Photosensitizer and Light Pave the Way for Cytosolic Targeting and Generation of Cytosolic CD8 T Cells Using PLGA Vaccine Particles

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J Immunol published online 27 May 2015
http://www.jimmunol.org/content/early/2015/05/27/jimmunol.1500431
Photosensitizer and Light Pave the Way for Cytosolic Targeting and Generation of Cytosolic CD8 T Cells Using PLGA Vaccine Particles

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The generation of CTLs is crucial in the immunological fight against cancer and many infectious diseases. To achieve this, vaccine Ags need to be targeted to the cytosol of dendritic cells, which can activate CD8 T cells via MHC class I (MHCI). Therefore, such targeting has become one of the major objectives of vaccine research. In this study, we aimed to bypass the unwanted and default MHCI presentation by using a photosensitizer that, upon light activation, would facilitate cytosolic targeting of codelivered Ag. Poly(lactide-co-glycolide) microparticles ~1 μm size were loaded with OVA and the photosensitizer tetraphenyl chlorine disulphonate (TPCS2a) and administered intradermally in mice, which were illuminated 1 d later for activation of the photosensitizer. Immunization in the presence of TPCS2a significantly increased activation of CD8 T cells compared with immunization without TPCS2a and as measured by CD8 T cell proliferation, production of proinflammatory IFN-γ, TNF-α, and IL-2, and prevention of tumor growth. Cytotoxicity was demonstrated by granzyme B production in vitro and by in vivo killing of CFSE-labeled targets. CD4-dependent Ab responses were abrogated in mice immunized with TPCS2a-containing particles, suggesting that photosensitization facilitated a shift from default MHCI toward MHCI Ag presentation. Hence, vaccine particles with Ag and photosensitizers proved an effective vehicle or adjuvant for stimulation of CTLs, and they may find potential application in therapeutic cancer vaccination and in prophylactic and therapeutic vaccination against intracellular infections. The Journal of Immunology, 2015, 195: 000–000.

Several approaches have been proposed for the delivery of Ags and drugs to the cytosol, such as cell-penetrating peptides that carry their cargo across the plasma membrane (2, 3), pH-sensitive and fusogenic liposomes that break up phagosomes (4–6), micelle-based immune-stimulating complexes that may facilitate Ag cross-presentation (7, 8), and recombinantly modified viruses (9–11) and bacteria (12–15) that trigger CTLs. Also, microparticles or nanoparticles made from biodegradable polymers, such as poly(lactide-co-glycolide) (PLGA), have been investigated for the cytosolic delivery of vaccines. PLGA particles may enable controlled and sustained Ag release and can, as particles in general, deliver large amounts of Ag to APCs at the single-cell level, thereby increasing the probability of reaching MHCI (19–23).

We recently suggested a photochemical approach for cytosolic targeting of Ags. By using a photosensitizer and light, soluble protein could be translocated to the cytosol of APCs in vitro (24) and in vivo (25, 26). This so-called “photochemical internalization” (PCI) represents a novel technology for controlled permeabilization of endosomes (27). PCI is based on photosensitizers that translocate from the plasma membrane to the endosomal membrane after endocytosis (Fig. 7). Upon subsequent light activation, the photosensitizer generates free radicals and reactive oxygen species, which rupture the endosomes and release the endocytosed material, such as Ags and adjuvants, into the cytosol. In the cytosol, Ags can enter the MHCI presentation for stimulation of CD8 T cells (28). In this study, we combined PCI technology with PLGA-based vaccine particles. The microparticles, loaded with an Ag and a photosensitizer, were tested in a mouse model for their capacity to trigger Ag-specific CD8 T cell responses. The hypothesized benefit of vaccine particles over that of soluble vaccines, apart from their better recognition by the APCs, is that both Ag and photosensitizer are...
jointly targeted to the APCs. This is important because the effect of PCI-based vaccines assumes that photosensitizer and Ag are taken up by the same individual cell that is subsequently illuminated for cytosolic delivery of the Ag. To our knowledge, this is the first study to demonstrate that PLGA vaccine particles can be efficiently used to target Ag to the cytosol for MHCI-restricted and strong stimulation of cytoxic CD8 T cells that prevent tumor growth.

