Research Article

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Efficient Induction of Antitumor Immunity by Synthetic Toll-like Receptor Ligand–Peptide Conjugates

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Abstract

Chemical conjugates comprising synthetic Toll-like receptor ligands (TLR-L) covalently bound to antigenic synthetic long peptides (SLP) are attractive vaccine modalities, which can induce robust $CD8^+$ T-cell immune responses. Previously, we have shown that the mechanism underlying the power of TLR-L SLP conjugates is improved delivery of the antigen together with a dendritic cell activation signal. In the present study, we have expanded the approach to tumor-specific $CD4^+$ as well as $CD8^+$ T-cell responses and *in vivo* studies in two nonrelated aggressive tumor models. We show that TLR2-L SLP conjugates have superior mouse $CD8^+$ and $CD4^+$ T-cell priming capacity compared with free SLPs injected together with a free TLR2-L. Vaccination with TLR2-L SLP conjugates leads to efficient induction of antitumor immunity in mice challenged with aggressive transplantable melanoma or lymphoma. Our data indicate that TLR2-L SLP conjugates are suitable to promote integrated antigen-specific $CD8^+$ and $CD4^+$ T-cell responses required for the antitumor effects. Collectively, these data show that TLR2-L SLP conjugates are promising synthetic vaccine candidates for active immunotherapy against cancer. *Cancer Immunol Res; 2(8); 756–64. ©2014 AACR.*

Introduction

Many therapeutic cancer vaccines are under clinical development. Although the first approved cancer immunotherapies increased survival in patients with prostate carcinoma and metastatic melanoma, the clinical effects observed are still moderate with a median increase in survival time of approximately 4 months (1, 2). We have previously demonstrated that long antigenic peptides can be used to induce strong T-cell–mediated antitumor immune responses. Vaccination trials with synthetic long peptides (SLP) in patients with human papillomavirus type 16 (HPV16)–induced premalignant high-grade vulvar lesions resulted in complete or partial clinical regression in >50% of the patients. Moreover, the strength of the vaccine-induced HPV-specific T-cell responses correlated with the clinical response in a variety of immune assays (3). This promising clinical study establishes a proof of concept for therapeutic vaccination with SLPs (4).

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Activation and induction of T-cell immunity rely on antigen processing and presentation by dendritic cells (DC). We showed in mouse models that SLPs required an obligatory DC processing step for efficient presentation of epitopes to the T-cell immune system (5, 6). In many clinical trials (including our own), the vaccine is applied in emulsions with mineral oils, resulting in local delivery and slow release of antigen, which seem to be important for the induction of effective antitumor immunity.

Despite our successful approach using peptides to treat premalignant lesions in patients, the efficacy of this vaccine can conceivably be further improved. SLP vaccination of patients with vulvar intraepithelial neoplasia (VIN) induced robust $CD4^+$ T-helper (Th) responses, but relatively low numbers of $CD8^+$ cytotoxic T lymphocytes (CTL) were elicited (3). Furthermore, patients with larger vulvar lesion sizes displayed higher frequencies of HPV16-specific $CD4^+CD25^+FoxP3^+$ T cells and a lower HPV16-specific IFNγ:IL10 ratio after vaccination (3).

Recent clinical studies applying peptide-based vaccination in incomplete Freund adjuvant (IFA) or Montanide ISA-51 demonstrated that the addition of Toll-like receptor ligands (TLR-L), either free Poly(I:C) or CpG, augmented T-cell responses in vaccinated patients with cancer (7, 8). We have studied different clinically feasible TLR-L targeting systems to overcome the suboptimal $CD8^+$ CTL induction, to counteract improper polarization and regulation of T-cell responses, and to enhance peptide delivery to DCs. Several studies show that random coupling of TLR-L to protein antigen is an attractive approach to deliver antigens to DCs and induces an antigen-specific T-cell immune response (9–13). Likewise, coupling of

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minimal CTL epitopes to a synthetic TLR2-L has been reported to be an efficient vaccine modality (14). In agreement with these studies, we found that targeting of well-defined SLPs via TLRs is a highly potent method for optimal DC activation, efficient and sustained antigen presentation, and strong induction of specific $CD8^+$ T cells (15–18). In our previous study, we have also examined the uptake and processing of the conjugated SLP in detail. A TLR2-L SLP conjugate was taken up independent of TLR2 expression, although TLR2 expression was necessary for DC maturation. Uptake was shown to be both clathrin dependent and caveolin dependent. Furthermore, processing of the conjugated SLP was dependent on endosomal acidification, transporter associated with antigen-processing (TAP) translocation, and proteasome-mediated cleavage, indicating that embedded MHC class I epitopes are readily processed and presented by DCs through the cross-presentation pathways (16).

In contrast with RNA- or DNA-based TLR-Ls such as Poly (I:C) and CpG, the well-defined lipopeptide Pam_3CSK_4 allows direct conjugation using in-line solid-phase peptide synthesis, which is of importance in view of GMP production. Moreover, in our previous study, we showed that Pam_3CSK_4-conjugated SLPs were similarly effective to CpG-conjugated SLPs in the induction of *in vivo* endogenous T-cell priming (16).

In this study, we investigated the potency of synthetic conjugates of the TLR2-L Pam_3CSK_4 and SLPs in inducing antitumor immune responses. Our data show that these improved vaccines induced more effective antitumor immunity against two different types of lethal experimental tumors than that induced by the mixtures of SLP and TLR2-L.

Materials and Methods

Synthesis of SLPs and TLR-L–coupled long peptides

Generation of Pam_3CSK_4 (TLR2-ligand) SLP conjugates was carried out as described previously (refs. 15, 16, and references therein). Three different TLR2-L SLP conjugates were tested in this study: conjugate of Pam_3CSK_4 and the ovalbumin-derived 24mer sequence DEVSGLEQLESIINFEKLAAAAAK (Pam–CTL conj) containing the CTL K^b epitope SIINFEKL, the 17mer Th ovalbumin epitope ISQAVHAAHAEINEAGR (Pam–Th conj), and the 19mer Moloney virus envelope (envH) Th epitope EPLTSLTPRCNTAWNRLKL (Pam–envH). The synthesis was done as described by Khan and colleagues (16) and only differs in the peptide sequences used for the generation of the conjugates. All three peptides are synthesized as C-terminal carboxamides. Where indicated, free SLPs consist of identical amino acid sequences as conjugated SLPs. See Fig. 1 for a schematic representation of applied TLR2-L SLP conjugates.

