Vaccine 30 (2012) 737–744

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Saponin-based adjuvants create a highly effective anti-tumor vaccine when combined with *in situ* tumor destruction

Martijn H. den Brok[a], Stefan Nierkens[a], Jori A. Wagenaars[a], Theo J. Ruers[b], Carla C. Schrier[c], Eric O. Rijke[c], Gosse J. Adema[a,*]

[a] *Department of Tumor Immunology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Geert Grooteplein 28, 6525 GA Nijmegen, The Netherlands*
[b] *The Netherlands Cancer Institute, Amsterdam, The Netherlands*
[c] *Intervet International BV, known as MSD Animal Health, Wim de Körverstraat 35, 5830 AA Boxmeer, The Netherlands*

ARTICLE INFO

Article history:
Received 2 September 2011
Received in revised form
15 November 2011
Accepted 18 November 2011
Available online 30 November 2011

Keywords:
ISCOMs
Saponin
Adjuvants
Cryo ablation
Cancer
Vaccination
Immunotherapy
Dendritic cell
Antigen presentation

ABSTRACT

Today's most commonly used microbial vaccines are essentially composed of antigenic elements and a non-microbial adjuvant, and induce solid amounts of antibodies. Cancer vaccines mostly aim to induce anti-tumor CTL-responses, which require cross-presentation of tumor-derived antigens by dendritic cells (DCs). Adjuvants that improve DC function and antigen cross-presentation are therefore advantageous for inducing anti-tumor immunity.

Previously, we have reported that *in situ* tumor destruction of established murine tumors by ablation efficiently delivers antigens to DC for the *in vivo* induction of anti-tumor immunity. Yet, tumor ablation alone resulted in only partial protection against a subsequent tumor-challenge. In this article, the ability of various non-microbial vaccine adjuvants to modulate the immune response following cryo-ablation was tested. The data show that tumor ablation with co-injection of saponin-based adjuvants, but not oil-in-water, water-in-oil or alum-based adjuvants, creates a highly effective *in situ* vaccine. Draining lymph node CD11c+ DCs acquire antigens more efficiently and become increasingly activated following ablation with saponin adjuvants relative to ablation alone. Moreover, our data reveal that the saponin-based adjuvants facilitate an in this model unprecedented level of antigen cross-presentation, induction of tumor-specific CTL and long-lasting tumor protection.

Collectively, combining saponin-based adjuvants with *in situ* tumor destruction leads to an extremely potent systemic anti-tumor response. This combination approach forms a powerful *in situ* DC vaccine for which no prior knowledge of tumor antigens is required. As saponin-based adjuvants are currently clinically available, they represent attractive tools for various human and veterinary settings where *in situ* tumor destruction is applied.

1. Introduction

Adjuvants used to enhance immunogenicity of vaccines comprise a very diverse group of compounds. Even though microbial-derived adjuvants like the Toll-like receptor agonists LPS or CpG have been studied in far more detail at the molecular level, the majority of vaccines is enforced with non-microbial adjuvants. For human vaccines, registered adjuvants are still limited and include aluminum-based adjuvants like AlOH and the oil/water-based adjuvant MF59. Both adjuvants are known for their robust antibody induction and their safety, but they appear poor adjuvants for building T cell-mediated immunity, which is thought to be crucial for anti-cancer-vaccines [1]. In addition, they primarily induce IgG1 antibodies over IgG2a and IgG2b, thereby generating mainly Th2 responses. In animal vaccines several additional adjuvants are in use, including saponins, saponin-containing immune-stimulating complexes (ISCOMs), oil-in-water, water-in-oil emulsions and carbopol formulations. The exact immunological mechanisms of many adjuvants are still poorly understood. For optimal immune activation, however, these classical adjuvants can be improved when combined with Pattern Recognition Receptor (PRR)-ligands [2,3].

Patients suffering from advanced cancer often have developed a compromised immune response to the tumor and its antigens. These antigens are usually (mutated) self-derived and as a result poorly immunogenic. Tumor-induced impairment of antigen presentation by dendritic cells and inhibition of CTL responses by for instance regulatory T cells will further contribute to an

* Corresponding author. Tel.: +31 24 3617600; fax: +31 24 3540339.
E-mail address: g.adema@ncmls.ru.nl (G.J. Adema).

0264-410X/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2011.11.080

immune-compromised state [4]. Cancer patients will therefore benefit from therapeutic treatments that not only induce a strong Th1-skewed cellular immunity, but also are able to counteract the immune suppressive tumor microenvironment.

The 'Immunostimulating complex' (ISCOM) or ISCOM Matrix adjuvants are composed of saponin, cholesterol and phospholipid, which auto-formulate into typical 40 nm-sized particles [5,6]. This class of adjuvants represents a unique group of compounds that facilitates Ag presentation to the immune system, but importantly, saponins are also thought to modulate the immune system without the need of adding extra PRR triggering components [7,8]. The immune responses generated have proven to be of broad specificity and to include robust antibody responses. Unlike the aluminum- or oil/water-based adjuvants, the saponin-based adjuvants are well-known for their Th1-skewing properties and solid CD4(+) and CD8(+) T-cell responses. Evaluation of ISCOM-containing vaccines in both murine and human studies start to show efficacy and safety and make this adjuvant an attractive modality for the use in anti-cancer vaccines [7]. Recently, Cebon and co-workers demonstrated strong humoral and T-cell-mediated immune responses, which reduced relapse rates in patients with fully resected melanoma. Conversely, advanced melanoma patients showed no clinical responses to vaccination, presumably due to the presence of large numbers of Tregs and the larger tumor-load [9]. These data illustrate the feasibility of anti-cancer vaccination with saponin-based adjuvants, but also demonstrate that combination with other techniques is instrumental.

Tumor ablative treatments, like cryo, laser, ultrasound or radiofrequency ablation, are successfully used in clinical settings to completely destruct different types of tumors, thereby leaving a residual tumor antigen fingerprint for the immune system [10–13]. Antigen-presenting cells (APCs), such as dendritic cells (DCs), are well-equipped to phagocytose dying cells and process tumor antigens for presentation to T lymphocytes [12]. Indeed, recent data demonstrated that DC in the tumor draining lymph nodes efficiently acquire tumor debris following *in situ* tumor destruction [10–12,14,15]. Although tumor ablation has been associated with the occurrence of immune activation in some patients [16], these responses are often weak and long-term survival is mostly limited by the appearance of novel local or distant lesions. This suggests the presence of micro-metastasis at the time of ablation and indicates the need for additive treatments inducing systemic responses.

Here, we show that from a panel of selected classical adjuvants only the saponin-based compounds can be effectively combined with *in situ* tumor ablation. Using a stringent murine model with established tumors we demonstrate that peri-tumoral injection of these adjuvants post-ablation results in superior anti-tumor immunity by modulating dendritic cell function. Co-injection of saponin-based adjuvants following *in situ* tumor-ablation therefore constitutes a powerful '*in situ* DC-vaccine' that effectively combines the favorable characteristics of both the ablation and the saponins.

