion of the rare endogenous, polyclonal, OVA-specific CD8+ T cells. Mice were vaccinated with Z12-OVALP combined with either the TLR3 agonist Hiltonol, the TLR4 agonist MPLA, or the TLR7 agonist R848 (Resiquimod) as adjuvants. In addition, Hiltonol was also tested in combination with an agonistic anti-CD40 antibody, which has been described to promote APC function (29). Z12-OVALP elicited cross-presentation in vivo when combined with any of these adjuvants, as evidenced by OVA257–264–specific CD8+ T–cell frequency measured by staining with H–2Kb–OVA257–264 multimer staining (Fig. 1B). Notably, this high magnitude immune response relied principally on Z12 action because vaccination with equimolar amounts of OVA257–264 long peptide, but without the CPP (OVALP; Supplementary Fig. S1), did not elicit detectable OVA-specific CD8+ T cells (Fig. 1B), even after a third vaccination (data not shown). The potency of Z12-OVALP to prime CD8+ T cells in vivo with only 10 nmol peptide is remarkable. Indeed, a comparable response could only be achieved by exploiting the unique synergistic properties of the α-Galactosylceramide adjuvant with the B subunit of Shiga toxin (8), or with TAT at a concentration of 100 μmol (30).

Z12 vaccination promotes persistent CD8+ T cells with potent cytotoxic function that home to the tumor site

Persistent effector cells are one of the critical requirements for a potent cancer vaccine. We therefore monitored the expansion and persistence of the pool of antigen specific T cells in the peripheral blood after multiple vaccinations (Fig. 2A). Multimer+ cells were either significantly increased when combined with MPLA as adjuvant, or maintained with four vaccinations over 6 months when combined with Hiltonol (Fig. 2B). This long-term persistence of OVA257–264–specific T cells without anti-CD40 is particularly noteworthy, because although vaccination with the OVA257–264 short synthetic peptide can be efficient, the response was reported to be short lived without anti-CD40 antibody (31). In our experiments, there was a reduction of the frequency of these OVA-specific CD8+ T cells in blood when anti-CD40 antibody was combined with Hiltonol (Fig. 2B), although this may be a consequence of the rat origin of this antibody. Despite the overall reduction in multimer+ cells, it is possible that anti-CD40 antibody induces more central or effector memory T-cell populations (32). Consistent with this hypothesis, we observed elevated proportions of a central memory-like population within the multimer+ CD8+ T cells (CD62L+ KLRG1−) in mice injected with Hiltonol and anti-CD40 antibody (Supplementary Fig. S4A); these cells would be expected to accumulate in secondary lymphoid tissues. As expected, most antigen-specific T cells displayed an effector phenotype with expression of KLRG1 and absence of the lymph node homing receptor CD62L (Supplementary Fig. S4B). Next, we assessed cytotoxic function of the persistent effector cells by performing an in vivo killing experiment 7 weeks after the last vaccination. Peptide-specific cytotoxicity of 47% to 90% was observed in the spleen after 16 hours (Fig. 2C and Supplementary Fig. S5). Thus, even mice with only modest proportions of specific CD8+ T cells measured by multimer analysis in the periphery (Fig. 2B) may exhibit robust cytotoxic function in the tissue (Fig. 2C).

Polyclonal immune response against gp100 self-antigen after Z13 vaccination

Next, we assessed whether our CPP could elicit an immune response against the self-/tumor-associated antigen gp100. On the basis of the results described in the previous section, we used three vaccinations when Hiltonol was used as adjuvant and four vaccinations with MPLA (Fig. 3A and B), using equimolar amounts of short peptides as control. At sacrifice, the gp100-specific immune response was first assessed by ELISPOT (data not shown), and then after one week of restimulation using MHC-peptide multimer staining. The short peptides did not elicit any gp100-specific immune response with any adjuvant used (Fig. 3), whereas all mice vaccinated with Z13-OVACD4gp100CD8 and Hiltonol were positive for gp100-specific T cells. Vaccination with Z13-OVACD4gp100CD8 and MPLA as adjuvant elicited still induced a gp100-specific immune response in 3 of 4 mice (Fig. 3B), but potency was less with this adjuvant. Thus, our CPP-based vaccine is able to elicit immune response against the challenging self-/tumor-associated-antigen gp100.
Z12 vaccination promotes effector CD8+ T cells homing to the tumor site

