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## Research paper

# Comparison of two different PEGylation strategies for the liposomal adjuvant CAF09: Towards induction of CTL responses upon subcutaneous vaccine administration



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## ABSTRACT

Using subunit vaccines, *e.g.*, based on peptide or protein antigens, to teach the immune system to kill abnormal host cells *via* induction of cytotoxic T lymphocytes (CTL) is a promising strategy against intracellular infections and cancer. However, customized adjuvants are required to potentiate antigen-specific cellular immunity. One strong CTL-inducing adjuvant is the liposomal cationic adjuvant formulation (CAF)09, which is composed of dimethyldioctadecylammonium (DDA) bromide, monomycoloyl glycerol (MMG) analogue 1 and polyinosinic:polycytidylic acid [poly(I:C)]. However, this strong CTL induction requires intraperitoneal administration because the vaccine forms a depot at the site of injection (SOI) after subcutaneous (*s.c.*) or intramuscular (*i.m.*) injection, and depot formation impedes the crucial vaccine targeting to the cross-presenting dendritic cells (DCs) residing in the lymph nodes (LNs). The purpose of the present study was to investigate the effect of polyethylene glycol (PEG) grafting of CAF09 on the ability of the vaccine to induce antigen-specific CTL responses after *s.c.* administration. We hypothesized that steric stabilization and charge shielding of CAF09 by PEGylation may reduce depot formation at the SOI and enhance passive drainage to the LNs, eventually improving CTL induction. Hence, the vaccine (antigen/CAF09) was post-grafted with a novel type of anionic PEGylated peptides based on GDGDY repeats, which were end-conjugated with one or two PEG<sub>1000</sub> moieties, resulting in mono- and bis-PEG-peptides of different lengths (10, 15 and 20 amino acid residues). For comparison, CAF09 was also grafted by inclusion of 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-methoxy (PEG)-2000 (DSPE-PEG<sub>2000</sub>) in the bilayer structure during preparation. Grafting of CAF09 with either type of PEG resulted in charge shielding, evident from a reduced surface charge. Upon *s.c.* immunization of mice with the model antigen ovalbumin (OVA) adjuvanted with PEGylated CAF09, stronger CTL responses were induced as compared to immunization of mice with unadjuvanted OVA. Biodistribution studies confirmed that grafting of CAF09 with DSPE-PEG<sub>2000</sub> improved the passive drainage of the vaccine to LNs, because a higher dose fraction was recovered in DCs present in the draining LNs, as compared to the dose fraction detected for non-PEGylated CAF09. In conclusion, PEGylation of CAF09 may be a useful strategy for the design of an adjuvant, which induces CTL responses after *s.c.* and *i.m.* administration. In the present studies, CAF09 grafted with 10 mol% DSPE-PEG<sub>2000</sub> is the most promising of the tested adjuvants, but additional studies are required to further elucidate the potential of the strategy.

## 1. Introduction

Guiding the immune system to kill infected or transformed cells constitutes a promising strategy for preventing so-called difficult-to-treat infectious diseases caused by obligate intracellular pathogens and

for treating cancer. It is well established that this requires stimulation of robust antigen-specific, major histocompatibility complex (MHC)-I-restricted cytotoxic T-lymphocyte (CTL) responses that can mediate subsequent killing of diseased cells [1]. The subunit vaccine technology, which exploits peptide or protein antigens consisting of CD8<sup>+</sup>

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T-cell epitopes, is promising for the design of vaccines that can induce CTL responses. However, the vast majority of peptide and protein antigens is not capable of inducing T-cell responses upon immunization. Hence, addition of adjuvants to the vaccines is crucial to stimulate or enhance induction and control of immune responses [2,3].

A prerequisite for priming of CTL responses is that the vaccine is targeted to cross-presenting dendritic cells (DCs), which present antigen-derived peptides on MHC-I to CD8<sup>+</sup> T cells [4,5]. In mice, two developmentally related DC subsets have been shown to be capable of cross-presenting antigens. These include lymph node (LN)-resident CD8 $\alpha^+$  DCs and migratory CD103<sup>+</sup> DCs [4,5]. However, it is not fully understood what the relative importance of each DC subset is for the induction of CTL responses [6–10]. Cross-presenting DCs express high levels of Toll-like receptor 3 (TLR3), and activation of TLR3 has been shown to promote priming of CTL responses [11]. Hence, ligands for TLR3, e.g., the synthetic double-stranded RNA polyinosinic:polycytidylic acid [poly(I:C)], are interesting adjuvants, and poly(I:C) has been exploited in a number of subunit vaccine formulations to enhance induction of CTL responses [12–14].

We recently demonstrated that strong, antigen-specific CTL responses are induced after intraperitoneal (i.p.) immunization with the liposomal cationic adjuvant formulation (CAF)09 in combination with a number of disease-relevant antigens [15]. CAF09 is based on dimethyldioctadecylammonium bromide/monomycolyl glycerol analogue 1 (DDA/MMG-1) liposomes with poly(I:C) adsorbed to the cationic DDA headgroups *via* attractive electrostatic interactions [15]. However, immunization with CAF09-adjuvanted antigens *via* clinically relevant administration routes [*i.e.* subcutaneous (s.c) or intramuscular (i.m.) immunization] results in weak CTL responses. This is probably a consequence of an unfavorable biodistribution profile of the vaccine, which impedes targeting of cross-presenting DCs [16]. Upon injection, the vaccine has been shown to aggregate due to interaction with interstitial proteins at the site of injection (SOI), eventually preventing free drainage to the peripheral LNs and passive targeting to LN-resident CD8 $\alpha^+$  DCs [15,16].

Induction of CTL responses after s.c. or i.m. immunization has been proposed to require passive drainage to the draining LNs (dLNs) *via* the lymphatics in order to passively target cross-presenting DCs localized in the dLNs [17]. Nanoparticles with a size below approximately 200 nm have been shown to passively drain *via* the lymphatics to the secondary lymphoid organs [17–19]. Therefore, aggregation of the vaccine at the SOI after administration should be avoided, as it may hinder passive drainage by preventing particle permeation through the endothelial cell wall of the lymphatics [17].

One strategy for reducing aggregation, and hence depot formation at the SOI with concomitantly enhanced clearance to the dLNs, is to surface-graft the liposomes with so-called stealth polymers, e.g., polyethylene glycol (PEG) [20]. PEGylation has been shown to increase the drainage of liposomes from the SOI upon s.c. immunization [21]. In addition, PEGylation of the liposomal vaccine adjuvant CAF01 based on DDA and trehalose 6,6'-dibehenate (TDB) resulted in decreased retention at the SOI and increased recovery of the liposomes in the dLNs upon s.c. administration, as compared to non-PEGylated CAF01 [22,23]. This effect was dependent on the PEG grafting density of the liposomes; liposomes with a grafting density of 25 mol% 1,2-distearoyl-

*sn*-glycero-3-phosphoethanolamine-N-methoxy(PEG)-2000 (DSPE-PEG<sub>2000</sub>) were drained to a higher extent than liposomes grafted with 10 mol% DSPE-PEG<sub>2000</sub> [22,23].

A major drawback of PEGylating vaccines is that the introduction of a shielding stealth layer on the surface also reduces binding and uptake of liposomes by antigen-presenting cells (APCs), causing loss of vaccine efficacy. The uptake level of PEGylated liposomes by APCs has been shown to be inversely proportional to the PEG grafting density [24,25]. Furthermore, PEGylation of liposomes reduces protein adsorption, which is inversely proportional to the PEG-chain length and the PEG grafting density [26,27]. It is well known that the reduced interaction with proteins like opsonins and lipoproteins results in increased blood circulation times of liposomes [28]. However, PEGylation may also interfere with antigen adsorption to adjuvants, as shown recently for the protein antigen Ag85B-ESAT6, which displayed reduced adsorption to CAF01 when the adjuvant was PEGylated [22].