2. Materials and methods

2.1. Mice and tumor cells

C57BL/6n mice (6–8 weeks old) were purchased from Charles River Wiga (Sulzfeld, Germany) and maintained under specific pathogen-free barrier conditions at the Central Animal Laboratory (Nijmegen, The Netherlands). Drinking water and standard laboratory food pellets were provided *ad libitum* and mice were allowed to settle for at least 1 week before random assignment into specific treatment groups. The experiments were performed according to the guidelines for animal care of the Nijmegen Animal Experiments Committee.

The murine melanoma cell line B16F10 (ATCC) was cultured in complete medium (MEM, 5% fetal bovine serum (Greiner Bio-one), 100 U/ml penicillin G sodium and 100 μg/ml streptomycin (Pen/Strep), MEM sodium pyruvate (1 mM), $NaHCO_3$, MEM vitamins, MEM non-essential amino acids (all from Gibco), 20 μM beta-mercaptoethanol). OVA-transfected B16F10 (B16OVA, clone MO5) was cultured in complete medium supplemented with 30 μg/ml hygromycin and 1 mg/ml G418.

2.2. Tumor model and cryosurgery

Tumor cells were suspended in a mixture of PBS and Matrigel (2:1, BD Biosciences), and 0.5×10^6 cells in a total volume of 50 μl were injected s.c. at the right femur. When tumor diameters measured between 6 and 8 mm (generally at days 9–10) they were randomly assigned to treatment groups. Cryo ablation (Cryo) was performed under isoflurane/O_2/N_2O anesthesia using a liquid nitrogen cryoablation system (CS76, Frigitronics, Shelton, CT) of which the tip is cooled by a continuous flow of circulating liquid nitrogen. During 2 treatment cycles of freezing and thawing the tumor was macroscopically frozen, while leaving surrounding healthy tissue intact. Mice were carefully examined for recurrent tumors at the site of ablation. When recurrences appeared (this study 9%), mice were excluded from further analysis. To monitor the induction of long-lasting tumor protection, mice were re-challenged with 15×10^3 B16F10 cells 40 days after cryo ablation. This number of cells was chosen after careful titration and ensures a solid 100% tumor take. Rechallenges were injected in 100 μl PBS s.c. on the contralateral leg or in the flank. Mice were sacrificed when tumor volume exceeded 1000 mm^3 or when occasionally tumors brake through the skin barrier.

2.3. Adjuvant injection

CpG 1668 (5′-TCCATGACGTTCCTGATGCT-3′) with total phosphorothioate-modified backbone was purchased from Sigma Genosys (Haverhill, UK). CpG was injected in PBS peritumorally (p.t., 30 μg divided over 2 injections of 20 μl lining the ablated tumor). The following non-microbial adjuvants were used (obtained from Intervet International BV, Boxmeer, The Netherlands): water-in-oil emulsion using mineral oil (Marcol 52); a water-in-oil emulsion using a non-mineral oil (Miglyol 840); an oil-in-water emulsion using mineral oil (Marcol 52), all prepared according to Jansen et al. [17]; an oil-in-water emulsion using squalene (MF59, prepared according to Ott et al. [18]); an oil-in-water emulsion using vitamin E acetate as described by Rijke et al. [19]; Matrix C (Isconova, Uppsala, Sweden) 750 μg/ml; Quil A saponin (Brenntag, Frederikssund, Denmark) 500 μg/ml; aluminum hydroxide (Brenntag, Frederikssund, Denmark) 0.75% in Tris–HCl or aluminum phosphate (Brenntag, Frederikssund, Denmark) 0.75% in phosphate buffer. For Fig. 1A, the two water-in-oil emulsions were mixed at a 1:1 ratio. The individual water-in-oil adjuvants were also tested separately in small groups of mice, with comparable results. Adjuvants (or mixes of them) were injected peri-tumorally (p.t.) (40 μl divided over 2 injections). All injections were done within 30 min after ablation.

2.4. BMDC culture and in vitro cross-presentation assays

Bone marrow cells were cultured for 8 days in RPMI medium, supplemented with 10% FCS, glutamine, antimycotics/antibiotics and β-mercaptoethanol in the presence of 20 ng/ml GM-CSF (PeproTech) to obtain bone marrow-derived dendritic cells (BMDCs). For the *in vitro* cross-presentation assays 100×10^3 DCs (B3Z assays) or 15×10^3 DCs (OT-I assays) were pulsed with indicated amounts of endotoxin-free chicken egg ovalbumin (OVA,

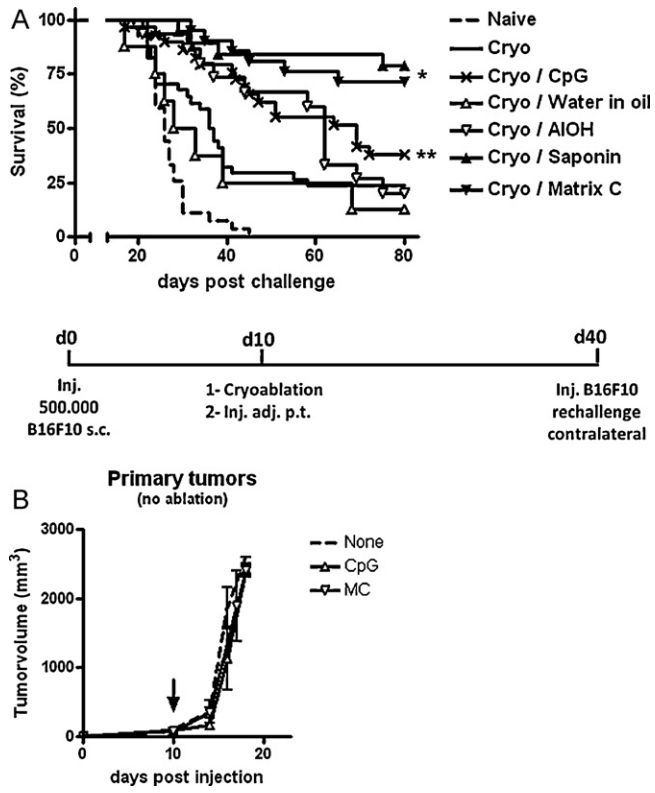

Fig. 1. Potent anti-tumor memory response following ablation combined with saponin-based adjuvants. (A) Established B16F10 tumors s.c. on the right femur were treated with cryo ablation alone, in combination with CpG-ODN, or in combination with the indicated non-microbial adjuvants. Forty days later, naïve and tumor-free mice received a re-challenge with tumor cells (15×10^3 B16F10 cells) s.c. at the flank. Growth of this re-challenge is depicted as a Kaplan–Meier survival curve demonstrating superior protection from tumor outgrowth after ablation combined with saponin-based adjuvants *Quil A saponin* or *Matrix C*. $^*p < 0.001$ compared to cryo/CpG, $^{**}p < 0.05$ compared to cryo. (B) Established B16F10 tumors received peri-tumoral injections like in A (arrow), but were not ablated by cryo. Data show growth of this primary tumor, demonstrating that combination with ablation is instrumental to cure the mice. Comparable data were obtained in three independent experiments.