Materials and Methods

Materials

PLGA 50:50 (Resomer RG503) was obtained from Evonik Industries. OVA, dichloromethane, DMSO, and chloroformic diacetate were obtained from Sigma-Aldrich (Buchs, Switzerland). Poly(vinyl alcohol) (PVA; Mowiol 8-88) was from Karuna Europe (Hattenheim, Germany). The photosensitizer tetraphenyl chlorin disulfonate (TPCS2a; Amphiix) was kindly provided by PCI Biotech (Lyssaker, Norway).

For immunization, female C57BL/6 mice were purchased from Harlan (Horst, The Netherlands) and used at 6–10 wk of age. Rag2-deficient OT-I mice, transgenic for the TCR that recognizes the MHCI H-2Kb epitope OVA257–264 (SIINFEKL), were originally purchased from Taconic (Micro BCA kit; Thermo Scientific Pierce). TPCS2a, because of fluorescence interference, by precipitation with Proteo-Solv (Infinite M200 PRO; Tecan). When OVA and TPCS2a were coencapsulated into PLGA microparticles, efficiencies (%) were calculated as (measured compound concentration/theoretical compound concentration) × 100.

Immunization and photosensitization of mice

Unless otherwise described, 2 × 10^6 purified and BCC-free spleen cells from female Rag2/OT-I mice were adoptively transferred by i.v. injection into recipient female C57BL/6 mice 1 d prior to immunization. Freeze-dried PLGA microparticles were carefully reconstituted in purified water on the day of immunization; formulations were kept light protected and used within 10 min of preparation. Mice were immunized intradermally in the abdominal region. Two injections (50 μl each) were given, and 25G needles were used. OVA was tested at 10–30 μg/dose, which corresponds to TPCS2a doses of 70–210 μg for the combined OVA and TPCS2a particles. Eighteen hours after immunization, the mice were anesthetized i.p. with ketamine (25 mg/kg body weight) and xylazine (4 mg/kg) and placed for 6 min on the light source (4.86 J/cm²). Blood was harvested at various time points for analysis of Ag-specific T cells by flow cytometry and for preparation of serum for later analysis of Abs. At the end of the experiments, mice were euthanized, and heart blood was isolated for analysis of anti-OVA IgGs by ELISA, whereas the splenocytes were analyzed ex vivo for Ag-specific T cell responses by flow cytometry and ELISA.

Analysis of immune responses by flow cytometry and ELISA

The proliferation and activation status of OVA-specific CD8 T cells in blood or spleen cells was monitored by staining the cells with PE-labeled H-2Kb–SIINFEKL, Pro5 pentamer (ProImmune, Oxford, U.K.) and anti-CD8 and anti-CD44 fluorescent Abs for analysis by flow cytometry, as previously described (25). Intracellular cytokine staining was performed after stimulation of the cells with 5 μg/ml SIINFEKL (6 h) and 5 μg/ml brefeldin A (last 4 h). The cells were fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences, Basel, Switzerland), according to the manufacturer’s instructions, and stained with anti-IFN-γ and anti–TNF-α for 35 min. All Abs were from eBioscience (Vienna, Austria). The stained cells were acquired using a FACSCanto (BD Biosciences, San Jose, CA) and analyzed using FlowJo 8.5.2 software (TreeStar, Ashland, OR). H-2Kb–SIINFEKL Pro5 pentamer–staining cells were gated on lymphocytes based on forward and side scatter properties, CD8, and CD44. Cytokine-producing cells were gated on lymphocytes based on forward and side scatter properties and on CD8. For analysis of cytokine secretion by ELISA, 2 × 10^6 splenocytes were restimulated in round-bottom 96-well plates with 0.1 μg/ml SIINFEKL, and supernatants were collected and analyzed for IL-2 (24 h) or IFN-γ, TNF-α, and granzyme B (96 h) secretion by ELISA (Ready-Set-GO! kits; eBioscience). For analysis of OVA-specific Abs in blood serum, MaxiSorp ELISA plates were coated with 2 μg/ml OVA. IgG1 and IgG2c were detected with biotin-conjugated rat anti-mouse Abs (BD Pharmingen, San Diego, CA). The plates were developed with streptavidin-conjugated HRP and TMB substrate (eBioscience). The OD at a given serum dilution was measured.