An effective cancer vaccine must induce T-cell homing to the tumor site. We tested whether this occurred after vaccination with the OVA-containing vector Z12-MultiE (Supplementary Fig. S1) in the orthotopic GL261 murine glioma model. Tumor-bearing mice were vaccinated twice with Z12-MultiE (Fig. 4A), and the BILs were then isolated and analyzed (Fig. 4B). Although a low but significant proportion of antigen-specific T cells was observed in peripheral blood (up to 1.2% of CD8+ T cells), there was a very high accumulation of antigen-specific T cells in the BILs, with a mean value of 39.8% of CD8+ T cells staining with the H-2Kb-OVA257-264-multimer. This efficient accumulation of T cells in the tumor bed could be related to the adjuvant we used, because Hiltonol was previously shown to promote T-cell homing to the brain in glioma-bearing mice through the expression of CXCL10 at the tumor site, and VLA-4 and CXCR3 on T cells (33, 34). In the GL261-QUAD glioma model, there is also a significant spontaneous antitumor immune response detectable in nonvaccinated mice (Fig. 4B). Nevertheless, after two vaccinations with Z12-MultiE the frequency of OVA257-264-specific cells CD8+ T cells in the BILs was doubled, with OVA-specific cells representing the majority of the CD8+ T cells in some vaccinated mice. Thus, as discussed for cytotoxicity measured in the spleen, immune responses measured in the target tissue (here the critical tumor site) may be orders of magnitude higher than those observed in the blood. However, although specific T cells infiltrating the tumor site are a necessary component of an efficacious cancer vaccine, they are not sufficient for antitumor function unless they can maintain their function within the immunosuppressive tumor microenvironment (19). We therefore assessed whether and how vaccination with Z12 influences the balance of tumor-infiltrating leukocytes. A significant change in the CD4:CD8 ratio was observed, with an increased infiltration of KLRG1+, granzyme B+ effector T cells (Supplementary Fig. S6A–S6C). Furthermore, Foxp3+ CD4+ regulatory T cells (Treg, Fig. 4C) were significantly reduced in BILs after Z12-mediated vaccination. In a recent study, poly-IC (as well as CpG-ODN) were reported to be favorable adjuvants to use with peptide vaccination to improve the antigen-specific T effector/Treg ratio in tumor-infiltrating lymphocytes using adoptively transferred monoclonal T cells (35). Thus, our results with a polyclonal immune response confirm the interest of a TLR3 agonist (Hiltonol in our study) in conjunction with the Z12 CPP-based vaccine. We validated the importance of these vaccination-induced phenotypic changes by measuring antigen-specific IFN-γ and TNFα expression by T cells in BILs from tumor-bearing mice; 53.3% of CD8+ T cells from vaccinated mice were cytokine positive, whereas only 7.3% of nonvaccinated mice expressed IFN-γ and/or TNFα (Supplementary Fig. S7A). Overall, the expression of effector cytokines was significantly lower in the control group as compared with the vaccinated mice (Supplementary Fig. S7B). Thus, although antigen-specific CD8+ T cells were clearly present in the BILs of nonvaccinated tumor-bearing mice (Fig. 4B), these cells were functionally impaired unless rescued by Z12-MultiE vaccination. An additional factor that could explain the potency of Z12 vaccination is the presence of protumoral myeloid cells within the brain tumor microenvironment. Indeed, we observed that the prominent CD45sh/CD11b+ population present in BILs of all nonvaccinated mice was often greatly diminished in vaccinated animals (Fig. 4D and Supplementary Fig. S6D); monocytic myeloid-derived suppressor cells (MDSC) were particularly affected by vaccination (Fig. 4E and Supplementary Fig. S6E). Intriguingly, the proportions of CD11bhigh cells were inversely correlated with H-2Kb-OVA257-264-multimer+ CD8+ T cells (Fig. 4F). Using the same glioma model, modulation of Gr1-expressing CD11b+ cells with anti-CD40 was recently reported to promote type I immunity and reduce Treg cells, but principally when combined with COX-2 blockade (36). Moreover, protumoral myeloid cells are considered to be associated with malignancy in human glioma (37). Our data suggest that a Z12-based vaccine adjuvanted with Hiltonol and anti-CD40 antibody impacts on protumoral myeloid cells in glioma. This mechanism may contribute to the robust and long-lasting effector response, and accumulation of tumor-specific CD8+ T cells within the tumor site that we observe.