Profos AG., Germany) in the presence of the various adjuvants. After 5 h, cells were washed and cultured overnight with 100×10^3 B3Z cells or for 72 h with 50×10^3 CFSE-labeled OT-I cells. B3Z cells and B6-mec-sigOVA were cultured as described elsewhere [20]. The presentation by DCs of SIINFEKL in H-2K^b results in production of β-galactosidase by B3Z cells [21], which can be detected by adding 0.15 mmol/l chlorophenolred-h-D-galactopyranoside (Calbiochem), 9 mmol/l $MgCl_2$, 0.125% NP40, and 100 mmol/l β-mercaptoethanol in PBS. Plates were incubated for 3–5 h at 37 °C and absorbance values were measured using a photo spectrometer (595 nm). For the readout of OT-I proliferation, cells were stained for CD44, a late stage activation marker, and CFSE dilution with CD44 expression were measured by FACS.

2.5. Ex vivo enrichment and analysis of dendritic cells

To analyze DC biology, DCs were isolated from the inguinal lymph nodes draining the tumor. Lymph nodes were excised, torn apart using needles, and digested in collagenase type II and DNase for 15 min at 37 °C. After addition of EDTA and re-suspending, cells were applied to nylon mesh to remove debris and DCs were enriched by positive selection using anti-CD11c-labeled (N418) magnetic micro beads (Miltenyi Biotec B.V.).

To study the fate of antigen and DC maturation after cryoablation, tumors were injected with fluorescently-labeled OVA protein (OVA-Alexa488, Molecular Probes, 20 μg/20 μl). CD11c-enriched cells were stained with anti-CD11c (HL3), anti-CD80 (1G10/B7) or isotype controls, all obtained from BD Biosciences. Expression of CD80 was analyzed in OVA$^+$ and OVA$^-$ cells gated CD11c+ cells by flow cytometry (FACS Calibur, Becton Dickinson).

2.6. In vivo cytox

Wildtype C57Bl/6 splenocytes were differentially labeled with the dye CFSE (1 μM vs 10 μM). $CFSE_{low}$ cells were pulsed with irrelevant CD8 HPV peptide (RAHYNIVTF, Kb), while the $CFSE_{high}$ cells were pulsed with CD8 OVA peptide (SIINFEKL, Kb). These two fractions were mixed 1:1 and injected i.v. into recipient mice that received the indicated ablation treatments with B16 tumors transfected with OVA (B16-OVA). All recipient mice received intratumoral injection of 20 μg OVA just prior to ablation to boost precursor frequencies. 20 h later, recipient spleens and LNs were isolated and analyzed for remaining target cells. Injection into an OT-I mouse served as a positive control. A timeline of this experiment is provided in Fig. 4A.

2.7. Statistical analyses

Data were analyzed using a two-tailed Student's *t*-test, while Kaplan–Meier survival curves were analyzed using a log rank test.

3. Results

3.1. Saponin-based adjuvants provide superior anti-tumor immunity

Radiofrequency- or cryo-ablation of established murine B16-OVA tumors previously resulted in weak, but tumor specific anti-tumor reactivity [11,22]. Peri-tumoral co-injection of the TLR ligand CpG-ODN directly after ablation resulted in a significant increase in immunity towards a subsequent tumor rechallenge [20,22,23]. Injection of other commonly used TLR-ligands (poly I:C, LPS, PAM_3cys) revealed that CpG-ODN was by far most effective in increasing anti-tumor immunity (data not shown). Here, we set out to explore the potency of various 'classical' non-microbial adjuvants, proven to be effective in various human or animal vaccines, in an orthotopic tumor ablation setting. To compare the induction of tumor-specific memory responses following administration of the various adjuvants in a stringent setting, wildtype B16-F10 tumor-bearing mice were treated with cryo ablation when the tumor measured 6–8 mm in diameter. Within 30 min following ablation, mice received peri-tumoral injections of either CpG-ODN 1668, water-in-oil adjuvant, aluminum hydroxide, Quil A saponin or Matrix C. Forty days after ablation the mice were re-challenged with B16-F10 cells (see detailed time schedule below Fig. 1A). As shown in Fig. 1A, ablation of B16-F10 resulted in a clear delay in the outgrowth of the B16-F10 rechallenge as compared to naïve controls and gave a low level of protection (24%). Co-injection of CpG-ODN resulted, as expected, in an additive effect and protected 38% of the mice. In contrast, no additive delay in outgrowth of the rechallenges was observed when ablation was accompanied by injection of water-in-oil or AlOH adjuvants. Additional data suggest that injection of three oil-in-water adjuvants or $AlPO_4$, yields similar results (see Section 2 for a detailed list, data not shown). Strikingly, peri-tumoral administration of both saponin-based adjuvants (Quil A saponin and Matrix C) resulted in a solid and superior protection of 79% and 71% respectively, thereby exceeding the efficacy of CpG-ODN injection. In a setting where the peritumoral area was injected with CpG-ODN or Matrix C, but the tumors were left untreated (no ablation), we observed that neither CpG nor Matrix C was able to significantly slow down the growth of the tumor (Fig. 1B). This