Analysis of in vivo cytotoxicity

In vivo cytotoxicity was analyzed on CFSE-labeled and SIINFEKL-pulsed naive spleen cells, as described (25). Briefly, mice adoptively transferred with 2 × 10^6 OT-I cells were immunized with PLGA–OVA or PLGA–OVA–TPCS2a or were left untreated; light treatment was done as described above. After 6 wk, 5 × 10^6 CFSE-labeled and SIINFEKL-pulsed and 5 × 10^6 CFSE-labeled and nonpulsed spleen cells were injected i.v. After 18 h, the mice were bled, and the cells were analyzed by flow cytometry for CFSE. Specific lysis or cytotoxicity was calculated for the cells using the following formula: percentage specific cytotoxicity = 100 – [100 × (CFSElo/CFSEhi)].

Analysis of tumor growth

Three weeks after the injection of CFSE-labeled cells, the same mice were challenged with 500,000 B16-OVA melanoma cells by intradermal injection into one of the flanks. A caliper was used to measure the size of the tumor, and the tumor volume was calculated as (length × width^2)/2.

Statistics

The two-sided Mann–Whitney U test was applied for nonparametric data analysis from two test groups. When three or more groups were compared, the Kruskal–Wallis test with the Dunn post hoc test was used. Differences in tumor size were analyzed using the Wilcoxon signed-rank test. The significance level was set to 95%. All calculations were done using the GraphPad Prism 5.04 software (Prism, La Jolla, CA).
Results

Physical characterization of PLGA microparticle formulations

PLGA microparticles were prepared with a mean size of 1 μm and a size range of 0.4–1.3 μm (data not shown). Upon lyophilization and reconstitution, the particle size range increased to 2–3.5 μm. The ζ-potential of the PLGA microparticles was in the range of −20 to −12 mV. The resulting microparticles contained 9.87 μg OVA/mg dry particles in the TPCS2a-free vaccine formulation and 8.30 μg/mg in the TPCS2a-containing vaccine; the loading efficiencies relative to the targeted loading were 69 and 58%, respectively. SDS-PAGE analysis of OVA extracted from microparticles demonstrated that the integrity of the protein was preserved during encapsulation and lyophilization (data not shown). An emulsion stabilizer was required for efficient encapsulation of TPCS2a. The addition of chlorhexidine diacetate in the inner aqueous phase demonstrated that the integrity of the protein was preserved during encapsulation and lyophilization (data not shown).

Photosensitization facilitated proliferation of Ag-specific CD8 T cells

To facilitate analysis of MHCI Ag presentation, 2 × 10⁶ Rag2/OT-I lymphocytes were adoptively transferred to wild-type C57BL/6 mice 1 d prior to immunization with PLGA-based vaccines. The particles were loaded with OVA (PLGA-OVA) or with OVA and TPCS2a (PLGA-OVA-TPCS2a). Eighteen hours later, the mice were anesthetized and euthanized. Two weeks later, the effect of immunization on the proliferation of splenocytes was measured ex vivo by pentamer staining and flow cytometry and expressed as the percentage of pentamer-staining cells among the total CD8 lymphocyte population (Fig. 1A). Significantly higher numbers of SIINFEKL-specific CD8 T cells were observed in mice immunized with PLGA-OVA-TPCS2a than with PLGA-OVA (Fig. 1B, p = 0.0006, Mann–Whitney test), with the average frequencies being 12.6 and 3.4%, respectively, and 0.9% for untreated mice. Seven weeks postimmunization, a reduction in the frequency of SIINFEKL-specific CD8 T cells was observed in the TPCS2a-free vaccine, whereas the frequency increased further after immunization with PLGA particles containing both OVA and TPCS2a. The frequencies of SIINFEKL-specific CD8 T cells were ~20.0% for PLGA-OVA-TPCS2a, 2.1% for PLGA-OVA, and 0.3% for untreated mice (Fig. 1C).