Hence, a promising strategy is to design liposomal vaccine adjuvants, which are capable of shedding the PEG layer prior to APC interaction. Grafting of liposomes with lipopolymers containing short lipid anchors, e.g., PEG-ceramides C<sub>8</sub> and C<sub>14</sub>, have been shown to decrease the dissociation half-lives to less than 1.2 min and ~1.1 h, respectively, as compared to a half-life of more than 13 days for dissociation of PEG-ceramide C<sub>20</sub> from dioleoylphosphatidylethanolamine (DOPE):dioleoyldimethylammonium chloride (DODAC) liposomes [29]. An alternative strategy is to insert degradable linkers between the lipid anchor and the PEG moiety, which can be cleaved by acid-catalyzed hydrolysis or *via* proteolysis. Thus, the linkers are cleaved in response to an environmental stimulus, e.g., the reduced pH in the endosomes (pH-sensitive linkers), or in the presence of specific proteolytic enzymes (peptide-based linkers) [30].

The purpose of this study was to investigate the effect of PEGylation on the ability of CAF09 to induce antigen-specific CTL responses after s.c. administration using net anionic ovalbumin (OVA) as model antigen. We compared two different PEGylation strategies. First, we tested a new approach for post-preparation surface grafting with PEG moieties. For this, a novel type of PEG-peptides, composed of an anionic peptide backbone (GDGDY repeats) and end-conjugated to one or two PEG<sub>1000</sub> moieties were used (Table 1). The rationale for this design was concomitant post-adsorption of anionic PEG-peptide and OVA to the net positively charged cationic liposomes *via* attractive electrostatic interactions. Our hypothesis was that surface grafting of CAF09 with such PEG-peptides would reduce depot formation upon s.c. injection and enhance vaccine clearance to the dLNs. We also envisaged that the PEG-peptide may subsequently desorb from the antigen-coated liposomes at a rate depending on: (i) the length of the anionic peptide chain, and (ii) the grafting density. This could potentially enhance vaccine interaction with APCs in the dLNs. The second strategy included conventional PEGylation of CAF09 by inclusion of DSPE-PEG<sub>2000</sub> in the liposomal bilayer structure during preparation, followed by OVA adsorption to the PEGylated CAF09.

**Table 1**  
Array of PEG-peptides. These analogues include mono- and bis-PEG-peptides.

Abbreviation	Class of PEG-peptide	Peptide length	Peptide sequence
aa-10-monoPEG <sub>1000</sub>	MonoPEG-peptide	10	GDGDY GDGDY
aa-15-monoPEG <sub>1000</sub>		15	GDGDY GDGDY GDGDY
aa-20-monoPEG <sub>1000</sub>		20	GDGDY GDGDY GDGDY GDGDY
aa-10-bisPEG <sub>1000</sub>	BisPEG-peptide	10	GDGDY GDGDY
aa-15-bisPEG <sub>1000</sub>		15	GDGDY GDGDY GDGDY
aa-20-bisPEG <sub>1000</sub>		20	GDGDY GDGDY GDGDY GDGDY

## 2. Materials and methods

### 2.1. Materials

DDA and MMG-1 were purchased from NCK A/S (Farum, Denmark), while DSPE-PEG<sub>2000</sub> was acquired from Avanti Polar Lipids (Alabaster, AL, USA).  $\gamma$ -Irradiated poly(I:C) was obtained from Sigma-Aldrich (St. Louis, MO, USA), and endograde OVA was from Hyglos GmbH (Bernried am Starnberger See, Germany). All other chemicals were used at analytical grade and purchased from commercial suppliers. Tris buffer (10 mM, pH 7.4) was used throughout the studies owing to the optimal stability of CAF01 in this buffer [31].

### 2.2. Synthesis of PEG-peptides

An array of PEG-peptides was designed to study the influence of peptide length [10, 15, and 20 amino acid (aa) residues, respectively] and degree of PEGylation (mono- versus bis-PEGylation) on the adsorption of the PEG-peptides to CAF09 (Table 1). The peptides were composed of blocks of five aa (GDGDY) repeated two, three and four times, respectively, and they were synthesized at > 95% purity by Genecust (Ellange, Luxembourg). The conjugates were synthesized by N-terminal PEGylation, as described elsewhere [32]. Briefly, 50 nmol of peptide was dissolved in acetate buffer (pH 5, NaOAc·H<sub>2</sub>O; 17 ml), and subsequently 5–6 equivalents of PEG-aldehyde (1101.31 g/mol, Iris Biotech GmbH, Marktredwitz, Germany) were added to the solution, followed by 50 equivalents of NaCNBH<sub>3</sub> in 3 ml acetate buffer. For the 10 aa peptide, the mixture was stirred for approximately 3 h, while the 15 aa peptide mixture was stirred for approximately 24 h. Then 17 equivalents of NaCNBH<sub>3</sub> were added, and the mixture was stirred for an additional 2 h. Analytical HPLC (see below) of the resulting mixture indicated peaks corresponding to both mono- and bis-PEG-peptides (results not shown).

Analytical HPLC of the PEG-peptides was performed using a Luna 2.5  $\mu$ m C18(2) High Speed Technology (HST) column (3.0  $\times$  100 mm) with a flow of 0.5 ml/min, or a Luna 3  $\mu$ m C18(2) column (4.6  $\times$  150 mm) (both Phenomenex, Torrance, CA, USA) at a flow rate of 0.8 ml/min. Peaks were UV-detected at 220 nm. Binary mixtures of eluent A [5% (v/v) MeCN in H<sub>2</sub>O with 0.1% (v/v) trifluoroacetic acid (TFA)] and eluent B [95% MeCN in H<sub>2</sub>O with 0.1% (v/v) TFA] were used. For the Luna 2.5  $\mu$ m column, the fraction of eluent B to eluent A was increased from 10 to 60% (v/v) in 10 min, while for the Luna 3  $\mu$ m column, the fraction of eluent B to eluent A was increased from 10 to 60% (v/v) in 30 min. The retention times (RT) of the PEG-peptides were: 10aa-monoPEG RT = 6.02 min (Luna 2.5  $\mu$ m), RT = 22.13 min (Luna 3  $\mu$ m); 10aa-bisPEG RT = 7.46 min (Luna 2.5  $\mu$ m), RT = 26.25 min (Luna 3  $\mu$ m); 15aa-monoPEG RT = 5.96 min (Luna 2.5  $\mu$ m), RT = 22.52 min (Luna 3  $\mu$ m); 15aa-bisPEG RT = 7.31 min (Luna 2.5  $\mu$ m), RT = 26.81 min (Luna 3  $\mu$ m). Purification was performed by preparative HPLC using a Luna 5  $\mu$ m C18(2) column (30  $\times$  250 mm) at a flow rate of 40 ml/min, and increasing the fraction of eluent B from 10 to 45% (v/v) in 20 min. All peptides were > 95% pure.

### 2.3. Preparation of liposomes

Liposomes were prepared by dissolving weighed amounts of lipids in 99% (v/v) EtOH. In a glass vial, DDA, MMG-1, DSPE-PEG<sub>2000</sub> and the fluorescent label 3,3'-dioctadecyloxycarbocyanine perchlorate (DiO, Life Technologies, Nærum, Denmark) were mixed and dried under a stream of N<sub>2</sub> for 2 h, followed by air-drying overnight. The dry lipid films were rehydrated in Tris buffer by high-shear mixing (HSM) using a Heidolph Silent Crusher equipped with a 6F shearing tool (Heidolph Instruments GmbH, Schwabach, Germany) at 60 °C and 26,000 rpm for 15 min. The poly(I:C) was added continuously during HSM using a peristaltic pump (Pharmacia Biotech, Stockholm, Sweden) over 30 min.

The concentrations of the components in the resulting dispersions were 2.5/0.5/0.5 mg/ml DDA/MMG-1/poly(I:C), eventually supplemented with 0.07–1.51 mg/ml DSPE-PEG<sub>2000</sub> (0.5–10 mol%) and/or 20  $\mu$ g/ml DiO, in a dose volume of 100  $\mu$ l. To prepare liposomes coated with PEG-peptides, CAF09 was added to PEG-peptides diluted to different concentrations in Tris buffer (1:1, v/v), and then formulations were mixed by continuous rotation for 30 min to ensure maximal adsorption of the PEG-peptides to the liposomes. The PEG-peptides (1–10 mol%) diluted in Tris buffer were added to CAF09 at 1:1 (v/v) (Suppl. Table S1).