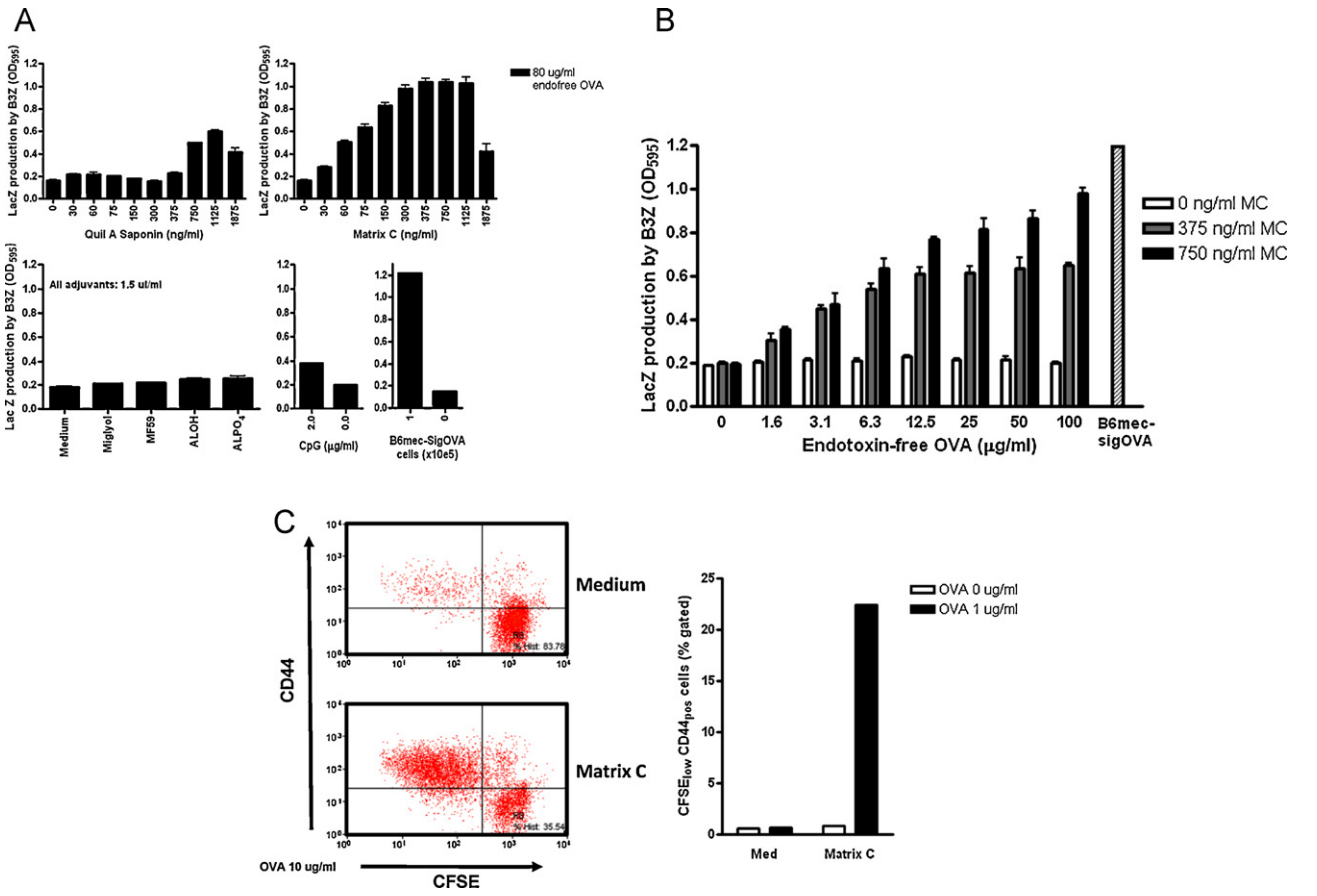

Fig. 2. Saponin-based adjuvants facilitate cross-presentation and T cell activation *in vitro*. Bone marrow dendritic cells were cultured with GM-CSF, and 1×10^5 cells were pulsed with one (A) or more (B) concentrations of endotoxin-free ovalbumin (OVA) in the presence of various adjuvants. The adjuvants presented in the lower-left panel have all been titrated, but shown is only the highest concentration. After 5 h, cells were washed and cultured overnight with 1×10^5 B3Z cells, which produce LacZ upon TCR recognition of OVA peptide 257–264 in the context of H-2K^b. Next, LacZ production by the B3Z cells, as a measure of cross-presentation, was evaluated using a colorimetric assay. As positive controls, CpG-ODN and B6Mec-SigOVA cells were used. Results are shown as means with SD. Similar results were obtained in three independent experiments. (C) 15×10^3 bone marrow dendritic cells were pulsed with endotoxin-free ovalbumin (OVA) in the presence of various adjuvants. After 5 h, cells were washed and cultured for 72 h with 50×10^3 CFSE-labeled OT-I cells. Next, cells were stained for CD44, a late T cell activation marker, and measured by FACS. Left panel shows some example FACS plots, demonstrating CD44 expression and cell proliferation (by dilution of the CFSE signal).

demonstrates that cryo-ablation as well as the adjuvant is necessary in this model and that the observed effects could not be reached using either treatment modality alone.

These data thus imply that the weak immune responses developing after *in situ* tumor destruction can be effectively improved by co-injection of saponin-based adjuvants.

3.2. Saponin-based adjuvants facilitate cross-presentation in vitro

As it is now well-recognized that dendritic cells acquire material from *in situ* destructed tumor-masses, we examined the contribution of these cells in the induction of the observed anti-tumor immunity. Previous studies revealed that next to antigen uptake, DC maturation and antigen cross-presentation are important in our model. Therefore we generated bone-marrow derived DCs with GM-CSF and exposed them to various concentrations of the adjuvants. Comparing the expression of maturation markers CD80, CD86 and CD40 we found that none of the adjuvants had any effect on DC maturation *in vitro* (not shown and Fig. 3B), which is in line with data obtained by others [24]. Toxicity was observed for the adjuvants Quil A saponin and Matrix C, at concentration higher than 1 μg/ml. We next determined the capacity of the various adjuvants to influence the DCs ability to cross-present exogenous antigens, using OVA as a model antigen and B3Z cells as a readout system. B3Z cells detect OVA-peptide presented in H-2K^b independent of co-stimulatory molecules. As shown in Fig. 2, little or no cross-presentation was observed upon provision of OVA alone (even up to 100 μg/ml). Upon co-addition of MF59 oil-in-water, AlOH, ALPO4, or Miglyol water-in-oil, no enhancement was observed (Fig. 2A). Quil A saponin and CpG-ODN could enhance cross-presentation significantly, but remarkably, Matrix C was able to induce cross-presentation of OVA to an unprecedented extend. This enhanced cross-presentation was already visible at concentrations as low as 1.6 μg/ml OVA (Fig. 2B). At the highest concentrations of Matrix C the DCs lost viability slowly (Fig. 2A and not shown). DCs treated with Matrix C were also able to cross-present to OVA-specific OT-I cells, even though OT-I cells are more co-stimulation dependent than B3Z cells (Fig. 2C). The capacity of BM-DCs to degrade OVA antigen (as measured by DQ-OVA that emits fluorescence upon degradation) was not altered in the presence of Matrix C, suggesting that the observed increase in cross-presentation is rather the result of changed antigen routing (Supplementary Figure 1).

These results thus demonstrate that only the saponin-based adjuvants efficiently induce cross-presentation in DCs.

3.3. Ablation with Matrix C leads to efficient in vivo antigen acquisition and DC maturation

Guided by our *in vitro* data we next explored the impact of the saponin-based adjuvants in the *in vivo* induction of anti-tumor

immunity. As we have previously found that following tumor ablation antigens are present in large amounts in the draining lymph node CD11c(+) fraction at one and even 3 days after ablation [11,20,23], we set out to explore the effect of *in situ* tumor destruction plus Matrix C administration on antigen uptake *in vivo*. In order to visualize uptake of antigens by flowcytometry, OVA conjugated to the fluorophore Alexa-488 (OVA-A488) was injected intra-tumoral (i.t.) prior to ablation. As shown in Fig. 3A, about 20% of all CD11c(+) DCs became OVA-A488 positive after cryo ablation, whereas much less antigen uptake was observed in non ablated mice (<5%). Co-administration of CpG-ODN did effect the loading of DCs in the draining LN only to a minor extend. Strikingly, when Matrix C was co-injected, over 40% of the CD11c+ cells became OVA-A488 positive, indicating an effective cooperation between ablation and Matrix C in loading of DCs.