Z12 delivers antigenic cargo to both MHC class I and MHC class II processing pathways

Optimal antitumor immunity is associated with both CD4+ and CD8+ T-cell responses (38, 39). To determine whether this could be achieved with our CPP-based vaccine, we used Z12 to deliver recombinant OVA protein (Z12-OVA, Supplementary Fig. S1) to BMDCs in a T-cell proliferation assay. We observed proliferation of both CD8+ and CD4+ OVA-specific T cells from OT-1 and OT-2 TCR transgenic mice, respectively. This indicates that Z12 is able to transport protein into both MHC class I and II processing compartments of BMDCs (data not shown). In order to target cancer cells with heterogeneous antigen expression, a cancer vaccine should ideally stimulate multiple specificities of tumor-specific CD4+ and CD8+ T cells; we therefore engineered Z12-MultiE carrying five different T-cell epitopes (Supplementary Fig. S1) to test this. We loaded BMDCs with Z12-MultiE and first assessed multi-epitopic presentation of the three CD8+ epitopes and two CD4+ epitopes in vitro. All epitopes were presented (albeit with varying efficiency), based on proliferative responses of TCR transgenic T cells specific for each of the antigens (Fig. 5A). The capacity to target multiple tumor antigens is essential to avoid outgrowth of antigen negative tumor cells, as has been reported in a clinical trial targeting a single epitope of EGFRVIII in glioblastoma (40).

To demonstrate Z12-mediated multi-epitopic cargo delivery to professional APCs in vivo, we adoptively transferred C57BL/6 mice (H-2Kb, Db, Iaα). Development of CPPs such as Z12 for human glioma therapy, with antigen cargoes restricted by different MHC alleles would be an attractive approach to circumvent the reported capacity of glioblastoma cells for MHC downregulation, in which selective HLA-A2 loss was observed in many tumors (41).

Vaccination of tumor bearing mice with Z12 induces therapeutic antitumor immunity with broad specificity

We evaluated polyclonal immune responses after vaccination with the Z12-MultiE recombinant protein. Vaccine-induced
responses were detected to all three CD8+ T cell epitopes incorporated in the Z12-MultiE protein (Fig. 6A), including the self/tumor-associated antigen gp100. The Z12-based vaccine elicited antigen-specific CD8+ and CD4+ T cells expressing TNFα and IFNγ (Fig. 6B; refs. 38, 42) but no IL-4 expression (data not shown), confirming induction of functional MHC class I- and II-restricted T cells with type I polarization. Evidence from animal models (38, 42) as well as clinical trials (43) points to the importance of type I immune response for brain tumor immunotherapy. To determine the therapeutic impact of our vaccination approach, we treated mice with three different tumor models: EG7 thymoma, B16 melanoma and GL261 astrocytoma. Although EG7 are OVA transfected, these tumors are aggressive (median survival of 20–23 days; Fig. 7A–C). We treated the mice in this challenging therapeutic window, vaccinations starting 5 days after tumor implantation. As expected from previous studies, the long peptide vaccine displayed a higher efficacy compared with short peptides, with an increase of the median survival to 24 days (44). Adding Z13 to the long peptide vaccine resulted in a significant increase of the median survival to 30 days (Fig. 7A). Similarly results were obtained by targeting the LCMV-GP33–41 viral antigen expressed by B16-LCMV-GP melanoma cells, with median survival extending from 31.5 days in nontreated mice to 38 days in mice receiving two vaccinations (Fig. 7C). Our CPP not only improved the modest efficacy of long-peptide based vaccines (Fig. 7A and B) but it also converted an inefficient protein vaccine into a therapy with highly significant clinical benefit (Fig. 7E and F). Moreover, in a challenging brain tumor model, we treated mice bearing orthotopic GL261-QUAD brain tumors with a Z12-based protein vaccine: this induced a very potent and durable therapeutic effect. Indeed, long-term survival up to 100 days was observed in vaccinated mice (Fig. 7G). Surviving vaccinated mice (7 of 8) sacrificed at this time point were totally asymptomatic and had no macroscopic evidence of tumor. Therefore, the in vitro predicted CPP properties of Z12, together with favorable modulation of multiple elements of the immune infiltrate that we observed, are fully validated in a therapeutic vaccination setting, with induction of durable antitumor immunity in an orthotopic glioma model.