Cell lines and mouse strains

The D1 cell line, a long-term growth factor–dependent immature splenic DC line derived from B6 (H-2^b) mice, was cultured as described (19), except that the glutamine addition to the medium was replaced by glutamax (Gibco, Invitrogen Life Technologies; ref. no. 35050–038). D1 cells have shown behavior similar to that of freshly isolated bone marrow–derived DCs (20). EG7 is an EL4-derived thymoma cell line transfected with the ovalbumin (OVA) protein (21). B16-OVA is the murine B16F10 melanoma cell line transfected with the ovalbumin protein (22). RMA is a mutagenized derivative of RBL-5, a Rauscher MuLV–induced T lymphoma cell line of C57BL/6 origin. The T-cell receptor (TCR) transgenic mouse strain MolH was generated on a C57BL/6 background at the Leiden University Medical Center (LUMC). MolH mice express a TCR$\alpha\beta$ recognizing the envH peptide Moloney $env_{119–137}$ presented by MHC I-A^b, under the control of K^b-promoter/IgG enhancer element (C.M. Britten and colleagues; submitted for publication). Female C57BL/6 (B6, H-2^b) mice that weighed 20 to 25 g were purchased from Charles River Laboratories. The TCR transgenic OT1 mouse strain expresses a TCR recognizing the OVA-derived K^b-associated epitope SIINFEKL, whereas the $CD4^+$ T cells from the TCR transgenic OT2 mouse strain express a TCR that recognizes the OVA-derived Th epitope ISQAVHAAHAEINEAGR in association with I-A^b. Both OT1 and OT2 mice are generated on a C57BL/6 background. All mice were kept at the LUMC animal facility under SPF conditions and used at 6 to 10 weeks of age in accordance with national legislation and under the supervision of the animal experimental committee of the Leiden University.

T-cell proliferation *in vitro*

D1 cells (15,000) were added to each well of a 96-well flat-bottom plate. Antigenic compounds (TLR2-L SLP conjugates, mixture of free SLP and TLR2-L, SLP alone, or Pam_3CSK_4 alone) were added in triplicates to reach various final concentrations of 0.128 nmol/L to 3 μmol/L. After incubation at 37°C for 24 hours, the cells were washed with PBS to remove all remaining antigens from the supernatant. Enrichment was performed for either $CD4^+$ or $CD8^+$ T cells (BD IMag) on single-cell suspensions of splenocytes and lymph node cells from OT1, OT2, or TCR MolH mice. Between 50,000 and 75,000 enriched T cells were added to the D1 cells and incubated for 24 hours (OT1 and OT2) or 72 hours (TCR MolH) at 37°C. Supernatant was taken for measurement of cytokine concentrations by ELISA (BioLegend). After 24 or 96 hours of incubation, Brefeldin A (Sigma-Aldrich) was added to a final concentration of 7.5 μg/mL to enable accumulation of newly synthesized proteins. After overnight incubation at 37°C, intracellular cytokine staining was performed by fixating the cells for 20 minutes in Fixation Buffer (BD Pharmingen) on ice. After washing, cells were permeabilized using Perm/wash buffer (BD Pharmingen). Fluorescently labeled antibodies directed against CD4, CD8, IL2, IFNγ, and/or CD40L (eBiosciences) were added to the cells, and incubated for 30 minutes on ice. After washing twice, cells were taken up in Perm/wash buffer and analyzed by flow cytometry.

DC maturation

Murine D1 DCs (50,000 per well) in triplicates were seeded into a 96-well plate and incubated with each TLR2-L SLP conjugate separately, or with free ovalbumin- and envH-derived SLPs at a concentration of 2 μmol/L. After 48 hours

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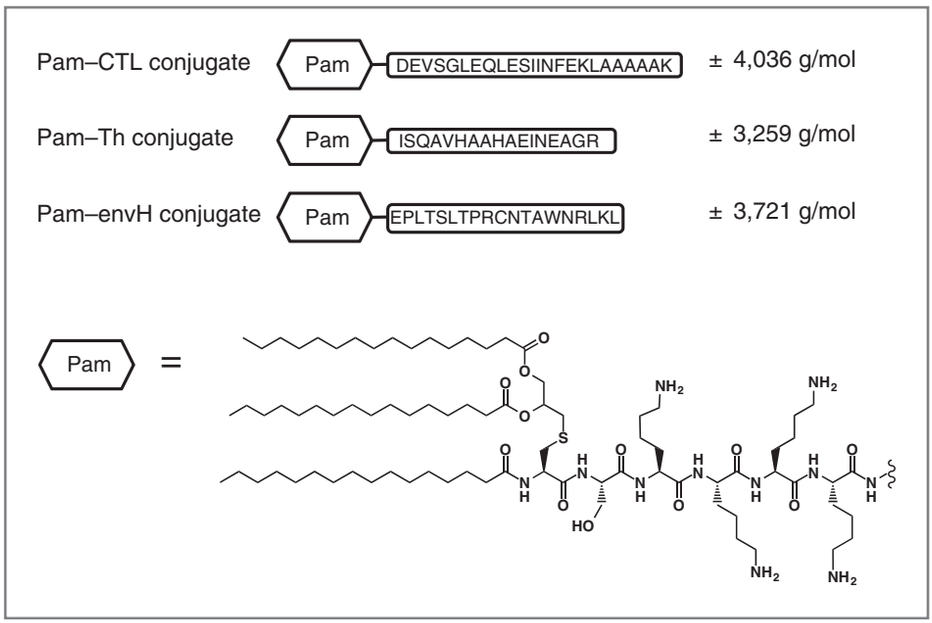

Figure 1. Schematic representation of applied TLR2-L SLP conjugates. The TLR-L peptide conjugates used for this study are represented as a cartoon. Pam–CTL conjugate and Pam–Th conjugate consist of Pam_3CSK_4 and synthetic peptide sequences derived from ovalbumin. The C-terminal part of the OVA CTL peptide is exchanged for alanine–lysine flanking (16). The Pam–envH conjugate consists of Pam_3CSK_4 and a Th peptide sequence derived from Moloney MuLV (21). Pam_3CSK_4 (structure is shown) is connected to the N-termini of the peptides via a peptide bond.

of incubation at 37°C, the supernatant was taken from the DCs and analyzed for IL12p40 production by ELISA (BioLegend). The cells were harvested and stained with fluorescently labeled antibodies directed against CD86, after which the expression levels were determined by flow cytometry.