Next, we studied the effect of cryo ablation with adjuvants on DC maturation *in vivo*. Hereto, total or OVA-A488(+) CD11c fractions were analyzed for expression of the maturation marker CD80. Fig. 3C shows that ablation of the tumor itself induces a two-fold increase in expression of CD80 as compared to untreated mice. Co-injections of CpG-ODN, Quil A saponin or Matrix C were all able to increase expression even further to MFI 1000. Maturation was primarily observed in the OVA-A488(+) cells [11]. In contrast, *in vitro* Matrix C administration to BMDCs did not seem to upregulate any maturation marker (Fig. 3B and data not shown).

These data thus demonstrate that released antigens preferentially accumulate in draining LN CD11c(+) DCs and that Matrix C is able to effectively enhance antigen accumulation. Maturation of these antigen-positive DCs occurs due to the ablation itself, but can be highly increased when combined with Matrix C *in vivo*.

3.4. Cryo ablation combined with Matrix C leads to efficient antigen-specific killing in vivo

To determine the role of cross-priming and CTL induction in the observed *in vivo* anti-tumor immunity, an *in vivo* cytotoxicity assay was performed. In this experiment we analyzed the effect of co-treatment with Matrix C. Co-treatment with AlOH, being the most often-used alternative adjuvant in commercial vaccines, was used as an additional control. A timeline of this experiment is provided in Fig. 4A. Splenocytes were differentially labeled with the dye CFSE. $CFSE_{intermediate}$ cells were pulsed with irrelevant CD8 HPV peptide (H-$2K^b$), while the $CFSE_{high}$ cells were pulsed with CD8 OVA peptide (H-$2K^b$). These two fractions were mixed 1:1 and injected into recipient mice that received the indicated ablation treatments with B16 tumors transfected with OVA (B16-OVA). 20 h later, recipient spleens were isolated and analyzed by FACS for remaining target cells. Fig. 4B shows some illustrative plots demonstrating increased killing of the OVA-pulsed target population. A quantification of this data is depicted in Fig. 4C. Specific killing was essentially absent when ablation was performed as a single treatment. Co-treatment with CpG-ODN resulted in a 2.5-fold increase in killing in the draining LNs as compared to ablation alone. Importantly, the percentage of killing in ablation/CpG was equal to the killing when AlOH was co-administered. However, when ablation was combined with Matrix C superior antigen-specific killing was observed, at levels comparable to the killing in a naïve OT-I mouse (carrying OVA-specific CD8 T cells).

Collectively, these data suggest that *in situ* tumor destruction plus immune activation by saponin-based adjuvants leads to a more potent systemic anti-tumor response than either treatment modality alone. This treatment regimen allows for direct antigen-loading of DCs *in vivo* without delivery of defined tumor antigens as in conventional DC vaccination (e.g. with tumor peptides). Moreover, the fact that adjuvants like ISCOMs or Matrix C are readily available for clinical application, makes them attractive tools in various human *in situ* tumor ablation settings.

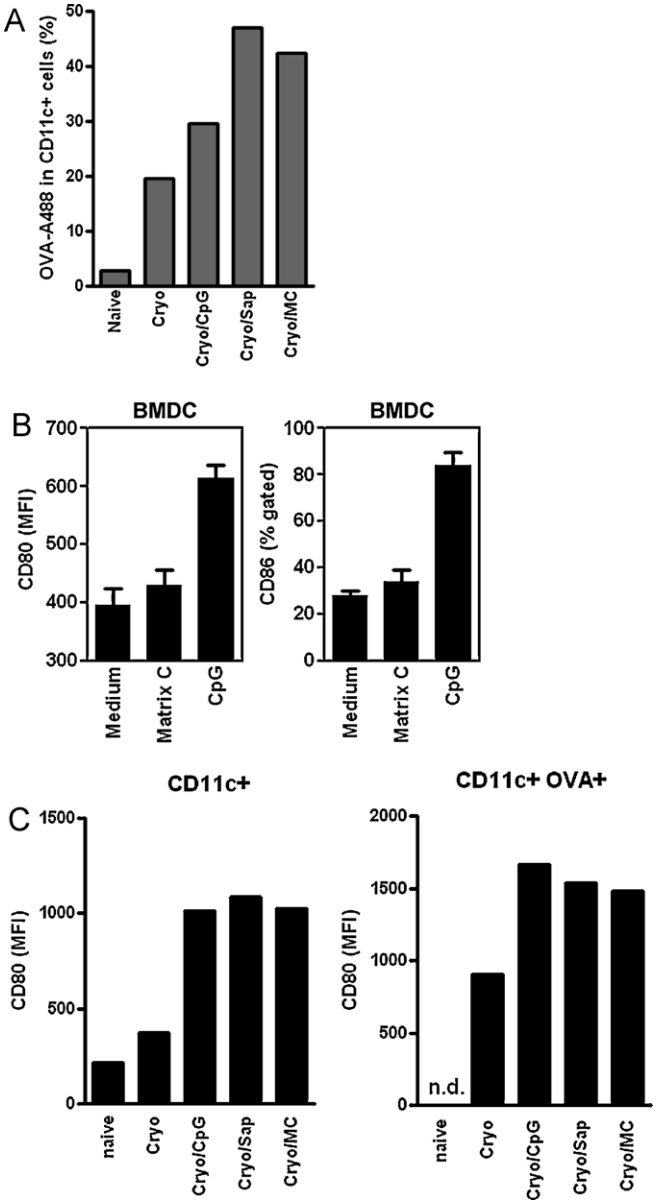

Fig. 3. *In vivo* OVA-Alexa488 uptake and DC maturation following combined ablation/saponin-adjuvant treatment. FACS analysis of sorted CD11c(+) cells isolated from pooled LN suspensions of naïve or tumor-ablated mice ($n = 6$ per group). Mice received 20 μg ovalbumin conjugated to Alexa-488 (OVA-Alexa488) i.t. just prior to the time point of ablation. Within 30 min post ablation, the indicated adjuvants were injected peri-tumorally. Two days after the indicated treatments the percentages of OVA-Alexa(+)/CD11c(+) DCs was determined (A) and separately analyzed for costimulatory molecule expression (C). CD80 and CD86 expression on *in vitro* cultured BMDCs was determined after 24 h exposure to 375 ng/ml Matrix C or 1 μg/ml CpG-ODN (B). Values shown in A are percentages of OVA-Alexa(+) cells within the CD11c(+) fraction, as observed in one of two independent representative experiments. Results depicted in B are shown as mean MFI (mean fluorescence intensity) or % cells gated positive, within the CD11c(+) fraction. Data in C are shown as mean fluorescence intensities, observed within the CD11c(+) or OVA-Alexa(+)/CD11c(+) fraction, as observed in one of two independent experiments. n.d.: not determined due to low number of cells.