### 2.4. Physicochemical characterization of liposomes

The average hydrodynamic diameter ( $z$ -average) of the liposomes and the polydispersity index (PDI) were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK) equipped with a 633 nm laser and 173° detection optics. The samples were diluted 10 times in Tris buffer, and the values of water were used for viscosity and refractive indexes. The zeta-potentials were measured by diluting the liposomes 100 times in milliQ water. Zetasizer Software v7.11 (Malvern Instruments Ltd) was used for acquisition and analysis of data. Adsorption of OVA to CAF09-PEG-lipid was evaluated at grafting densities of 0, 1, 2.5, 5, and 10 mol% DSPE-PEG<sub>2000</sub> and final OVA concentrations in the range of 0.05–1.5 mg/ml. The liposome dispersions and OVA were mixed for 30 min to ensure adsorption prior to ultracentrifugation at 137,500g for 30 min. at 4 °C. The amount of non-adsorbed OVA in the supernatant was determined by using the bicinchoninic acid (BCA) assay (Pierce BCA protein assay kit, ThermoFisher Scientific Inc. Waltham, MA, USA). The adsorption of OVA to CAF09 grafted with 10 mol% aa15-bisPEG<sub>1000</sub> was evaluated in Tris buffer at pH 7.4 by mixing 10.0  $\mu$ l 2 mg/ml AlexaFluor (AF) 647-conjugated OVA (ThermoFisher Scientific Inc.) with 15.4  $\mu$ l 10 mg/ml PEG-peptide and 100  $\mu$ l CAF09 in a total volume of 200  $\mu$ l in 1.5 ml low adsorption Eppendorf tubes. The mixture was left to equilibrate at room temperature (rt) for approximately 10 min and ultracentrifuged at 137,500g for 30 min. at 4 °C. The concentration of non-adsorbed OVA in the supernatant was determined by fluorescence spectroscopy using a Fluostar Optima (BMG Labtech, Ortenberg, Germany). The percentage of liposome-adsorbed protein was calculated by subtracting the amount of protein remaining in solution from the amount of protein initially added to the liposome dispersion.

### 2.5. Biodistribution studies

The animal experiments were conducted in accordance with the national Danish guidelines for animal experiments, as approved by the Danish Council for Animal Experiments and in accordance with EU directive 2010/63/EU for animal experiments. All efforts were made to ensure maximum comfort for the animals, and they were allowed free access to food and water. The biodistribution of the antigen and the adjuvant, respectively, and the influx of immune cells to the SOI and the dLNs upon i.m. immunization were compared for the vaccines adjuvanted with non-PEGylated and PEGylated CAF09, respectively. The i.m. administration route was applied, as it enabled removal of the SOI for analysis. The vaccines adjuvanted with non-PEGylated CAF09 and CAF09 grafted with 10 mol% DSPE-PEG<sub>2000</sub>, respectively, were prepared by mixing the adjuvant and the model antigen OVA (10  $\mu$ g/dose) in Tris buffer for 30 min with intermittent vortexing. For the formulations with 20 mol% aa15-bisPEG<sub>1000</sub>, OVA and the PEG-peptide were diluted in Tris buffer prior to addition of CAF09, and the three components were mixed for 30 min. Female, C57BL/6 mice aged 8–10 weeks were immunized i.m. in both quadriceps with vaccines prepared as described above using the DiO-containing adjuvant and AF647-conjugated OVA (15  $\mu$ g/dose) in a dose volume of 50  $\mu$ l/muscle. The mice were euthanized 3, 24, 48, 96 and 168 h after immunization, respectively, and the dLNs and quadriceps were isolated.

## 2.6. Immunization studies

The immune responses to the vaccines OVA/CAF09 and OVA/CAF09 with 10 mol% aa15-bisPEG<sub>1000</sub> (OVA/CAF09-PEG-peptide) and 10 mol% DSPE-PEG<sub>2000</sub> (OVA/CAF09-PEG-lipid), respectively, were evaluated by immunization of female, 7–9-week-old C57BL/6 mice (Harlan, Horst, The Netherlands). The mice were immunized two times s.c. at the base of the tail with a two-week interval, and the inguinal LNs (ILN), the spleen and the blood were harvested one week after the final immunization. The s.c. administration route was chosen for the immunization studies, because it inflicts less pain to the mice, as compared to i.m. immunization.

## 2.7. Preparation of organs

Single cell suspensions of the LNs and the spleens were prepared by passage of the organs through a nylon mesh cell strainer, followed by washing in phosphate-buffered saline (PBS) or RPMI 1640 (Gibco Invitrogen, Carlsbad, CA, USA) or FACS-buffer [PBS with 10% fetal calf serum (FCS)]. The muscles were treated with the enzymes A, D and P from the Skeletal Muscle Dissociation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions, and 200 µl of the supernatants were removed prior to washing, which was performed as described above. Single cell suspensions used for re-stimulation studies were resuspended in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS,  $5 \times 10^{-6}$  M β-mercaptoethanol, 1% (v/v) penicillin–streptomycin, 1% (v/v) sodium pyruvate, 1 mM L-glutamine, and 10 mM HEPES. The blood was harvested in EDTA-tubes and loaded onto Lympholyte (Cedarlane, Burlington, Canada) followed by centrifugation at 900g for 20 min. The lymphocytes were harvested and treated as described above.

## 2.8. Intracellular and surface flow cytometry

For the biodistribution studies, immune cells in the muscle tissue and the dLNs were identified by surface staining the cells with anti-Ly6G:PE (1A8, BD Biosciences, San Jose, NJ, USA), anti-CD11b:PerCP-Cy5.5 (M1/70, BD Biosciences), anti-F4/80:PE-Cy7 (BM8, eBioscience, San Diego, CA, USA), anti-Ly6C:APC-Cy7 (HK1.4, Biolegend, San Diego, CA, USA), anti-CD11c:BV421 (HL3, BD Biosciences), anti-CD86:BV510 (GL1, BD Biosciences), and anti-CD19:BV786 (1D3, BD Biosciences). Cellular subsets were identified by the presence or absence of surface markers, essentially as described previously [33] (Suppl. Fig. S1 and S2). The data were acquired by using a BD Fortessa or a BD Canto flow cytometer (BD Biosciences), and they were analyzed by using the FlowJo vX software (Tree Star, Ashland, OR, USA).

The frequency of OVA-specific CD8<sup>+</sup> T cells was determined by SIINFEKL-pentamer flow cytometry. A number of  $10^6$  cells/well was stained with PE-labelled H-2 K<sup>b</sup>-SIINFEKL (ProImmune, Oxford, UK) for 15 min at rt, followed by treatment with Fc-block (anti-mouse CD16/CD32, BD Biosciences) for 10 min and staining with anti-CD62L:FITC (MEL-14, BD Biosciences), anti-CD8:PerCP-Cy5.5 (53–6.7, eBioscience), anti-CD19:PE-Cy7 (1D3, BD Biosciences), anti-CD44:APC (IM7, BD Biosciences), and anti-CD4:eFluor780 (RM4-5, eBioscience), respectively, for 30 min.

## 2.9. Meso scale discovery (MSD) assay

Induction of pro-inflammatory cytokines and chemokines in the muscles following immunization were evaluated by MSD (Meso Scale Diagnostics LLC, Rockville, MD, USA). A seven-spot pro-inflammatory plate [Interferon (IFN)-γ, IL-1β, IL-6, keratinocyte chemoattractant/human growth-regulated oncogene (KC/GRO), IL-10, IL-12p70, and tumor necrosis factor (TNF)-α] and a separate monocyte chemoattractant protein (MCP)-1 plate were used. A volume of 25 µl undiluted muscle supernatant was pipetted into each well and diluted with 25 µl

Diluent 41, followed by incubation for 2 h on a shaking table at rt. The plates were washed with PBS supplemented with 0.05% (v/v) Tween 20, followed by incubation with detection antibodies for 2 h on a shaking table at rt. Finally, read buffer was added to the plates, and they were read using a Meso Sector S600 with Discovery Workbench 4.0. MSD software (Meso Scale Diagnostics LLC).