Priming of endogenous naïve CD8$^+$ T cells

To analyze the endogenous CTL response, naïve C57BL/6 mice were injected s.c. with different amounts of the TLR2-L SLP conjugates (Pam–CTL conj; 0.04–5 nmol) or the free 24mer CTL SLP mixed with the free TLR2-L (Pam_3CSK_4; both at 1–40 nmol) in 200 μL of saline. Fourteen days later, mice received the same vaccination formulations. After 28 days, spleen cells were restimulated *in vitro* by coincubating 10×10^6 splenocytes with 1×10^6 Mitomycin C–treated (Kyowa; 50 μg/mL 1 hour at 37°C) and irradiated (4,000 rad) EG7 cell line (EL4-OVA), in the absence of additional cytokines. After 7 days, viable splenocytes were isolated over a Ficoll gradient and stained for H-2K^b tetramer (TM)-$OVA_{257–264}$, CD8β2 (clone 53–5.8), and propidium iodide to exclude dead cells.

Vaccination and tumor challenge

C57BL/6 mice were vaccinated either with a single dose of the TLR2-L SLP conjugates, or the SLP (5 nmol/mouse) in 50% (vol/vol) emulsion with IFA and PBS administered in a 0.2-mL depot s.c. (Fig. 5), or in a prime-boost setting (Fig. 4). Control groups were similarly injected with a 50% (vol/vol) emulsion of PBS and IFA. In the experiment depicted in Fig. 4, 5 nmol of Pam_3CSK_4 was coinjected in the group of mice injected with the single TLR2-L SLP conjugate, and 10 nmol of Pam_3CSK_4 in the group of mice injected with the mixture of free SLP to compensate for the 10 nmol of Pam_3CSK_4 injected in the group of mice injected with the two TLR2-L SLP conjugates at the same time. Fourteen days after immunization, mice were challenged subcutaneously with 6×10^4 B16-OVA melanoma cells in PBS containing 0.1% BSA (Fig. 4), or 2.5×10^3 RMA tumor cells in 0.2 mL PBS/0.1% BSA (Fig. 5).

To test the efficacy of the TLR2-L SLP conjugates, we have used, in contrast with our previous study (23), the subcutaneous route for inoculation of RMA cells and vaccinated with 10-fold lower doses of SLP. Tumor growth was followed over time, and mice were sacrificed when the tumor reached 1,000 mm^3, according to the regulations of our local ethical committee for animal experimentation.

Results

TLR2-L SLP conjugates induce T-cell activation *in vitro*

To evaluate the potency of TLR2-L SLP conjugates as a vaccine modality, we determined the capacity of three different conjugates in facilitating MHC class I and class II antigen presentation to naïve specific T cells *in vitro*. Schematic representations of the TLR2-L SLP conjugates used in this study are depicted in Fig. 1.

To confirm that the conjugates have retained the capacity to induce DC maturation, DCs were incubated with the conjugates used in this study for 48 hours, after which IL12p40 production by the DCs was measured by ELISA (Supplementary Fig. S1A). The expression of costimulatory markers was determined by flow cytometry (Supplementary Fig. S1B). All three conjugates showed capacities to induce maturation of DCs similar to that by free Pam_3CSK_4.

MHC class I antigen presentation was analyzed by loading DCs with either a TLR2-L SLP conjugate consisting of an ovalbumin-derived CTL-24mer and Pam_3CSK_4 (Pam–CTL conj; Fig. 2A), compared with a mixture of nonconjugated SLP and Pam_3CSK_4, to nonconjugated SLP only and Pam_3CSK_4 only (Fig. 2A). The next day, TCR transgenic OT1 CD8$^+$ T cells were added to the DCs after washing, and specific activation of these T cells was analyzed for the production of the type I cytokine