4. Discussion

By studying the innate and adaptive immune response following cryoablation with co-injection of various classical adjuvants

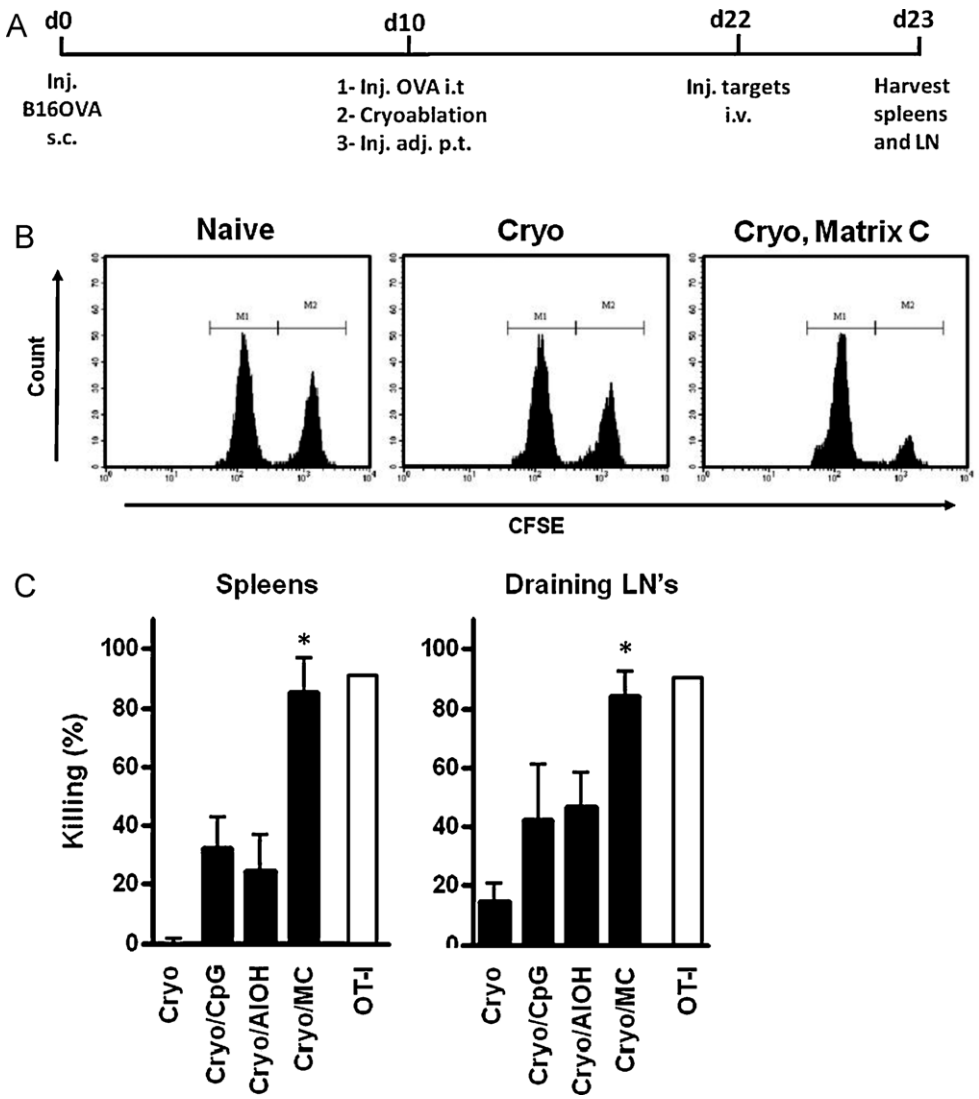

Fig. 4. *In vivo* cytolytic function of ablation-induced CD8+ T-cells. Established B16OVA tumors were treated with cryo ablation alone, or in combination with the indicated adjuvants. After 10 days, the treated mice, or one OT-I mouse, received 3×10^6 $CFSE_{high}$ splenocytes pulsed with the target peptide (OVA; SIINFEKL), along with 3×10^6 $CFSE_{low}$ splenocytes pulsed with the control peptide (HPV; RAHYNIVTF)(both K^b). Spleens and sup. ing. LNs of these mice were harvested 20 h later and the relative numbers of $CFSE_{high}$ and $CFSE_{low}$ target cells were determined by flow cytometry. Panel A depicts a detailed experimental timeline, while B shows some example FACS histograms. Data in C are depicted as mean percentages of target cell killing ($n = 6$ mice), corrected for background cell-death in naïve mice. $^*p < 0.05$ compared to cryo or cryo/CpG.

we have uncovered the unique ability of saponin-based adjuvants to synergize with *in situ* tumor ablation in generating anti-tumor responses. Upon combination of ablation with saponin-based adjuvants we observed enhanced dendritic cell-mediated cross-presentation, DC maturation, antigen-specific T cell killing and subsequent induction of superior anti-tumor immunity.

Vaccines designed for a given application require a matching combination of selected antigens and immunopotentiator(s) that selectively drives the required immune response, accompanied by minimal adverse reactions. Although the working mechanisms of existing immunopotentiators like AlOH are intensively studied [25], the exact immunological requirements for protective immunity are still poorly understood. Most of the adjuvants used in this study are presumed to facilitate residence time, location and dose of the antigen after injection, thereby enabling a sustained immune response. Recently, various authors have suggested that particulate adjuvants like the alum-based adjuvants exert their effect via NALP3 [26–28]. Uptake of microparticles by DCs induced NALP3-dependent enhancement of IL-1β secretion, indicative of inflammasome activation. It will be interesting to find out if the efficacy of saponin-based adjuvants depends on inflammasome activation and/or IL-1 production as well.

While many adjuvants have been tested for usage in novel vaccine design over the last few decades, the vast majority has not been approved for human use, with limitations including lack of efficacy, unacceptable toxicity, difficulty of manufacture, poor stability or exorbitant costs. Alum (aluminum-based minerals) was one of the first adjuvants discovered and, remarkably, is still the most widely used adjuvant in nowaday's human vaccines. In addition, alum is a moderate adjuvant for antibody induction to protein subunits and a poor adjuvant for cell-mediated immunity. In mice, alum mainly induces the increases of IgG1, instead of IgG2a and IgG2b, thereby producing mainly Th2 responses. MF59, consisting of emulsified squalene, one of the few other adjuvants licensed for human use in addition to alum, induces similar Th2 immune responses. Although this seems to be less critical in anti-microbial vaccination, cancer immunotherapy in general benefits most from a Th1 type immunity, accompanied by a strong cellular response. We here show that only the saponin-based adjuvants stood out among the other adjuvants in our *in situ* anti-tumor therapy. We obtained

superior anti-tumor protection in one of the most stringent tumor models available (B16F10 melanoma), even exceeding the effects of CpG-ODN, the TLR ligand previously yielding the best results in this model [22,23].