## 2.10. Statistical analysis

One-way or two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test were used to analyze the difference between the individual groups using GraphPad Prism version 6.05 for Windows (GraphPad Software, La Jolla, CA, USA).

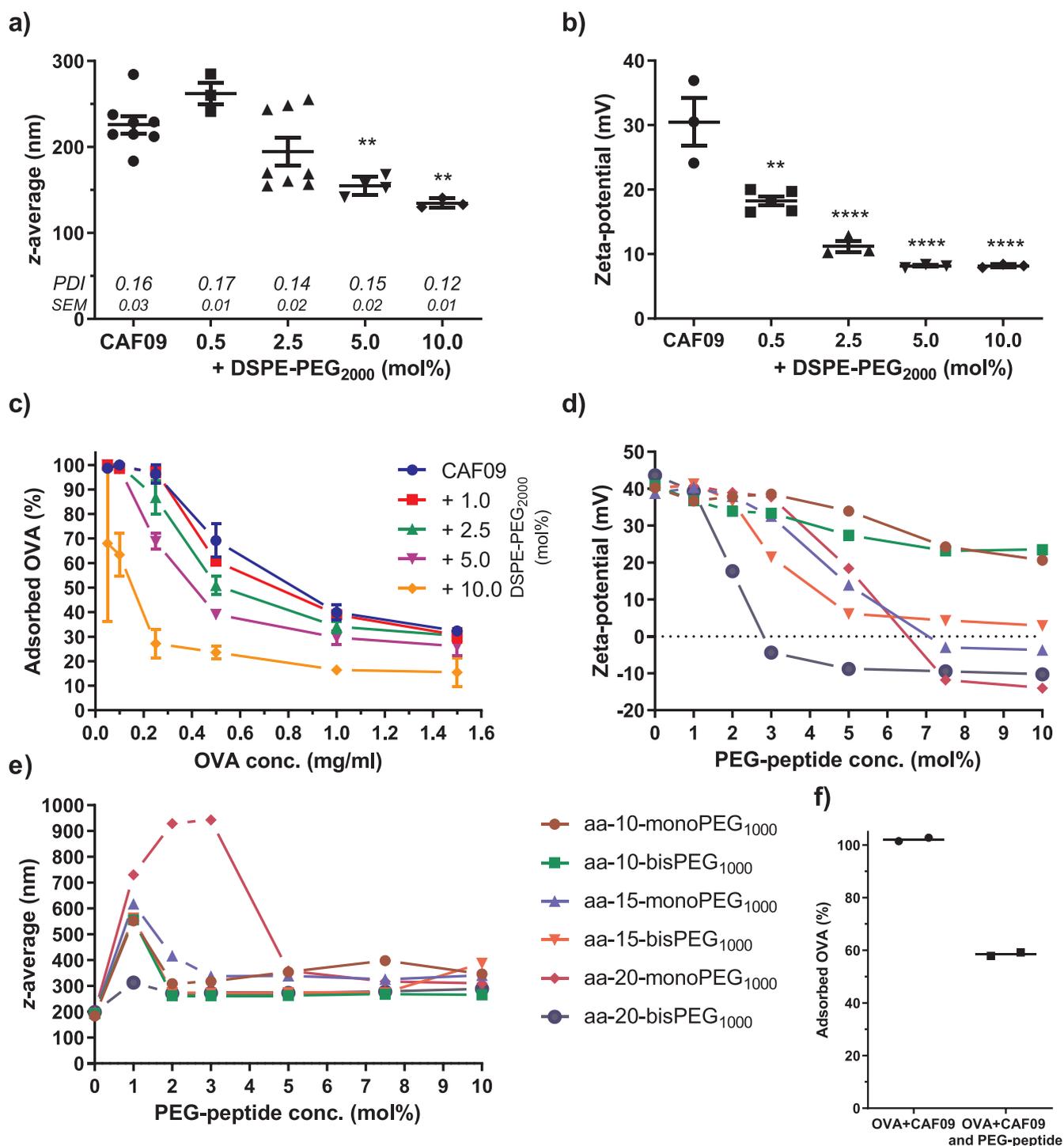
## 3. Results

### 3.1. Pegylation of CAF09 reduces the particle size and zeta-potential

CAF09 was PEGylated using 2.5, 5.0, or 10 mol% of DSPE-PEG<sub>2000</sub>, and the average hydrodynamic particle diameter and PDI were measured. At 5 and 10 mol% DSPE-PEG<sub>2000</sub>, the z-average was significantly smaller than the z-average of the non-PEGylated CAF09, while the PDI was not significantly affected (Fig. 1a). As expected, the zeta-potential was reduced as a function of the PEG grafting density, resulting in near-neutral zeta-potential at PEG grafting densities of 5 and 10 mol% (Fig. 1b).

The degree of adsorption of the model antigen OVA to PEGylated CAF09 was determined at different DSPE-PEG<sub>2000</sub> grafting densities (Fig. 1c). For grafting densities up to 5 mol%, OVA was 100% adsorbed to CAF09 at concentrations below 0.2 mg/ml, which covers the concentration range usually used in vaccines (0.05–0.075 mg/ml). At increasing OVA concentration, the amount of OVA adsorbed to the liposomes decreased as a function of the PEG-grafting density. However, it was difficult to sediment CAF09 with 10 mol% DSPE-PEG<sub>2000</sub> during ultracentrifugation, especially at low OVA concentrations, maybe as a combined result of steric repulsion between the PEG chains. The morphology of CAF09 grafted with 10 mol% DSPE-PEG<sub>2000</sub> was imaged using cryo-transmission electron microscopy (cryoTEM). The observed liposomes displayed varying degrees of lamellarity and sizes (Suppl. Fig. S3), as shown previously for non-PEGylated CAF09 [16].