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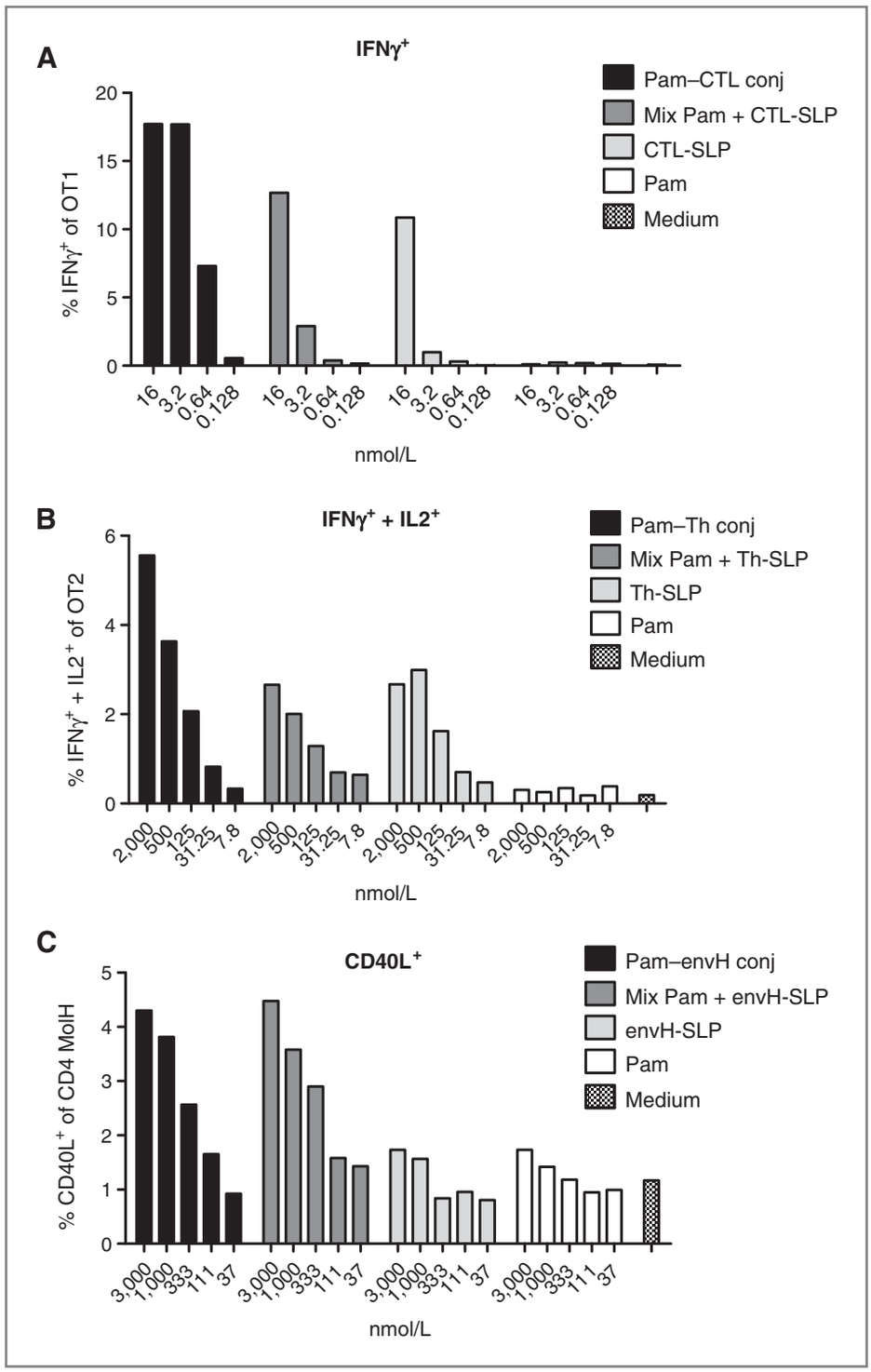

Figure 2. Activation of TCR transgenic T cells. A, $CD8^+$ T cells derived from an OT1 TCR transgenic mouse were cocultured with DCs loaded with a conjugate of Pam_3CSK_4 and OVA-derived CTL SLP (Pam–CTL conj), a mixture of Pam_3CSK_4 and OVA-derived CTL SLP (Mix Pam + CTL-SLP), the OVA-derived CTL SLP only (CTL-SLP), or Pam_3CSK_4 only (Pam). The amount of intracellular IFNγ was measured by ICS after 48 hours of coculture. B, $CD4^+$ T cells derived from an OT2 TCR transgenic mouse were cocultured with DCs loaded with a conjugate of Pam_3CSK_4 and OVA-derived Th SLP (Pam–Th conj), a mixture of Pam_3CSK_4 and OVA-derived Th-SLP (Mix Pam + Th-SLP), the OVA-derived Th-SLP only (Th-SLP), or Pam_3CSK_4 only. The percentage of $CD4^+$ T cells producing both IFNγ and IL2 was determined by ICS after 48 hours. C, $CD4^+$ T cells derived from a CD4 MolH TCR transgenic mouse were cocultured with DCs loaded with a conjugate of Pam_3CSK_4 and Moloney MuLV–derived Th-SLP (Pam–envH conj), a mixture of Pam_3CSK_4 and MuLV-derived Th-SLP (Mix Pam + envH-SLP), the MuLV-derived Th-SLP only (envH-SLP), or Pam_3CSK_4 only. The percentage of $CD4^+$ T cells producing CD40L was determined by ICS after 120 hours of coculture. Data shown are representative of at least three independent experiments.

IFNγ by intracellular cytokine staining (ICS). The conjugate induced more $IFN\gamma^+$ OT1 T cells than both the mixture of free SLP and TLR2-L, and the free SLP alone. Flow cytometry dot displays of cytokine-producing OT1 cells are shown in Supplementary Fig. S2A. The actual amount of secreted IFNγ measured by ELISA was higher in OT1 T cells that were activated by DCs loaded with the conjugate (Fig. S2B).

To evaluate the potency of a conjugate of ovalbumin-derived Th peptide and Pam_3CSK_4, DCs were similarly loaded either with a conjugated ovaH-$17mer_{323–339}$ (Pam–Th conj; Fig. 2B) or

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with control compounds. The next day, after the antigens were removed by washing, activation of TCR transgenic OT2 T-cells was determined. MHC class II antigen presentation was likewise improved when antigen was delivered to the DCs in conjugated form, giving rise to increased amounts of OT2 T cells that produce both IFNγ and IL2. (Fig. 2B). The conjugate induced CD40L (CD154) expression on $CD4^+$ T cells to a similar extent as that induced by the mixture, but higher than that induced by free SLP (Supplementary Fig. S2C).

A newly generated TCR transgenic mouse strain (MolH), which expresses a TCR recognizing the Moloney $env_{138-156}$ Th peptide (envH) presented by $I\text{-}A^b$ (C.M. Britten and colleagues; submitted for publication), was used to analyze the potency to facilitate MHC class II presentation of the Pam–envH conjugate. DCs were loaded with a conjugate of envH-SLP and Pam_3CSK_4 (Pam–envH conj; Fig. 2C) or control compounds as described above. The next day, splenocytes from the MolH mouse enriched for CD4 were added to the DCs and cocultured for 5 days. We observed that conjugating Pam_3CSK_4 to an envH-19mer SLP in this model strongly induced $CD4^+$ T-cell activation as shown by the accumulation of CD40L. The $CD4^+$ MolH T cells showed similar activation when cocultured with DCs loaded with either the conjugate or the mixture of free SLP and Pam_3CSK_4. Analysis of IL2 secretion after 4 days of culture showed similar results (Supplementary Fig. S2D). Taken together, these data show that the conjugated SLPs used in this study are taken up and processed by DCs and efficiently induce $CD8^+$ and $CD4^+$ T-cell activation. Both conjugates of the ovalbumin-derived SLPs induced higher Th1-type cytokine-producing cells.