Photosensitization facilitated production and secretion of cytokines and cytotoxic mediators from Ag-specific CD8 T cells

The splenocytes were restimulated in vitro with SIINFEKL and analyzed by flow cytometry for surface expression of CD8 and the effector/memory marker CD44, as well as for intracellular production of IFN-γ and TNF-α (Fig. 1D). Immunization with both PLGA-OVA and PLGA-OVA-TPCS2a resulted in activation of CD8 T cells with a CD44 phenotype, which produced both IFN-γ and TNF-α. However, the frequencies of IFN-γ– and TNF-α–producing CD8⁺ CD44⁺ T cells were significantly higher after immunization with the TPCS2a-containing particles than with the TPCS2a-free particles at 2 wk (Fig. 1E, 53.7 versus 1.6%, p = 0.0033) and 7 wk (Fig. 1F, 9.9 versus 1.1%, p = 0.0006) after immunization. Hence, increases in both proliferation (Fig. 1B, 1C) and cytokine production (Fig. 1E, 1F) from weeks 2 to 7 indicate a persistent Ag presentation and immune stimulation by the TPCS2a-containing PLGA microparticles. Also, when no adoptive transfer with OT-I cells was done, a strong benefit of TPCS2a-containing PLGA-OVA vaccines was observed. The frequencies of pentamer-binding (Fig. 1G) and IFN-γ–producing (Fig. 1H) CD8 T cells were much lower than after immunization of OT-I transferred mice (Fig. 1B, 1E), but they were significantly higher in mice immunized with the photosensitizer-containing particle vaccine TPCS2a than in mice that were not immunized with TPCS2a.

The effect of photosensitization on the immunogenicity of protein-containing PLGA microparticles also was studied by measuring cytokine secretion in vitro after restimulation of splenocytes with SIINFEKL for CD8 T cell–specific immune responses. SIINFEKL–specific cytokine secretion was enhanced in mice immunized with PLGA-OVA-TPCS2a compared with mice that received PLGA-OVA (p < 0.01). Seven weeks after immunization, the secretion of IL-2 (Fig. 2A, p = 0.0006), IFN-γ (Fig. 2B, p = 0.0006), and TNF-α (Fig. 2C, p = 0.0012) was significantly higher in splenocytes from mice immunized with PLGA-OVA-TPCS2a than from mice immunized with PLGA-OVA. Nonetheless, PLGA-OVA microparticles stimulated significantly stronger secretion of IFN-γ and TNF-α (both p = 0.0167), but not IL-2 (p = 0.116), compared with nonimmunized mice.

![](image)
PCI-based vaccines stimulated cytotoxic CD8 T cells

To further test the effector function of the stimulated CD8 T cells, cytotoxicity was analyzed. First, the release of the cytotoxic mediator granzyme B was analyzed in the supernatants of cultured and SIINFEKL-restimulated splenocytes from immunized mice. Although immunization with PLGA-OVA caused a small increase in granzyme B production (Fig. 2D, p = 0.0167 compared with untreated mice), a very strong secretion of granzyme B was observed after immunization with concomitant photosensitization. The effect was significantly stronger than after immunization without TPCS2a (p = 0.0021). This effector function was further confirmed in vivo using an in vivo cytotoxicity assay with CFSE-labeled targets (Fig. 3A, 3B). None of the nonimmunized mice showed SIINFEKL-specific cytotoxicity (mean 6.2%), whereas all seven mice immunized with PLGA-OVA-TPCS2a exhibited specific killing of target cells (mean, 98.0%; range: 93.5–99.5%). Five of seven mice immunized with PLGA-OVA showed specific killing (mean, 60.7%; range: 5.1–92.1%), but the effect was significantly weaker than after immunization with photosensitization (p = 0.0021).