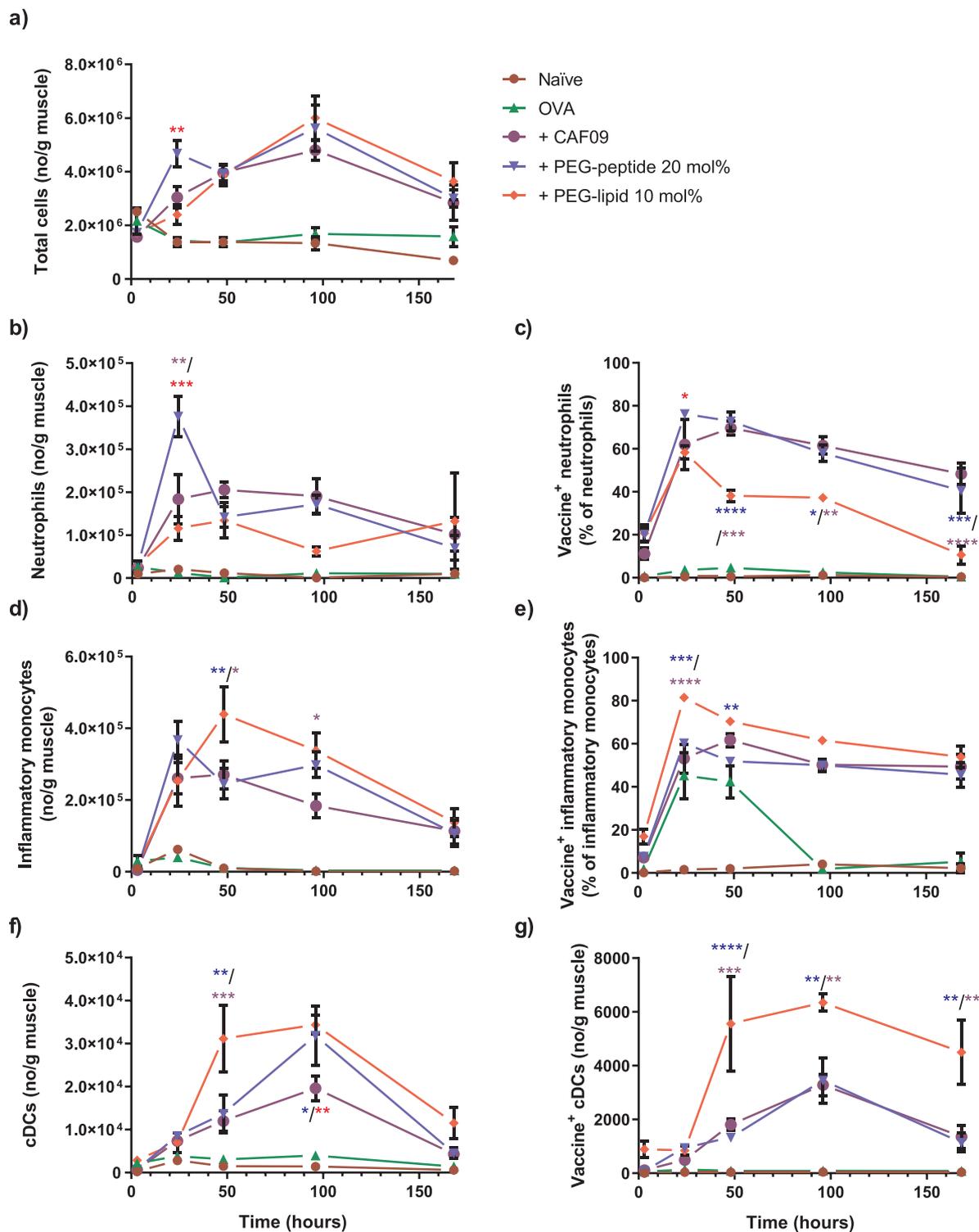
In addition, CAF09 was PEGylated using the PEG-peptides of different peptide chain lengths (10, 15 and 20 aa), which were mono- and bis-PEGylated, respectively (Table 1). The PEG-peptides and CAF09 were mixed at different molar ratios. The mixture was incubated for 30 min with intermittent agitation to allow complete adsorption of the anionic PEG-peptides to the cationic liposomal surface prior to measuring the z-average and the zeta-potential of the formulations. As expected, the zeta-potentials decreased at increasing molar ratios of PEG-peptides. At 10 mol%, the zeta-potentials depended on the length of the peptide; surface grafting with 10 mol% 10 aa PEG-peptide resulted in a zeta-potential of approximately 20–30 mV, whereas coating with the 15 aa PEG-peptide resulted in a near neutral zeta-potential, and CAF09 grafted with the 20 aa PEG-peptides displayed a zeta-potential of approximately –20 mV (Fig. 1d). The zeta-potential was not dependent on the degree of PEGylation (mono- versus bis-PEGylation) in the examined concentration range. The effect on zeta-potential suggests that the PEG-peptides are capable of shielding the cationic surface charge. When the 20 aa peptide was used at high molar ratios, the negative charge of the peptides might exceed the cationic charge of the liposomes. The z-average of the liposomes was largely influenced by the molar ratio, as PEGylation at the lowest molar ratios tested resulted in aggregation of the liposomes, which was not evident at higher molar ratios. It is likely that the presence of the PEG chains prevents aggregation at the higher molar ratios tested, while bridging attractive electrostatic interactions exist between the peptide and liposomes at the lower molar ratios tested, eventually resulting in aggregation (Fig. 1e).



**Fig. 1.** Physicochemical characterization of PEGylated CAF09. (a) z-average and PDI of CAF09-PEG-lipid. Data represent mean values  $\pm$  SEM, n = 3–8. \*\* $p \leq 0.01$ , as compared to CAF09. (b) Zeta-potential of CAF09 grafted with DSPE-PEG<sub>2000</sub>. Data represent mean values  $\pm$  SEM, n = 3–5. \*\* $p \leq 0.01$  and \*\*\*\* $p \leq 0.0001$ , as compared to CAF09. (c) Relative adsorption in percent of OVA to CAF09 grafted with DSPE-PEG<sub>2000</sub>. Data represent mean values  $\pm$  SEM, n = 3. (d) Zeta-potential and (e) z-average of CAF09 PEGylated with different PEG-peptides, n = 2. (f) Adsorption of AF647-conjugated OVA to non-PEGylated CAF09 and CAF09 grafted with 10 mol% aa-15-bisPEG<sub>1000</sub>, n = 2.

The liposome dispersions grafted with PEG-peptides were relatively monodisperse displaying PDI values below 0.2, except for CAF09 grafted with aa20-monoPEG<sub>1000</sub>, which had PDIs of approximately 0.35 (Suppl. Fig. S4). The adsorption of the model antigen OVA labelled with AF647 was only determined for the adjuvant tested in the subsequent immunogenicity studies (CAF09 grafted with 10 mol% aa15-bisPEG<sub>1000</sub>). The adsorption of OVA to CAF09 grafted with aa15-bisPEG<sub>1000</sub> was approximately 59% (Fig. 1f). In contrast 100% OVA

was adsorbed to non-PEGylated CAF09. The reduction in OVA adsorption to CAF09 after grafting with 10 mol% aa15-bisPEG<sub>1000</sub> may be due to attractive electrostatic interactions between the cationic DDA headgroups and the PEG-peptide at this concentration, evident from the neutral zeta-potential (Fig. 1d). Hence, shielding of the cationic surface charge by grafting with PEG-peptide may reduce the available cationic surface charge responsible for adsorption of negatively charged OVA, which has an isoelectric point of 4.5.



**Fig. 2.** Cellular composition in the quadriceps following i.m. immunization with unadjuvanted OVA, OVA adjuvanted with non-PEGylated CAF09, CAF09-PEG-peptide (20 mol% aa-15-bisPEG<sub>1000</sub>) and CAF09-PEG-lipid (10 mol% DSPE-PEG<sub>2000</sub>). (a) Total influx of cells into the quadriceps. (b) Number of neutrophils in quadriceps per g muscle. (c) The frequency of vaccine<sup>+</sup> neutrophils. (d) Number of inflammatory monocytes in quadriceps per g muscle. (e) The frequency of vaccine<sup>+</sup> inflammatory monocytes. (f) The number of cDCs in the quadriceps. (g) The number of vaccine<sup>+</sup> cDCs in the quadriceps. Data represent mean  $\pm$  SEM, n = 2 (naïve mice and mice immunized with unadjuvanted OVA) and 4 (mice immunized with adjuvanted OVA). 2-way ANOVA followed by Tukey's multiple comparisons test comparing the groups; CAF09, CAF09 + PEG-peptide 20 mol% and CAF09 + PEG-lipid 10 mol%, \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , \*\*\*\* $p \leq 0.0001$ . Significance markers are colored according to involved groups.

### 3.2. Biodistribution of vaccines following i.m. immunization

Mice were immunized i.m. in the quadriceps with AF647-conjugated OVA adjuvanted with DiO-labelled formulations (i) CAF09, (ii)

CAF09 with 20 mol% PEG-peptide (aa15-bisPEG<sub>1000</sub>, referred to as CAF09-PEG-peptide) and (iii) CAF09 with 10 mol% PEG-lipid (DSPE-PEG<sub>2000</sub>, referred to as CAF09-PEG-lipid), respectively. The choice of the specific molar PEG grafting densities was based on the

physicochemical characteristics of the resulting liposome dispersions: CAF09 grafted with 20 mol% PEG-peptide displayed particle sizes and zeta-potentials (219 nm and 7 mV, respectively) comparable to those of CAF09 grafted with 10 mol% PEG-peptide. Hence, grafting densities between 0 and 10 mol% were investigated further. The number of immune cells present in the quadriceps increased significantly within the first 48 h after immunization, as compared to the number of cells for naïve mice (Fig. 2a), whereas there was no significant change in the number of immune cells in the dLNs throughout the study (results not shown). The identity of the immune cells and their relative degree of vaccine association at the SOI and in the dLNs were evaluated by flow cytometry (Suppl. Fig. S1 and S2).