Quillaja saponin used in our study however also has some drawbacks, limiting its use as adjuvant in human vaccination, like toxicity, undesirable hemolytic effect and instability in aqueous phase. Matrix C is an adjuvant based on ISCOM (Immunestimulating complex) matrices. These matrices are small (40 nm) cage-like structures that formulate when cholesterol, phospholipid and saponins are specifically mixed in a certain ratio [6,29]. The cholesterol interacts irreversibly with saponin, thereby protecting the saponin from hydrolysis, which adds substantially to the stability of the adjuvant. Moreover, the ability of the saponin to interact with membranes is largely reduced in these matrices, thereby reducing its hemolytic and toxic side-effects [30,31]. This was also reflected in our *in vitro* work where Quil A saponin appeared vulnerable to instability and was more toxic for cells *in vitro*. Higher concentrations were needed to obtain similar effects as with Matrix C, while at the same time these concentrations decreased cellular integrity (Fig. 2). Due to its reduced toxicity and enhanced stability, ISCOM containing adjuvants are currently successfully being tested in various human applications [8,9,32]. In our experiments we did not see any side effects following peri-tumoral application of the saponin-based adjuvants.

The only study up to now using ISCOMs in a human cancer setting is using full-length NY-ESO-1 protein formulated in ISCOMs adjuvant. Although Cebon and co-workers could demonstrate strong humoral and T-cell-mediated immune responses, which reduced relapse rates in patients with fully resected melanoma, advanced melanoma patients showed no clinical responses to vaccination [9]. Unfortunately, patients with advanced melanoma had a significantly higher proportion of circulating regulatory T cells compared with those with minimal residual disease. Surprisingly, our study shows that it is not mandatory to formulate tumor antigens within the ISCOM matrices, as co-injection of Matrix C into the ablated tumor material appears to be sufficient. It is tempting to speculate that lipophylic antigens readily diffuse into the matrices, or that the matrices along with the antigens are transported passively towards the lymph nodes and taken up by lymph node-resident APCs.

One benefit of using *in situ* tumor destruction is that it provides an unbiased antigen source for the immune system. The broad repertoire of antigens released can be processed into multiple B and T-cell epitopes. In this way immunity is not solely depending on a single epitope from a single protein like in antigen-specific-vaccinations. Although we elucidated some aspects of the cellular working mechanism, it remains unclear which molecular mechanisms underlie the observed improved DC functions. In line with our data, recent work from Schnurr and coworkers demonstrated that ISCOMs could facilitate cross-presentation of exogenous antigen *in vitro* [33–35]. The authors suggest that ISCOMs enable cytosolic escape of the endocytosed antigens, after which the exposure of these antigens to the cytosolic protein degradation enzymes subsequently forces cross-presentation of these antigens.

Interestingly, the addition of Matrix C to *in vitro* cultured DCs did not induce upregulation of any tested maturation marker, which is in line with other research [24]. This might imply that *in vivo* T cells miss a substantial part of co-stimulation, which could possibly even lead to the induction of regulatory T cells. On the contrary, we did observe maturation of *in vivo* DCs when *in situ* tumor destruction was combined with Matrix C alone. This *in vivo–in vitro* discrepancy is most likely the result of endogenous mediators released by tumor ablation that mature the DCs. Consistently, we showed in Fig. 3 that cryoablation in itself could already mature especially the antigen positive DCs. Alternatively, *in vivo* DC maturation could be the result of the interaction between various cell-types, a situation not present *in vitro*.

Clinically applied *in situ* tumor ablation techniques, like cryo, laser, ultrasound or radiofrequency ablation, have shown to induce (weak) immune responses that are only occasionally strong enough to lead to spontaneous regressions [16,36–38]. Clinical data additionally show that ablation alone does poorly protect against secondary growth, and thus encompasses a high rate of metastases and recurrent disease. The weak immune responses following ablation suggest that local enforcement by immune interventions can be helpful to boost systemic immunity. In line with this, we have previously demonstrated reduced growth of injected secondary 'metastases' upon ablation of the primary tumor with peritumoral co-injection of CpG-ODN [23]. Additional stimulation with TLR ligands, adjuvants or immune stimulating antibodies might therefore be a valuable tool to combine with local ablation, especially when the stimulating compound is already approved for clinical use. The immune modulating antibody CTLA4 is an example of a tool that is now available for clinical use and could be easily combined with *in situ* tumor destruction techniques [11,39], but also Matrix C might be a promising candidate.

In conclusion, our data demonstrate that anti-tumor immunity following cryo-ablation can be greatly enhanced by co-injection of saponin-based adjuvants. We showed that they promote adaptive immunity via an improved functioning of dendritic cells and increased CTL induction, making these adjuvants, and particularly Matrix C, outstanding tools to combine with *in situ* tumor destruction.

Acknowledgement

This research was supported by Intervet International BV.

Conflict of interest: The authors state that C.S. and E.R. are full time employees of MSD Animal Health.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.11.080.

References

[1] Lindblad EB. Aluminium compounds for use in vaccines. Immunol Cell Biol 2004;82(October (5)):497–505.
[2] Harandi AM, Medaglini D, Shattock RJ. Vaccine adjuvants: a priority for vaccine research. Vaccine 2010;28(March (12)):2363–6.
[3] Wickelgren I. Immunology Mouse studies question importance of toll-like receptors to vaccines. Science 2006;314(December (5807)):1859–60.
[4] Figdor CG, de VI, Lesterhuis WJ, Melief CJ. Dendritic cell immunotherapy: mapping the way. Nat Med 2004;10(May (5)):475–80.
[5] Sun HX, Xie Y, Ye YP. ISCOMs and ISCOMATRIX. Vaccine 2009;27(July (33)):4388–401.
[6] Morein B, Sundquist B, Hoglund S, Dalsgaard K, Osterhaus A. Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. Nature 1984;308(March (5958)):457–60.
[7] Drane D, Gittleson C, Boyle J, Maraskovsky E. ISCOMATRIX adjuvant for prophylactic and therapeutic vaccines. Expert Rev Vaccines 2007;6(October (5)):761–72.
[8] Maraskovsky E, Schnurr M, Wilson NS, Robson NC, Boyle J, Drane D. Development of prophylactic and therapeutic vaccines using the ISCOMATRIX adjuvant. Immunol Cell Biol 2009;87(July (5)):371–6.
[9] Nicholaou T, Ebert LM, Davis ID, McArthur GA, Jackson H, Dimopoulos N, et al. Regulatory T-cell-mediated attenuation of T-cell responses to the NY-ESO-1 ISCOMATRIX vaccine in patients with advanced malignant melanoma. Clin Cancer Res 2009;15(March (6)):2166–73.
[10] den Brok MH, Sutmuller RP, van der Voort R, Bennink EJ, Figdor CG, Ruers TJ, et al. In situ tumor ablation creates an antigen source for the generation of antitumor immunity. Cancer Res 2004;64(June (11)):4024–9.
[11] den Brok MH, Sutmuller RP, Nierkens S, Bennink EJ, Frielink C, Toonen LW, et al. Efficient loading of dendritic cells following cryo and radiofrequency ablation in combination with immune modulation induces anti-tumour immunity. Br J Cancer 2006;95(October (7)):896–905.