| FIGURE 2. | Secretion of cytokines and cytotoxic mediators was measured by ELISA upon in vitro restimulation of splenocytes with H2-Kb-SIINFEKL. Secretion of IL-2 (A), INF-γ (B), TNF-α (C), and granzyme B (D). Analysis was performed on splenocytes harvested 7 wk after immunization of mice with 2 × 106 adoptively transferred OT-I cells. Immunization with PLGA particles loaded with OVA or loaded with OVA and TPCS2a was done as described in Fig. 1. Control mice were left untreated. Mean and SEM are reported for each group. The p values were calculated using the Mann–Whitney test.

| FIGURE 3. | In vivo cytotoxicity and inhibited tumor growth. Mice received adoptive transfer of OT-I cells the day before immunization with PLGA-OVA or PLGA-OVA-TPCS2a (30 μg OVA, 210 μg TPCS2a). All mice were light treated 1 d later; control mice were left untreated (n = 7–8/group). (A and B) After 6 wk, splenocytes from naive mice were purified and split in two: one part was loaded with SIINFEKL and CFSE, the other was loaded with CFSE and no Ag. The cells were mixed 1:1 and transferred (i.v.) to the immunized mice. One day later, the mice were bled, and the Ag-specific killing of CFSE-labeled cells was analyzed by flow cytometry. Representative CFSE line graphs (A) and cytotoxicity bar graphs (mean ± SEM) (B). The p value was calculated by the Mann–Whitney test. (C) Three weeks later, all mice received 500,000 OVA-expressing B16 melanoma cells (intradermal), and tumor growth was monitored. The tumor size on day 16 after challenge is shown. The p value was calculated using the Wilcoxon signed-rank test.

Growth of tumor was prevented by vaccination with PLGA particles and photosensitization

The mice used in the in vivo cytotoxicity assay (Fig. 3A) were challenged 3 wk later with OVA-expressing B16 mouse melanoma cells and monitored for growth of tumor. The earliest onset of tumor was observed after 11 d for nonimmunized mice (data not shown). On day 16, six of eight nonimmunized and five of seven mice immunized with PLGA-OVA had solid tumors (Fig. 3C). None of the mice immunized with particulate Ag and photosensitizer had tumors.

Photosensitization abrogated MHCI presentation of Ag and production of Abs

Exogenous Ags, such as vaccines, are primarily presented via MHCI molecules and stimulate CD4 T cell–dependent Ab responses. To investigate the potential of PCI to deliver the Ag to cell cytosol, thus redirecting the Ag from the default MHCI pathway, mice were immunized with PLGA-OVA or PLGA-OVA-TPCS2a, and blood was collected for analysis of OVA-specific Abs. Immunization with PLGA-OVA particles stimulated high titers of anti-OVA IgG1, but an Ab response was not detected in mice that had received TPCS2a-containing PLGA microparticles (Fig. 4). Comparable results were obtained when IgG2 subclasses were measured (data not shown). Indirectly, this result suggests that the PCI-mediated cytosolic and MHCI targeting were so effective that the Ag did not reach the MHCI pathway of Ag presentation.

The adjuvant effect of the photosensitizer TPCS2a was dose and light dependent and dependent on codelivery with Ag

The adjuvant effect of the photosensitizer on the microencapsulated Ag is assumed to be light dependent. To test this hypothesis or to test any intrinsic and light-independent adjuvant effects of TPCS2a, mice were immunized with TPCS2a-containing PLGA microparticles loaded with 10 or 30 μg OVA and tested again for stimulation of SIINFEKL-specific CD8 T cell responses as a function of light activation. In mice that received 10 μg OVA and 70 μg TPCS2a in PLGA microparticles, but were not subsequently light treated, a baseline level of CD8 T cell proliferation was detected, with an average frequency of 0.3% SIINFEKL-
specific CD8 T cells in the spleen and as assessed 2 wk after immunization (Fig. 5A). When light was applied to the same vaccine, a significantly increased proliferation was observed (mean, 2.1%, \( p = 0.0079 \), Mann–Whitney test, \( n = 5 \)). The adjuvant effect of light-activated TPCS2a also was evident at the higher doses of 30 \( \mu \)g OVA and 210 \( \mu \)g TPCS2a, with the average frequency of specific CD8 T cells increasing from \( \sim 2.0 \) to 9.0% upon exposure to light (\( p = 0.0079, \ n = 5 \)). Intracellular staining for secretion of IFN-\( \gamma \) and TNF-\( \alpha \) revealed that immunization with TPCS2a- and OVA-containing PLGA microparticles elicited double-producing cells and that the degree of elicitation was dependent on light activation of TPCS2a, with the light causing a 5–10-fold increase in the frequencies of cytokine-producing CD8 T cells (Fig. 5B).Restimulation of splenocytes with SIINFEKL and analysis of granzyme B secretion by ELISA confirmed that light activation of the photosensitizer was needed to obtain adjuvant effects (Fig. 5C). Also, the increase in Ag-specific secretion of IL-2, IFN-\( \gamma \), and TNF-\( \alpha \) was dependent upon light irradiation of the photosensitizer (Fig. 5D–F).