Effective *in vivo* priming of $CD8^+$ T cells by TLR2-L SLP conjugates

To determine the capacity to prime naïve $CD8^+$ T cells, groups of mice were immunized with increasing doses (0.04–5 nmol) of the Pam–CTL conjugate containing the ovalbumin-derived CTL epitope SIINFEKL (Conjugate; Fig. 3A) or increasing doses (1–40 nmol) of a mix composed of the same non-conjugated long 24mer CTL peptide and equimolar amounts of the free TLR2-L Pam_3CSK_4 (Mixture; Fig. 3A), and the resulting frequency of vaccine-induced $CD8^+$ T cells was determined by K^b-SIINFEKL tetramer staining. Mice were injected s.c. with different doses of the conjugated and nonconjugated vaccines in saline, and boosted with the same formulations 14 days later. The splenocytes were harvested 2 weeks after the last immunization and restimulated for 1 week, followed by immunologic analysis. As shown in Fig. 3A and B, induction of specific $CD8^+$ T cells was detected in mice injected with 0.2 nmol of the TLR2-L SLP conjugate, whereas even 40 nmol of the nonconjugated SLP did not lead to a detectable response. Similarly, direct *ex vivo* ICS analysis of spleen-derived $CD8^+$ T cells revealed a higher percentage of IFNγ-producing $CD8^+$ T cells in mice vaccinated with the conjugate (Fig. 3C). These results show the strong CTL priming potency of the TLR2-L SLP conjugate, which not only induces higher numbers of specific CTL, but also requires antigen doses one to two logs lower for induction of detectable CTL numbers than those for the nonconjugated peptide.

Induction of antitumor immunity by TLR-L peptide conjugates

To investigate the potency of the TLR2-L SLP conjugates in protecting against the outgrowth of the aggressive melanoma tumor cell line B16-OVA (Fig. 4), mice were injected s.c. with 5 nmol of the conjugate containing the SIINFEKL CTL epitope mixed with 5 nmol of the conjugate containing the ovaH Th epitope (Pam–CTL conj + Pam–Th conj) or the single conjugates and mixture controls. All vaccines were administered in IFA emulsions. After 2 weeks, mice were boosted with the same vaccines. Again, 14 days later, all mice were challenged with B16-OVA melanoma cells by s.c. injection. As shown in Fig. 4A, the average onset of tumor growth was significantly delayed in the group of mice receiving the Pam–CTL + Pam–Th conjugates compared with those receiving the mixture of free SLPs and Pam_3CSK_4 (Fig. 4A; $P < 0.001$), whereas vaccination with either one of the conjugates only induced a minor delay in tumor outgrowth (Fig. 4B). As shown by the survival curves in Fig. 4C, this tumor model required the inclusion of conjugates containing both a CTL and a Th epitope into the vaccine to induce an optimal antitumor immune response. The reduced tumor growth resulted in a long-term survival rate of 66% (4 of 6 mice) in the group of mice injected with the two TLR2-L SLP conjugates. Strikingly, the vaccine composed of the two free SLP and Pam_3CSK_4 only protected 1 of 7 mice against tumor outgrowth (Fig. 4C; $P = 0.009$). A moderate survival rate (15%–25%) was observed in the group of mice injected with the individual conjugates (Fig. 4C). These data are in line with our previous published results and those of others, showing that it is optimal to provide the antigen and the adjuvant in a conjugated form (8, 12, 14).

In an independent tumor model, we investigated the capacity of the TLR2-L SLP conjugates in protecting mice against the outgrowth of murine leukemia virus (MuLV)–induced lymphoma. Viral tumor antigens are becoming increasingly important as targets for cancer immunotherapy as, according to numbers released by the World Health Organization, infectious agents are now estimated to be the cause of approximately 18% of all cancers worldwide (24). We have shown previously that controlling tumor outgrowth of Moloney MuLV leukemia is critically dependent on the induction of $CD4^+$ T cells (19). Therefore, we used the TLR2-L SLP conjugate of Pam_3CSK_4 conjugated to Moloney envH-SLP, as shown in Fig. 2C. Groups of mice were injected with the Pam–envH TLR2-L SLP conjugate, the free nonconjugated SLP (envH-SLP) either alone or together with Pam_3CSK_4. All vaccines were administered in IFA emulsions. After 14 days, mice were challenged with the MuLV-induced tumor cell line RMA. As shown in Fig. 5A, vaccination with the conjugate significantly delayed tumor outgrowth compared with that in mice vaccinated with the free SLP ($P = 0.038$). Seventy days after tumor challenge, 5 of 8 mice in the group vaccinated with the conjugate were tumor free, whereas only 2 of 8 mice in the mixture group and 1 of 8 mice in the SLP group remained tumor free (Fig. 5B). All mice in the group vaccinated with vehicle alone grew large tumors within 23 days after tumor injection and had to be sacrificed.