In the quadriceps, the OVA/CAF09-PEG-peptide vaccine caused the largest influx of neutrophils ( $\text{Ly6G}^+$ ) 24 h after immunization, while the OVA/CAF09-PEG-lipid vaccine induced a slightly larger influx of inflammatory monocytes ( $\text{F4/80}^+ \text{Ly6C}^+$ ) (Fig. 2b/c). Interestingly, the frequency of vaccine-positive neutrophils was lower for the group immunized with the OVA/CAF09-PEG-lipid vaccine at late time points, as compared to the other vaccines (Fig. 2d). In contrast, the CAF09-PEG-lipid vaccine was associated with inflammatory monocytes to a slightly higher extent than the other vaccines (Fig. 2e). Furthermore, unadjuvanted OVA was only associated with inflammatory monocytes and not with neutrophils (Fig. 2e).

Influx of conventional DCs (cDCs;  $\text{CD11c}^+$ ,  $\text{CD11b}^+$ ) was detectable at the SOI 48 h after administration, and was thus delayed, as compared to the influx of neutrophils and inflammatory monocytes to the SOI (Fig. 2f). Immunization with the OVA/CAF09-PEG-lipid vaccine caused a higher influx of cDCs into the quadriceps and resulted in a significantly higher number of vaccine<sup>+</sup> cDCs at the late time points, as compared to immunization with the other adjuvants (Fig. 2g).

### 3.3. Immunization with CAF09-PEG-peptide induces a pro-inflammatory environment at the SOI

The cytokine responses at the SOI after immunization with OVA/CAF09, OVA/CAF09-PEG-lipid, and OVA/CAF09-PEG-peptide were evaluated by MSD (Fig. 3). The cytokines and chemokines IFN- $\gamma$ , IL-1 $\beta$ , KC/GRO, MCP-1 and TNF- $\alpha$  were all induced by the adjuvants, while there was no significant induction of IL-10, IL-12p70 and IL-6. For KC/GRO, induction was observed 3 h after immunization, which suggests that a minor dose fraction of the vaccine is phagocytosed at that time point (results not shown). The cytokine levels were increased at 24 h p.i. for the immunized groups, and the OVA/CAF09-PEG-peptide vaccine induced significantly higher IFN- $\gamma$  and IL-1 $\beta$  responses than OVA/CAF09 (only IFN- $\gamma$ , Fig. 3a) and OVA/CAF09-PEG-lipid (Fig. 3b), and induced higher levels of TNF- $\alpha$ , MCP-1 and KC/GRO, than the OVA/CAF09 vaccine (Fig. 3c–e). Hence, the OVA/CAF09-PEG-peptide vaccine appears to have a greater pro-inflammatory potential than the other vaccines, possibly due to desorption of poly(I:C) from the vaccine after administration.

### 3.4. CAF09 PEGylated with DSPE-PEG2000 associates with cDCs in the dLNs

The vaccine recovery in the dLNs was measured as the frequency of OVA<sup>+</sup> and DiO<sup>+</sup> single-positive lymphocytes (Fig. 4a/b). The frequency of OVA<sup>+</sup>DiO<sup>+</sup> double-positive cells was negligible (Suppl. Fig. S2), hence this cell population was not considered further. Immunization with OVA adjuvanted with CAF09-PEG-lipid resulted in significantly larger association of OVA with cells 24 p.i., as compared to the other groups, whereas immunization with OVA adjuvanted with CAF09-PEG-peptide resulted in less OVA association with the cells (Fig. 4a). The presence of OVA<sup>+</sup> cells might indicate that a fraction of the antigen is desorbed from the PEGylated adjuvants and drains passively to the dLNs.

Only immunization with OVA adjuvanted with CAF09-PEG-lipid

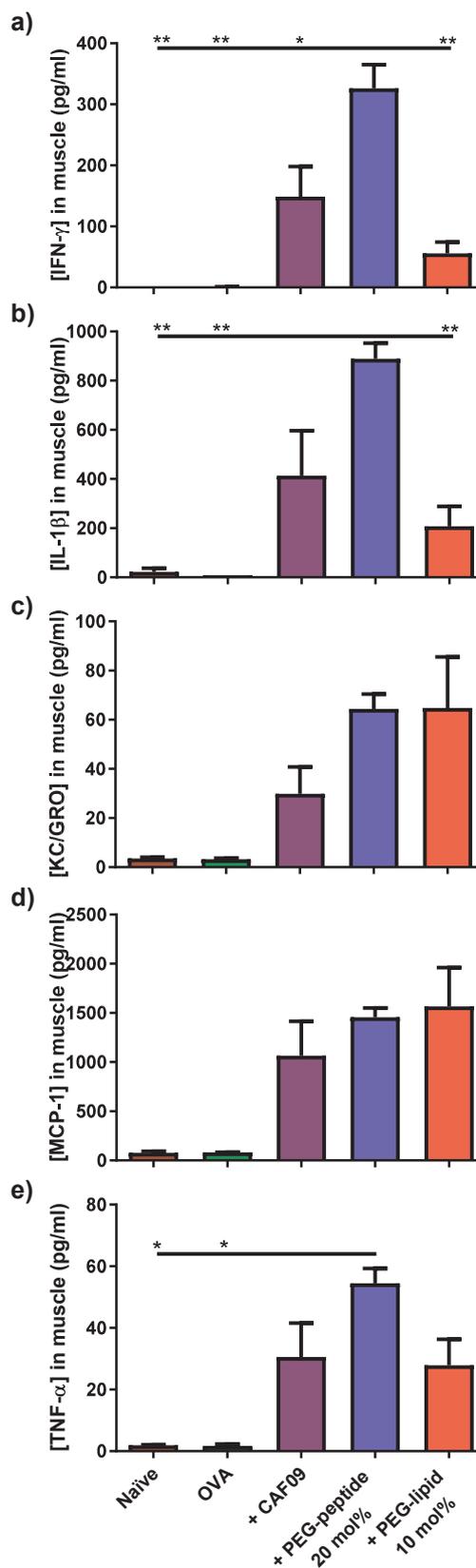
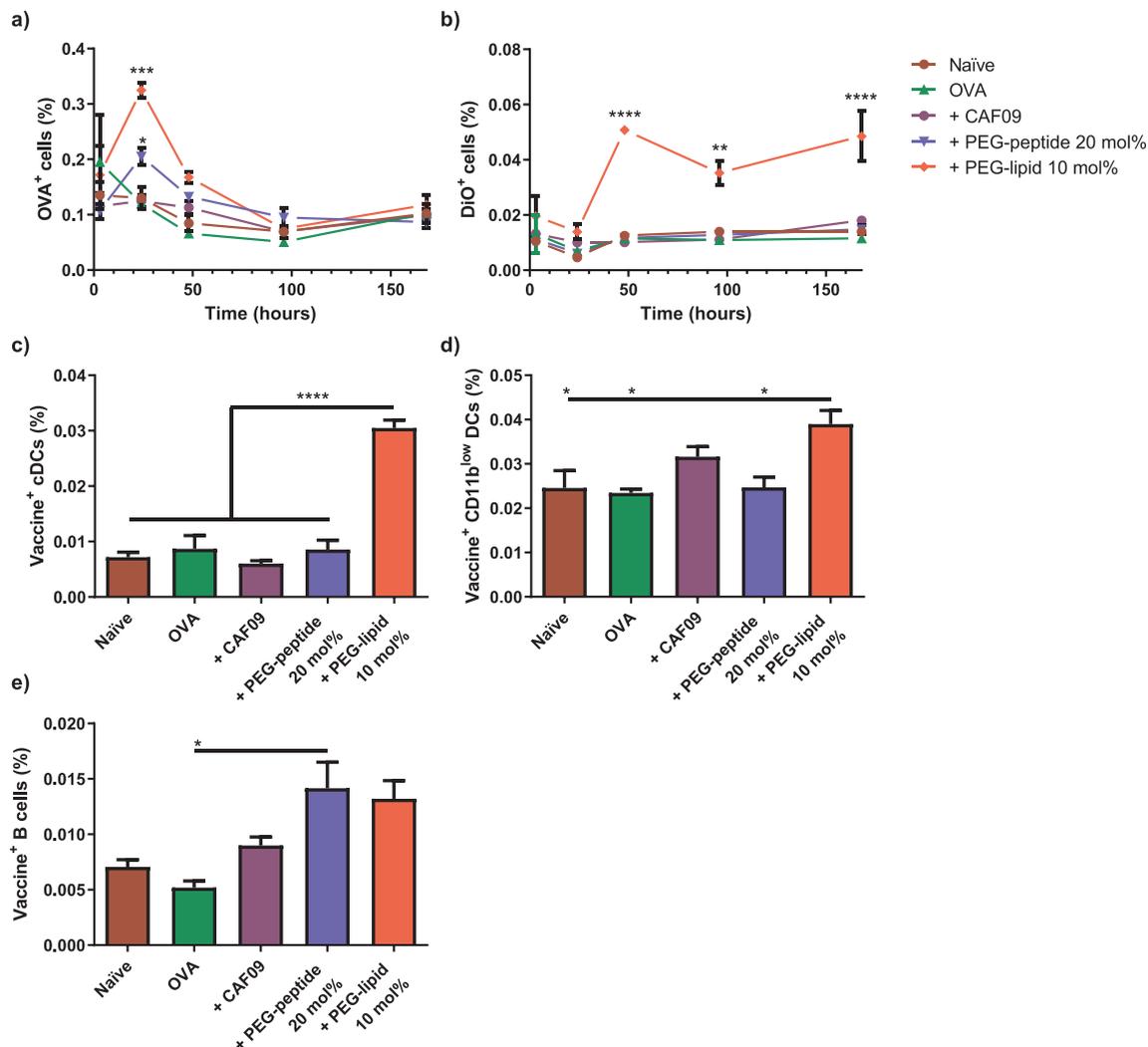