Concluding Remarks

Therapeutic cancer vaccines must all exploit the potential of DCs to elicit tumor-specific T cells. Our design and development of a therapeutic cancer vaccine based on a Z12 or Z13 fused to a multi-epitopic protein has overcome many shortcomings of previous CPPs (28) or protein-based vaccines. Namely, the Z12/Z13 facilitates induction of a robust integrated immune response, comprising both CD4+ and CD8+ T cells. For anticancer immune responses, this is a major advance over the previously reported CD4+ T cell-bias for protein vaccines (45). Moreover, eliciting a multi-epitopic response with a protein rather than minimal peptide epitopes offers the opportunity for generating peptides binding to multiple MHC alleles. An additional strength of protein-based vaccines in contrast to short peptides is the requirement for antigen processing, thus reducing the risk of peripheral tolerance induction (2). Moreover, providing antigen in a protein form may promote sustained stimulation of T cells through intracellular antigen depots in DCs (5). On the basis of our in vitro data, Z12/Z13 is strongly immunostimulatory. Indeed, our Z12 CPP-based vaccine induces durable immune responses and shows therapeutic efficacy even in different tumor models, including a challenging orthotopic brain tumor model. Moreover, this robust therapeutic effect did not require additional treatment modalities such as checkpoint blockade, cell transfer, local delivery to the tumor bed, or approaches to dampen local or systemic immunosuppression (46). Although ultimate clinical efficacy may benefit from such approaches, it will be advantageous to move toward rapid clinical development with a vaccine vector that is sufficiently potent to have clinical impact even as a monotherapy. The unique properties of Z12/Z13 and its capacity to stimulate powerful and broad antitumor immunity against an aggressive glioma in the challenging site of the CNS confirms its potential to be a key component of next generation cancer immunotherapies.

Disclosure of Potential Conflicts of Interest

M. Derouazi is the CEO of Amal Therapeutics and has ownership interest (including patents) in Amal Therapeutics and patents related to Z12 (University of Geneva). A.M. Salazar is a CEO, Scientific Dir. at Oncovir, Inc. and has ownership interest (including patents) in the same. P.-Y. Dietrich has ownership interest in patents related to Z12 (University of Geneva) and is a consultant/advisory board member for Amal Therapeutics. P.R. Walker reports receiving a commercial research grant from Joint funding by Swiss Confederation Commission for Technology and Innovation and Amal Therapeutics; has ownership interest in patents related to Z12 (University of Geneva); and is a consultant/advisory board member for Amal Therapeutics. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: M. Derouazi, A.M. Salazar, P. Y. Dietrich, P. R. Walker Development of methodology: M. Derouazi, P.-Y. Dietrich Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W. Di Berardino-Besson, S. Hoepner, Y. Dufour, C. Yacoub Maroun, P.-Y. Dietrich, P.R. Walker Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Derouazi, W. Di Berardino-Besson, E. Belnoue, P. Teta, P.-Y. Dietrich, P. R. Walker Writing, review, and/or revision of the manuscript: M. Derouazi, E. Belnoue, S. Hoepner, A.M. Salazar, P.-Y. Dietrich, P.R. Walker Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W. Di Berardino-Besson, R. Walther, M. Benkhoucha, P. Teta, C. Yacoub Maroun, D. Martinvalet, P.R. Walker Study supervision: M. Derouazi, P.-Y. Dietrich, P. R. Walker

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Novel Cell-Penetrating Peptide-Based Vaccine Induces Robust CD4+ and CD8+ T Cell–Mediated Antitumor Immunity

Madiha Derouazi, Wilma Di Berardino-Besson, Elodie Belnoue, et al.


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