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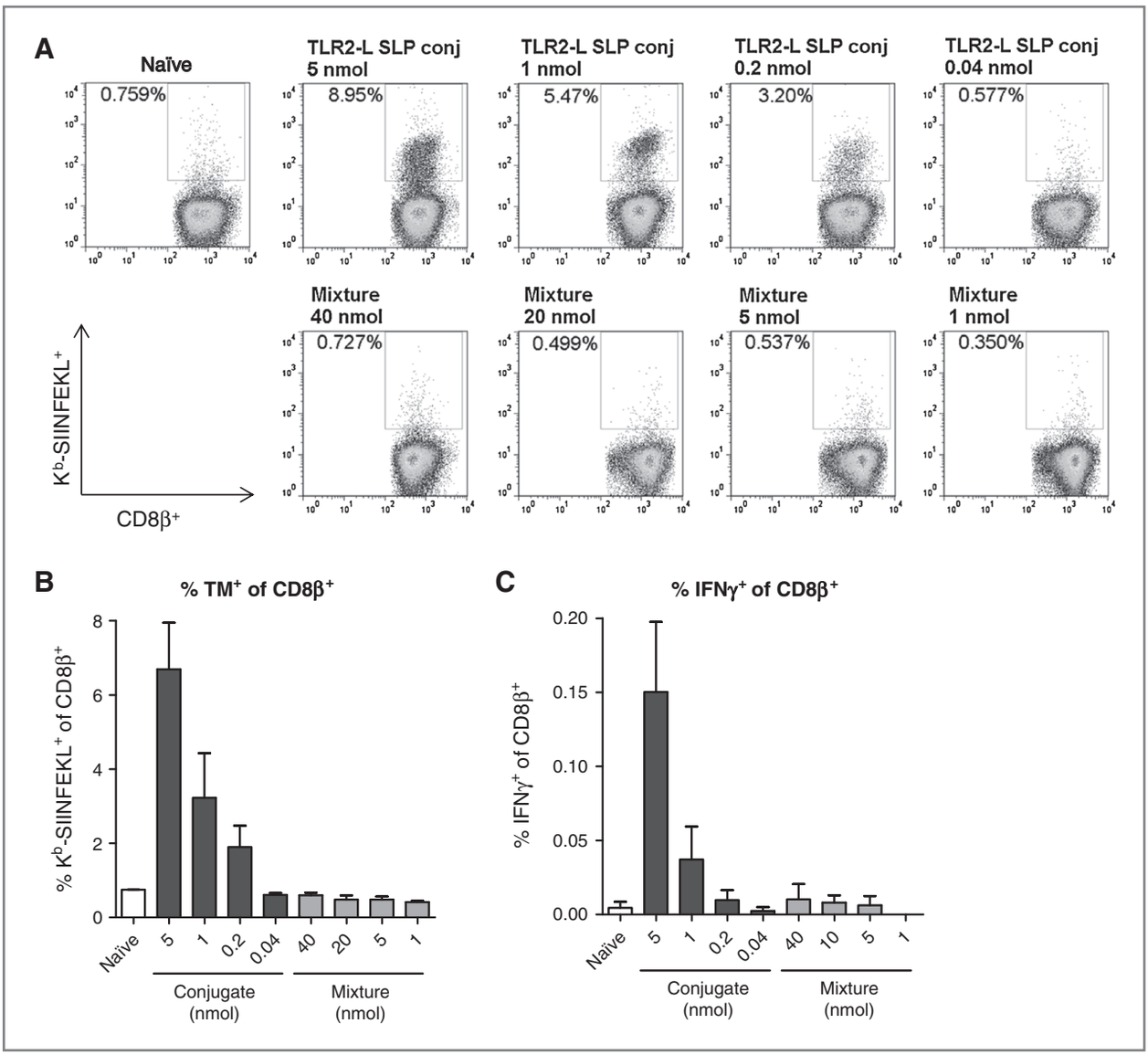

Figure 3. Effect of vaccine dose on induction of specific $CD8^+$ T cells. A, a representative FACS plot of each of the groups is shown; cells were gated on $CD8\beta^+$ and the percentage given in the top of the right quadrant are the percentages of tetramer positive cells of total $CD8^+$ T cells. B, naïve C57BL/6 mice (4 mice per group) were injected and boosted with either Pam_3CSK_4–24mer CTL conjugated SLP (conjugate) or Pam_3CSK_4 mixed with 24mer CTL-SLP (mixture), at the indicated dose. After restimulation *in vitro*, cells were analyzed for the presence of $CD8\beta^+$ T cells capable of interacting with H-$2K^b$-$OVA_{257–264}$ tetrameric complexes. The *y*-axis displays the percentages of tetramer-positive cells in total $CD8\beta^+$ cells. Similar results were obtained in two independent experiments. C, percentage of $CD8^+$ T cells producing IFNγ in the spleen of vaccinated mice, measured by ICS *ex vivo*.

Discussion

We have shown previously that synthetic TLR-L peptide conjugates containing a CTL epitope are efficiently targeted to DCs *in vivo* and are superior in inducing a strong $CD8^+$ T-cell immune response (15, 16). In the present study, we report the effectiveness of the TLR2-L SLP conjugates in two aggressive tumor models.

In vitro T-cell activation analysis (Fig. 2) showed strong T-cell responses upon exposure to conjugate-loaded DCs. $CD8^+$ T cells produce much larger amounts of IFNγ in response to the conjugate than to a mixture of the same free ligand and SLP. Naïve OT2 $CD4^+$ T cells showed enhanced production of cytokines IFNγ and IL2 upon stimulation with conjugates. This enhanced IL2 production may explain the importance of $CD4^+$ T-cell help in the B16-OVA melanoma model (Fig. 4), as $CD4^+$ T cells play an important role in the recruitment and cytolytic function of tumor-infiltrating $CD8^+$ T cells (25). Although $CD4^+$ MolH T-cell activation *in vitro* is similarly enhanced by conjugates or mixtures and the dual cytokine effect was not observed (Fig. 2C), conjugation of a Th-SLP does have a large impact on tumor control *in vivo* (Figs. 4 and 5). This discrepancy may be