Fig. 3. Inflammatory cytokines in the quadriceps, as measured by MSD, 24 h following i.m. immunization with unadjuvanted OVA, OVA adjuvanted with non-PEGylated CAF09, CAF09-PEG-peptide (20 mol% aa-15-bisPEG<sub>1000</sub>) and CAF09-PEG-lipid (10 mol% DSPE-PEG<sub>2000</sub>). (a) IFN- $\gamma$ , (b) IL-1 $\beta$ , (c) KC/GRO, (d) MCP-1, and (e) TNF- $\alpha$ . \*\* $p \leq 0.01$  and \* $p \leq 0.05$  CAF09-PEG-peptide as compared to the other groups. Data represent mean  $\pm$  SEM,  $n = 2$  (naïve mice and mice immunized with unadjuvanted OVA) and 4 (mice immunized with adjuvanted OVA).



**Fig. 4.** Recovery of vaccine in the dLNs as measured by flow cytometry, following i.m. immunization with unadjuvanted OVA, OVA adjuvanted with non-PEGylated CAF09, CAF09-PEG-peptide (20 mol% aa-15-bisPEG<sub>1000</sub>) and CAF09-PEG-lipid (10 mol% DSPE-PEG<sub>2000</sub>). (a) Frequency of OVA<sup>+</sup> cells. \*\*\* $p \leq 0.001$  CAF09-PEG-lipid as compared to the other groups, \* $p \leq 0.05$  CAF09-PEG-peptide as compared to CAF09. (b) Frequency of DiO<sup>+</sup> cells. \*\*\*\* $p \leq 0.0001$  and \*\* $p \leq 0.001$  CAF09-PEG-lipid as compared to the other groups. (c) Frequency of vaccine<sup>+</sup> cDCs and (d) frequency of vaccine<sup>+</sup> CD11b<sup>low</sup> DCs of cell population 48 h after i.m. immunization. \*\*\*\* $p \leq 0.0001$  and \* $p \leq 0.05$  CAF09-PEG-lipid as compared to the other groups. (e) Frequency of vaccine<sup>+</sup> B cells of cell population 48 h after i.m. immunization. \* $p \leq 0.05$  CAF09-PEG-peptide as compared to unadjuvanted OVA. Data represent mean  $\pm$  SEM,  $n = 2$  (naïve mice and mice immunized with unadjuvanted OVA) and 4 (mice immunized with adjuvanted OVA).

resulted in significant frequencies of DiO<sup>+</sup> cells, indicating that the vaccine is either transported or passively drained to the dLNs (Fig. 4b). None of the other adjuvants were detected in the dLNs above baseline level, however, they may have been degraded prior to entry into the dLNs. Similarly, the absence of OVA in the DiO<sup>+</sup> cells might indicate that the antigen is degraded.

After 48 h, the CAF09-PEG-lipid vaccine (either OVA<sup>+</sup> or DiO<sup>+</sup>) was associated with cDCs and CD11b<sup>low</sup> DCs in the dLNs to a significantly higher extent than the other vaccines (Fig. 4c/d), whereas there was little association with B cells (Fig. 4e). In combination, these results indicate that CAF09-PEG-lipid is drained to the dLNs and associates with the cDCs much more efficiently than CAF09 and CAF09-PEG-peptide.

### 3.5. CAF09 PEGylated with DSPE-PEG2000 and PEG-peptide, respectively, induces antigen-specific CD8<sup>+</sup> T-cell responses

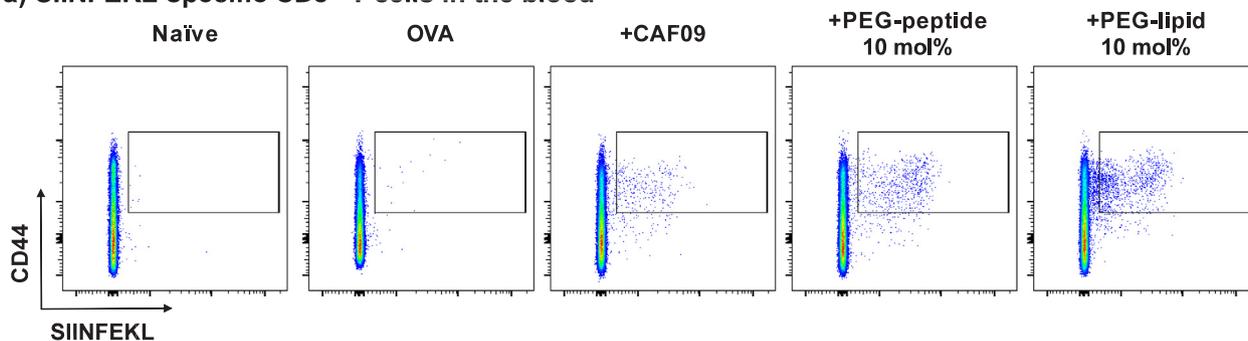
The immunogenicity of PEGylated CAF09 was evaluated in C57BL/6 mice with the model antigen OVA. CAF09 was PEGylated with 10 mol % PEG-peptide (aa15-bisPEG<sub>1000</sub>, CAF09-PEG-peptide) and 10 mol%

PEG-lipid (DSPE-PEG<sub>2000</sub>, CAF09-PEG-lipid), which were administered s.c. two times with a 2-week interval.

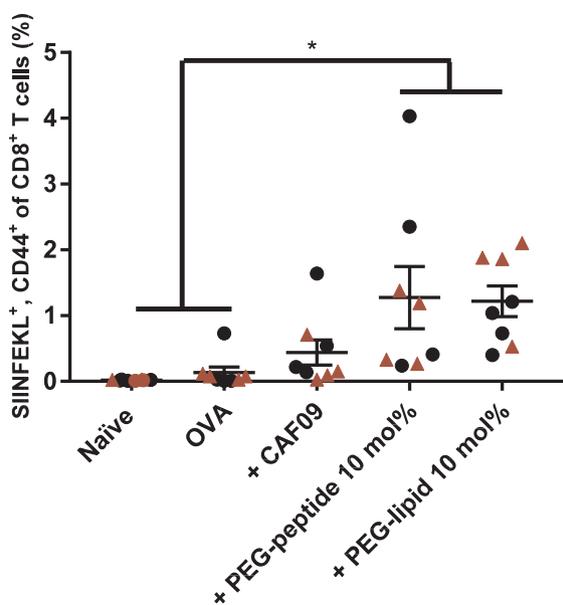
The levels of antigen-specific CD8<sup>+</sup> T cells in the blood and dLNs of immunized mice were evaluated by staining with the specific CD8 epitope SIINFEKL-loaded MHC-I-pentamer and relevant fluorescent antibodies followed by flow cytometry (Fig. 5a). In the blood, mice immunized with OVA/CAF09-PEG-peptide and OVA/CAF09-PEG-lipid displayed significantly higher levels of antigen-specific CD8<sup>+</sup> T cells as compared to the naïve and OVA-immunized mice (Fig. 5b). The levels of antigen-specific CD8<sup>+</sup> T cells in the mice immunized with OVA/CAF09 were comparable to the levels found in previous immunization studies [16]. Furthermore, the levels of antigen-specific CD8<sup>+</sup> T cells in the dLNs were significantly higher in the mice immunized with CAF09-PEG-lipid as compared to the other groups in the study (Fig. 5c).