Because the hypothesized basis of PCI-mediated immunization is that light activation of photosensitizers in the endosomes causes these to get leaky and to release their content of Ag, it is important that both photosensitizer and Ags are taken up by the same cells. To test this, PLGA-OVA was compared with the same vaccine mixed with soluble TPCS2a, with TPCS2a and OVA contained in separate particles (PLGA-OVA mixed with PLGA-TPCS2a), and, finally, with TPCS2a and OVA contained in the same particles (PLGA-OVA-TPCS2a). The OVA and TPCS2a doses were kept constant. Only when OVA and TPCS2a were coencapsulated were significant Ag-specific proliferation and cytokine secretion observed (Fig. 6).

**Discussion**

The majority of vaccines fail to generate strong CD8 T cell responses because exogenous proteins are mainly processed in lysosomes and presented by the MHCI molecules to stimulate proliferation and differentiation of CD4 Th cells, which again can trigger B cells for production of Abs. However, malignancies and infections by intracellular pathogens are better controlled by cytotoxic CD8 T cell responses (30–33). Therefore, strategies to stimulate CTLs are central in modern vaccine development.

For more than two decades, the induction of CTLs by biodegradable PLGA microparticles has been investigated (17, 21, 34–37). However, despite the fact that the particles are easily recognized by APCs (38, 39), that PLGA itself is immunologically inert (i.e., not recognized by specific Abs and eliminated by phagocytes), and that the particles can contain a high load of Ag that would facilitate CTLs and Th1-like immune responses, PLGA microparticles have not lived up to the initial expectations with regard to CD8 T cell activation. The problem is that particles typically remain contained in the APC phagosomes and are consequently shuffled to the lysosomes for loading on MHCI molecules. If phagosomal maturation could be blocked and the Ag released into cytosol, it could enable correct processing of antigenic peptides by proteasomes and loading of peptides on MHCI for CTL induction. In this regard, it was shown that photosensitizers can mediate cytosolic delivery of chemotherapeutic drugs (28) and, more recently, of soluble Ags (25, 26). Briefly, the photosensitizer binds to the plasma membrane of APCs, and upon endocytosis of extracellular photosensitizer and Ag, the photosensitizer is translocated to the endosomal or phagosomal membranes. Upon subsequent light activation of the photosensitizer, the endosomal membranes are disrupted, and the endosomal content is released into the cytosol as illustrated in Fig. 7.
molecules in ER and that are exported for stimulation of CD8 T cells. If light is applied, the vaccine-containing phagosomes rupture, which causes Ag release to cytosol followed by Ag processing in proteasomes to yield short linear peptides that bind to MHC-I and the Ags are digested, and the processed Ag peptides bind MHC-II. In these phagolysosomes, the particles containing Ag and photosensitizer (PhotoSens) are taken up by APCs by phagocytosis. If light is not applied, the vaccine-containing phagosomes may function as particles and, thereby, facilitate concerted targeting to individual APCs.