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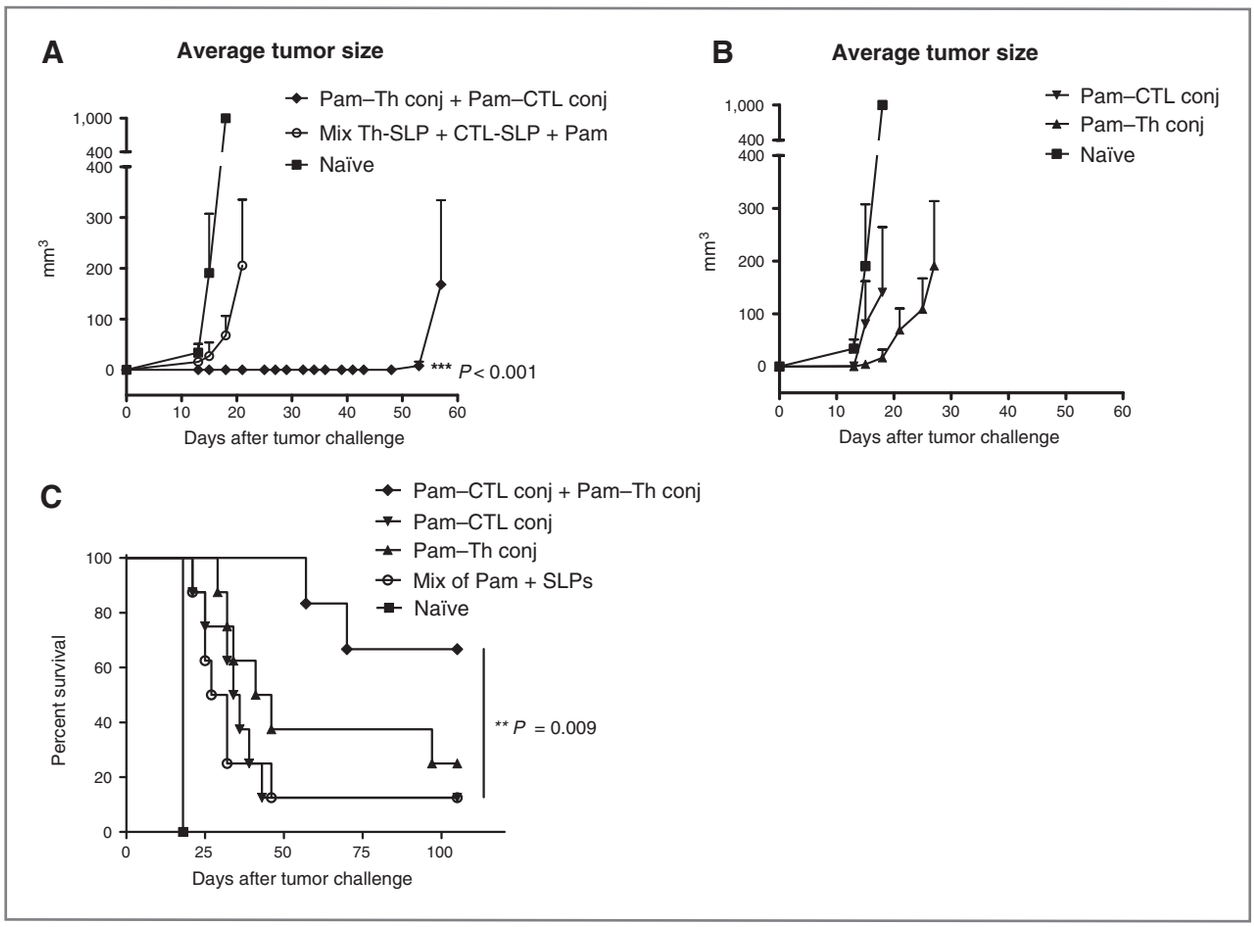

Figure 4. Tumor-specific TLR2-L SLP conjugate vaccination increases long-term survival of mice challenged with tumor cells. Naïve C57BL/6 mice were injected s.c. with IFA (naïve; $n = 4$), 5 nmol TLR2-L SLP conjugate containing the SIINFEKL CTL epitope mixed with 5 nmol TLR2-L SLP conjugate containing the ovaH Th epitope (Pam–CTL conj + Pam–Th conj; $n = 6$), 5 nmol of 24mer containing the SIINFEKL CTL epitope mixed with 5 nmol of 17mer containing the ovaH Th epitope and 10 nmol of Pam_3CSK_4 (Mix of Pam + SLPs; $n = 7$), TLR2-L SLP conjugate containing the SIINFEKL CTL epitope (Pam–CTL conj) and 5 nmol of Pam_3CSK_4 ($n = 8$), and 5 nmol of TLR2-L SLP conjugate containing the ovaH Th epitope (Pam–Th conj) and 5 nmol of Pam_3CSK_4 ($n = 8$). Data from one representative experiment are split up in two plots, showing the average tumor sizes per group until the first tumor in a group reached 1,000 mm^3 (A and B) and survival curves of treatment groups (C). Significance of differences (log-rank test): Pam–CTL conj + Pam–Th conj versus naïve, $P = 0.0027$; Pam–CTL conj + Pam–Th conj versus Mix of Pam + SLPs, $P = 0.009$; Pam–CTL conj + Pam–Th conj versus Pam–CTL conj, $P = 0.008$; Pam–CTL conj + Pam–Th conj versus Pam–Th conj, $P = 0.085$. Similar results were obtained in two additional independent experiments.

explained by the finding that conjugated SLPs are efficiently targeted to DCs *in vivo*, associated with the formation of an antigen depot within DCs (17), and thus antigen presentation can be sustained over a longer period. This may be crucial for DC migration and effective T-cell priming in the lymphoid organs (18). This property of conjugated SLP does not fully surface in short-term *in vitro* T-cell activation assays, in which all compounds are present in one well. The specific *in vivo* targeting properties of the conjugates may therefore play an important role. As a consequence, the vaccine dose can be reduced and still evoke efficient antitumor immunity. Accordingly, we show that immunization with the conjugate induced T-cell responses starting from doses as low as 0.2 nmol, whereas no specific T-cell responses could be detected after immunizing mice with a 40-nmol dose of free TLR-L and SLP (Fig. 3).