[12] Goldszmid RS, Idoyaga J, Bravo AI, Steinman R, Mordoh J, Wainstok R. Dendritic cells charged with apoptotic tumor cells induce long-lived protective CD4+ and CD8+ T cell immunity against B16 melanoma. J Immunol 2003;171(December (11)):5940–7.
[13] Mulier S, Ni Y, Jamart J, Michel L, Marchal G, Ruers T. Radiofrequency ablation versus resection for resectable colorectal liver metastases: time for a randomized trial? Ann Surg Oncol 2008;15(January (1)):144–57.
[14] Apetoh L, Ghiringhelli F, Zitvogel L. Calreticulin dictates the immunogenicity of anti-cancer chemotherapy and radiotherapy. Med Sci (Paris) 2007;23(March (3)):257–8.
[15] Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med 2007;13(September (9)):1050–9.
[16] Sanchez-Ortiz RF, Tannir N, Ahrar K, Wood CG. Spontaneous regression of pulmonary metastases from renal cell carcinoma after radio frequency ablation of primary tumor: an in situ tumor vaccine? J Urol 2003;170(July (1)):178–9.
[17] Jansen T, Hofmans MP, Theelen MJ, Manders F, Schijns VE. Structure- and oil type-based efficacy of emulsion adjuvants. Vaccine 2006;24(June (26)):5400–5.
[18] Ott G, Radhakrishnan R, Fang JH, Hora M. The adjuvant MF59: a 10-year perspective. In: O'Hagan DT, editor. Vaccine adjuvants: preparation methods and research protocols. Totowa, NJ: Humana Press Inc.; 2000. p. 211–28.
[19] Rijke EO, Cornelius L, Schrier C. Experiences with a Tocopherol-based adjuvant in chickens. In: Davison TF, Bumstead N, Kaiser P, editors. Advances in avian immunology research. Oxfordshire, UK: Carfax Publishing Ltd.; 1994. p. 265–71.
[20] Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, et al. In vivo colocalization of antigen and CpG [corrected] within dendritic cells is associated with the efficacy of cancer immunotherapy. Cancer Res 2008;68(July (13)):5390–6.
[21] Karttunen J, Sanderson S, Shastri N. Detection of rare antigen-presenting cells by the lacZ T-cell activation assay suggests an expression cloning strategy for T-cell antigens. Proc Natl Acad Sci USA 1992;89(July (13)):6020–4.
[22] Nierkens S, den Brok MH, Roelofsen T, Wagenaars JAL, Figdor CG, Ruers TJ, et al. Route of administration of the TLR9 agonist CpG critically determines the efficacy of cancer immunotherapy in mice. PLoS ONE 2009;4(12):e8368.
[23] den Brok MH, Sutmuller RP, Nierkens S, Bennink EJ, Toonen LW, Figdor CG<ET-AL. Synergy between in situ cryoablation and TLR9 stimulation results in a highly effective in vivo dendritic cell vaccine. Cancer Res 2006;66(July (14)):7285–92.
[24] Maraskovsky E, Sjolander S, Drane DP, Schnurr M, Le TT, Mateo L, et al. NY-ESO-1 protein formulated in ISCOMATRIX adjuvant is a potent anticancer vaccine inducing both humoral and CD8+ t-cell-mediated immunity and protection against NY-ESO-1+ tumors. Clin Cancer Res 2004;10(April (8)): 2879–90.
[25] Lambrecht BN, Kool M, Willart MA, Hammad H. Mechanism of action of clinically approved adjuvants. Curr Opin Immunol 2009;21(February (1)): 23–9.
[26] Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature 2008;453(June (7198)):1122–6.
[27] Kool M, Petrilli V, de Smedt T, Rolaz A, Hammad H, van Nimwegen M, et al. Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome. J Immunol 2008;181(September (6)):3755–9.
[28] Sharp FA, Ruane D, Claass B, Creagh E, Harris J, Malyala P, et al. Uptake of particulate vaccine adjuvants by dendritic cells activates the NALP3 inflammasome. Proc Natl Acad Sci USA 2009;106(January (3)):870–5.
[29] Demana PH, Davies NM, Vosgerau U, Rades T. Pseudo-ternary phase diagrams of aqueous mixtures of Quil A, cholesterol and phospholipid prepared by the lipid-film hydration method. Int J Pharm 2004;270(February (1–2)): 229–39.
[30] Ronnberg B, Fekadu M, Morein B. Adjuvant activity of non-toxic Quillaja saponaria Molina components for use in ISCOM matrix. Vaccine 1995;13(October (14)):1375–82.
[31] Ronnberg B, Fekadu M, Behboudi S, Kenne L, Morein B. Effects of carbohydrate modification of Quillaja saponaria Molina QH-B fraction on adjuvant activity, cholesterol-binding capacity and toxicity. Vaccine 1997;15(December (17–18)):1820–6.
[32] Schnurr M, Chen Q, Shin A, Chen W, Toy T, Jenderek C, et al. Tumor antigen processing and presentation depend critically on dendritic cell type and the mode of antigen delivery. Blood 2005;105(March (6)):2465–72.
[33] Schnurr M, Orban M, Robson NC, Shin A, Braley H, Airey D, et al. ISCOMATRIX adjuvant induces efficient cross-presentation of tumor antigen by dendritic cells via rapid cytosolic antigen delivery and processing via tripeptidyl peptidase II. J Immunol 2009;182(February (3)):1253–9.
[34] Duewell P, Kisser U, Heckelsmiller K, Hoves S, Stoitzner P, Koernig S, et al. ISCOMATRIX adjuvant combines immune activation with antigen delivery to dendritic cells in vivo leading to effective cross-priming of CD8+ T cells. J Immunol 2011;187(July (1)):55–63.
[35] Wilson NS, Yang B, Morelli AB, Koernig S, Yang A, Loeser S, et al. ISCOMATRIX vaccines mediate CD8(+) T-cell cross-priming by a MyD88-dependent signaling pathway. Immunol Cell Biol 2011;(September).
[36] Machi J, Bueno RS, Wong LL. Long-term follow-up outcome of patients undergoing radiofrequency ablation for unresectable hepatocellular carcinoma. World J Surg 2005;29(November (11)):1364–73.
[37] Sabel MS, Nehs MA, Su G, Lowler KP, Ferrara JL, Chang AE. Immunologic response to cryoablation of breast cancer. Breast Cancer Res Treat 2005;90(March (1)):97–104.
[38] Widenmeyer M, Shebzukhov Y, Haen SP, Schmidt D, Clasen S, Boss A, et al. Analysis of tumor antigen-specific T cells and antibodies in cancer patients treated with radiofrequency ablation. Int J Cancer 2011;128(June (11)): 2653–62.
[39] Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 2010;363(August (8)):711–23.