The experiments described in this study were designed to exploit the potential of Ag-containing PLGA microparticles combined with a photosensitizer (TPCS2a) to trigger cytosolic Ag delivery and generation of CTLs. Immunization of mice with PLGA microparticles loaded with both Ag and photosensitizer strongly facilitated CD8 T cell responses compared with immunization with PLGA particles containing Ag only. Proliferation of Ag-specific CD8 T cells in vivo and their function in vivo, ex vivo, and in vitro were strengthened by using the photosensitizer TPCS2a. Compared with immunization with soluble OVA and TPCS2a (25) or with in vitro–generated DCs loaded with OVA and TPCS2a (26), immunization with the PLGA-based vaccine produced much stronger proliferation of Ag-specific CD8 T cells; the beneficial immunological effect of particulate Ag–delivery systems over soluble Ags was shown earlier for formulations without photosensitizers (40, 41). In the current study, as much as 20–30% of the CD8 T cells in the spleen were specific for the immunodominant SIINFEKLI epitope, whereas ∼2–10% were obtained after immunization with soluble OVA (25, 26) or after autologous immunization with OVA– and PCI-treated DCs (24). Moreover, although the soluble Ag and photosensitizer produced a proliferation peak ∼10–12 d after immunization, after which the frequency of specific CD8 T cells decreased to ∼10–20% of the peak response within 2–4 wk, the PLGA-based and TPCS2a-containing vaccine, but not the TPCS2a-free vaccine, provided a sustained immune stimulation. The frequency of Ag-specific CD8 T cells increased over ∼7 wk reaching as much as 30–35% of all CD8 T cells.

Compared with our previous reports on soluble OVA and TPCS2a (25, 26), no additive effect was observed when combining soluble TPCS2a with particulate OVA in PLGA particles or when combining TPCS2a-containing particles with OVA-containing particles. At first glance, this may appear to be a contradiction. However, when both Ag and photosensitizer are administered as soluble compounds, their local biodistribution in the skin are expected to be similar or at least comparable, whereas when the Ag is contained in PLGA particles and the TPCS2a is given as a solute or contained in different particles, the biodistribution and migratory properties are expected to be less similar and comparable. Hence, the probability that the two compounds reach the same APC is lower when both are given as solutes than when both are contained in the same particles. Moreover, we observed that soluble OVA and TPCS2a can form colloidal complexes in vitro (B. Gander, unpublished observations). TPCS2a has two anionic sulfonate substituents, and it can undergo several protonation steps (B. Gander, unpublished observations). TPCS2a has two anionic sulfonate substituents, and it can undergo several protonation steps (B. Gander, unpublished observations).}

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cine. Finally, in contrast to soluble OVA and TPCS2a, particulate OVA-TPCS2a stimulated granzyme B production in CD8 T cells; when infected target cells or Ag-expressing tumor cells are recognized by CTLs, granzyme B is secreted into the target cells along with the pore-forming perforin to mediate cell death. The potential effector function of this granzyme B was tested in an in vivo cytotoxicity test: all mice immunized with PLGA-OVA-TPCS2a produced strong positive killing of Ag-loaded target cells, whereas this function was impaired in mice immunized with particles that did not contain the photosensitizer. Hence, the quantity of potential effector cells, as well as their effector quality, was found to be improved after immunization with photosensitizer-containing PLGA particles.

Interestingly, medium-control cultures of splenocytes from TPCS2a-immunized mice also showed significant production of IFN-γ (data not shown). The secretion was orders of magnitude lower than that after restimulation of the cells with Ag, but it suggests that 7 wk after the immunization, the spleen still contained APCs with Ag-containing PLGA particles that caused in vivo re-stimulation of the in vivo–primed T cells.

We recently suggested that the mechanism of action by which photosensitization mediates stimulation of CD8 T cell responses is an endosome-to-cytosol translocation of Ag and a subsequent TAP1-dependent and proteasome (trypsin- and caspase-like)–dependent MHCI Ag presentation (25). If a large fraction of Ag is funneled to MHCI, by consequence, less Ag should be available for MHCI. We tested this indirectly by measuring MHCI- and CD4-dependent Ab production. Immunoization without photosensitizer led to strong Ab production with anti-OVA IgG1 titers ∼1×10^3–1×10^5 after a single injection with 30 µg OVA in PLGA. In contrast, anti-OVA Abs were not detectable in mice immunized with TPCS2a-containing vaccines. This finding supports the hypothesis that photosensitizers can inhibit default phagosomal maturation toward lysosome fusion and drive the localization of Ag toward MHCI by cytosolic delivery. Although not aimed for explicitly, the consequence of highly effective cytotoxic targeting is suppression of the humoral response.