The aforementioned SLP vaccine consisting of overlapping HPV16 E6- and E7-derived SLPs has proved to be clinically effective in the treatment of premalignant $HPV16^+$ vulvar lesions. Because all possible Th and CTL epitopes of the E6 and E7 protein are present in the formulation, the SLP vaccine can potentially induce a specific immune response in an outbred population. However, as opposed to premalignant lesions, cancer often coincides with a more immunosuppressive microenvironment within and around the tumor, characterized by prevalence of immunosuppressive cytokines and high infiltration of regulatory T cells (Treg; refs. 26, 27). As supported by other studies, we believe that coadministration of SLPs with a strong TLR-L may enhance the cellular immune response in such a way that this tolerance barrier can be overcome (28, 29). Our data show that the potency of SLPs to trigger $CD8^+$ as well as $CD4^+$ T-cell responses, which are both critical for effective

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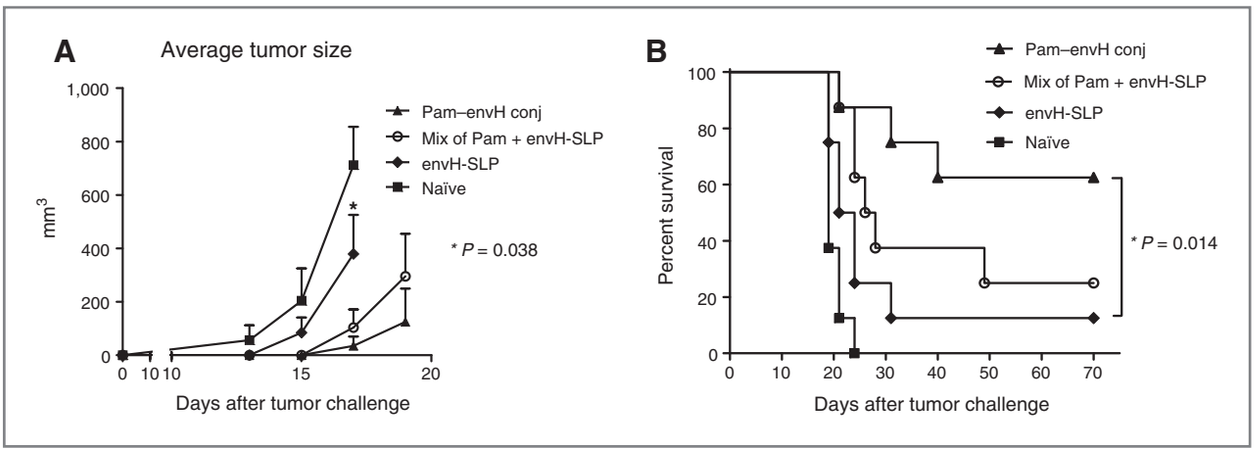

Figure 5. TLR2-L SLP conjugates induce antitumor responses against a natural viral antigen. Naïve C57BL/6 mice were vaccinated s.c. on day 14 with IFA (naïve), 5 nmol of Pam_3CSK_4-envH Th conjugate (Pam–envH conj), 5 nmol of MolH-Th SLP mixed with 5 nmol of Pam_3CSK_4 (Mix Pam + envH-SLP), or 5 nmol of MolH-Th SLP (envH-SLP) and challenged with 2.5×10^3 RMA cells s.c. at day zero. A, the tumor size of each individual mouse was monitored every other day and the average tumor size per group is depicted until the first tumor in a group reached 1,000 mm^3 ($n = 8$/group). B, vaccination with tumor-specific Th SLP conjugated to Pam_3CSK_4 increases long-term survival of mice challenged with tumor cells; the percentage of surviving mice is depicted. Significance of differences (log-rank test): Pam–envH conj versus naïve, $P = 0.0002$; Pam–envH conj versus Mix of Pam + envH-SLP, $P = 0.093$; and Pam–envH conj versus envH-SLP, $P = 0.014$. Data shown are from one representative experiment out of three independently performed experiments with similar outcomes.

antitumor immunity, is not only maintained but markedly increased by the TLR2-L SLP conjugates. The combinatory effect of integrated $CD8^+$ and $CD4^+$ T-cell response induction, together with the strong TLR signaling, thus constitutes a highly efficient vaccination format.

Triggering of TLRs has been implicated in both beneficial and detrimental effects in antitumor immunity, with the detrimental effects mostly being inflicted by direct interactions of TLR-Ls with TLRs expressed on tumor cells (29–31). We and others have observed strong immune-potentiating effects of TLR2 triggering (16, 32) as well as its effects in tumor eradication (14, 33). Indeed, the *in vivo* experiments in the present study show strong induction of antitumor immunity when a TLR2-L is covalently conjugated to the antigen (Figs. 3C, 4 and 5). Although TLR2 has been reported to be expressed on Tregs, we have not observed any suppressive effects of our TLR2-L SLP conjugates. Even so, TLR2 triggering on Tregs by TLR2-L Pam_3CSK_4 has been shown to transiently abrogate the suppressive capacity of naturally occurring Tregs (34), suggesting that Pam_3CSK_4 may have additional beneficial effects in immune therapy of tumors.

In summary, this study shows that synthetic TLR-L SLP conjugates are superior to free SLP mixed with free TLR-L in inducing immunity against two aggressive tumor cell types. These findings can be explained by the targeting of TLR2-L conjugates to antigen-presenting cells *in vivo*, combined with the delivery of a Th1-type maturation signal. Upon uptake, conjugated SLPs are efficiently processed and presented to T cells, while codelivering a strong T-cell activating signal. Because TLR-Ls are well-defined and nontoxic chemical compounds, they lend themselves readily for GMP production. Chemically well-defined TLR2-L SLP conjugates are therefore promising vaccine candidates in a variety of clinical settings ranging from persistent virus infections to cancer. In the coming years, this concept will be tested in humans. We will perform a phase I/II clinical trial using TLR2-L SLP conjugates in patients with HPV16-induced cancer to determine their safety profile and the optimal dose range for T-cell immunogenicity and proper T-cell polarization.

Disclosure of Potential Conflicts of Interest

C.J.M. Melief is chief scientific officer (CSO) of the biotech company ISA (Immune System Activation), aiming at development of synthetic peptide-based cancer vaccines, including conjugates between a proprietary TLR ligand and synthetic long peptides. As CSO, C.J.M. Melief receives a salary from ISA and is in possession of stock appreciation rights. No potential conflicts of interest were disclosed by the other authors.

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