The adjuvant potential of TPCS2a was dependent on light activation and not on some other unknown intrinsic adjuvant effect of the compound, because enhanced immune responses were measured in mice only after exposure of mice to light. This observation is in agreement with previous studies in which cytosolic drug release from photosensitive liposomes was triggered by light activation (43, 44). Moreover, it could be argued that photosensitizer and light are solely producing damage and, thereby, providing danger signals that adjuvate cross-presentation and stimulation of CD8 T cell responses. This was described as one of the immunological effects of photodynamic therapy (45). However, in vitro experiments with Ag- and TPCS2a-loaded DCs did not support this, because DCs treated with high TPCS2a doses were not able to present Ag and stimulate CD8 T cell responses (data not shown). Therefore, we conclude that the adjuvant effect of TPCS2a is based on its light activation, with the consequence being the release of free radicals causing phagosomal unloading and the release of Ag or Ag-containing particles into the cytosol of viable APCs that are able to direct presentation de novo Ag to and present peptides to a TAP-dependent and MHCI-restricted manner to CD8 T cells.

Compared with soluble Ags, PLGA microparticles offer the chance to deliver high loads of entrapped Ag and adjuvant to APCs at a single-cell level. The polymer type, the surface decoration of the particles, as well as further excipients and adjuvants all represent factors that enable the modulation of the immunological properties of a particle-based Ag delivery system (35, 46–52). In studies in which PLGA microparticles were shown to stimulate APCs associated with the release of Ags into the cytosol as consequence of destabilization of the endosomal membrane (53–55). Although this destabilization was produced by the polymer itself, we show that a photosensitizer can be included in the particles for the purpose of destabilizing the endosomes, targeting of Ag to cytosol and MHCI, and generation of high numbers of CTLs. To achieve highly effective antitumor effects, reports indicate that vaccines should generate a certain threshold frequency of Ag-specific CD8 T cells (56, 57). Moreover, the polyfunctionality of Ag-specific CD8 T cells (e.g., their capacity to secrete multiple cytokines and release cytotoxic mediators), was reported to represent a predictive factor for the efficacy of immunotherapeutic vaccines (58, 59) and for disease progression in HIV patients (60). The current work demonstrated that the combination of PLGA microparticle–based Ag delivery and photosensitization was an effective immunization strategy for stimulation of high levels of polyfunctional Ag-specific CD8 T cells in a mouse model. These findings encourage further evaluation and development of PLGA microparticles as an Ag-delivery system to promote the treatment of or protection against CD8 T cell–dependent diseases and cancer, which remain among the most urgent unmet medical and societal needs.

Acknowledgments

We thank Jennifer Sand for technical assistance. The photosensitizer TPCS2a (Amphinex) and the light source (LumiSource) were gifts from PCI Biotech.

Disclosures

M.H. is an employee of PCI Biotech, which has field patents on the use of photosensitizers in vaccination. M.H. and P.J. received financial support from PCI Biotech. P.J. is listed as an inventor on patents describing the use of photosensitizers in vaccination. The other authors have no financial conflicts of interest.

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In conclusion, the use of PLGA-based vaccines has shown promise in the induction of both humoral and cellular immune responses. The ability to control release of the antigen at the site of injection is a significant advantage, as it allows for sustained exposure of the immune system to the antigen. Additionally, the use of photosensitizers in conjunction with PLGA-based vaccines offers the potential for enhanced immune response through photodynamic activation.

However, there are still several challenges that need to be addressed. Further research is required to optimize the formulation of the PLGA-based vaccines and to understand the mechanisms by which they induce immune responses. The development of appropriate delivery systems and the identification of optimal adjuvants will be crucial in realizing the full potential of these vaccines.

In summary, the use of PLGA-based vaccines and photosensitizers offers a promising approach for vaccine development. Continued research is needed to fully exploit the potential of these systems in the generation of effective vaccines that can provide long-lasting immunity against various infectious diseases.