## 4. Discussion

PEGylation of cationic adjuvants is an attractive approach for reducing depot formation at the SOI and increasing passive drainage to the dLNs. PEGylation of CAF09 with DSPE-PEG<sub>2000</sub> resulted in

a) SIINFEKL-specific CD8<sup>+</sup> T cells in the blood

## b) Blood



## c) dLN

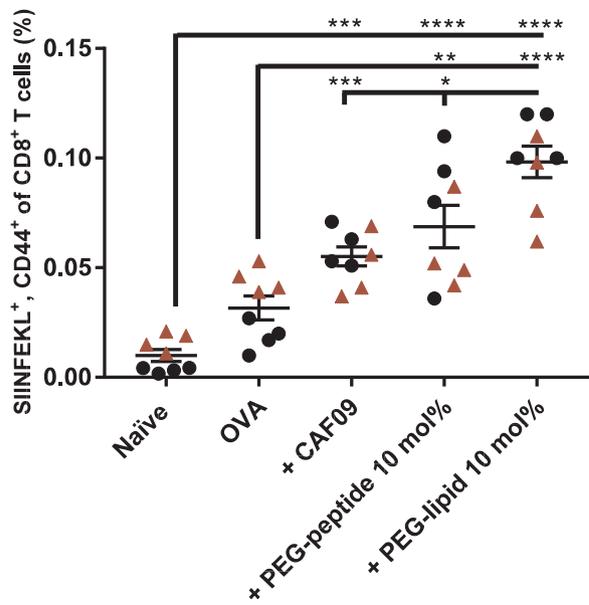


Fig. 5. Immune responses following s.c. immunization with unadjuvanted OVA, OVA adjuvanted with non-PEGylated CAF09, CAF09-PEG-peptide (10 mol% aa-15-bisPEG<sub>1000</sub>) and CAF09-PEG-lipid (10 mol% DSPE-PEG<sub>2000</sub>) as evaluated one week after the final of two immunizations. (a) Plots of antigen-specific CD8<sup>+</sup> T cells in the blood of different immunization groups. Antigen-specific CD8<sup>+</sup> T cells in (b) the blood and (c) the dLNs were measured by flow cytometry identifying CD44<sup>+</sup>, CD8<sup>+</sup> T cells binding fluorescently labeled SIINFEKL-MHC-I complexes. Data represent results from two identical studies (black circles and red triangles, respectively), mean  $\pm$  SEM, n = 8. \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$  and \*\*\*\*  $p \leq 0.0001$ , one-way ANOVA followed by Tukey's post test.

relatively small liposomes (approx. 130 nm for CAF09 with 10 mol% DSPE-PEG<sub>2000</sub>), and the apparent surface charge was reduced as a function of the PEG grafting density. The zeta-potentials of CAF09 mixed with PEG-peptides decreased as a function of the length of the peptide and concentration of the PEG-peptide. The zeta-potentials appeared to start forming a plateau for CAF09 with the aa-15 and aa-20 PEG-peptides at higher concentrations, which was not observed for the aa-10 PEG-peptide. The mechanisms of shielding of the liposomal surface are apparently very different for the two PEGylation strategies. Hence, when using a lipid-anchor for PEGylation, the surface charge is shielded via the formation of a stealth PEG layer on the particle surface [34]. The PEG-moieties adopt different conformations, depending on the grafting density; at a low density (< 5 mol%) the PEG moieties are expected to adopt a mushroom-like conformation [20,34]. Increasing the grafting density induces a change to a brush-border conformation of the PEG moieties due to steric repulsion. At higher grafting densities (above approx. 10 mol%), the liposomes become destabilized due to increased steric repulsion [20,34]. Furthermore, the reduced particle size observed at increasing mol% of DSPE-PEG<sub>2000</sub> is likely a result of colloidal stabilization, which prevents aggregation during liposome preparation due to steric repulsion between the PEG moieties.

Despite PEGylation with DSPE-PEG<sub>2000</sub>, poly(I:C) and OVA are adsorbed to CAF09 to a certain extent. On the other hand, PEGylation

with PEG-peptides involves the direct attractive electrostatic interaction between the DDA headgroups and the negatively charged peptides, and this process is in direct competition with the adsorption of poly(I:C) and OVA (that the peptides might also interact with). However, this opens the possibility that the PEG-peptides may desorb *in vivo* after immunization, perhaps due to dissociation or competition with interstitial proteins. For this strategy to be successful, the kinetics of desorption is key. Hence, the desorption kinetics of the injected liposomes should be optimized to allow diffusion of the liposomes into the lymphatics before dissociation of the PEG-peptides takes place.

While all the vaccine formulations caused a significant influx of immune cells into the SOI, the adjuvants attracted different types of cells, as the OVA/CAF09-PEG-peptide attracted more neutrophils than the other adjuvants, whereas the OVA/CAF09-PEG-lipid attracted more inflammatory monocytes. Interestingly, neutrophils and inflammatory monocytes took up the vaccine particles to the same extent, indicating that the adjuvants do not target specific immune cell subsets. The OVA/CAF09-PEG-peptide induced higher levels of IFN- $\gamma$  and IL-1 $\beta$ , which was in accordance with the influx of neutrophils and inflammatory monocytes peaking earlier and at higher levels for this adjuvant, as compared to the other adjuvants. Furthermore, KC/GRO was induced already after 3 h, which is in accordance with the early peak of neutrophil influx, as compared to inflammatory monocyte influx.

The influx of DCs into the SOI was maximal and reached a plateau at 48 h for the OVA/CAF09-PEG-lipid vaccine, whereas the influx peaked at 96 h for the OVA/CAF09 vaccine and the OVA/CAF09-PEG-peptide vaccine. Simultaneously, the frequency of vaccine<sup>+</sup> cDCs was highest for mice vaccinated with the OVA/CAF09-PEG-lipid and lowest for the mice vaccinated with OVA/CAF09-PEG-peptide. Interestingly, the OVA/CAF09-PEG-lipid vaccine displayed the highest frequency of association with cDCs, despite the lipid-anchored PEG-moieties. This adjuvant might be believed to have the lowest capability of interacting with APCs due to the lipid anchor of the PEG-moieties that has been shown to play a role in preventing cellular absorption of PEGylated liposomes [24,25].

The dLNs from mice immunized with OVA/CAF09-PEG-lipid displayed significantly higher frequencies of association with total lymphocytes and cDCs in particular, as compared to those from mice immunized with OVA/CAF09 and OVA/CAF09-PEG-peptide. Interestingly, only single-positive (*i.e.*, either OVA<sup>+</sup> or DiO<sup>+</sup>) immune cells were observed in the dLNs. This is in contrast to the SOI where the vaccine<sup>+</sup> cells were almost exclusively double-positive. Possibly, this is because the components of the vaccine drain separately from the SOI, or that the fluorescent labels are degraded at different rates in the cells. From this study, it is not possible to deduce, whether the vaccine<sup>+</sup> cells in the dLNs had migrated from the SOI, or if the vaccine particles drained passively to the dLNs prior to phagocytosis by APCs. The kinetics of vaccine association with lymphocytes in the dLNs indicate migration by APCs from the SOI, as other studies have shown that passive drainage of CAF09 takes place within 6 h following *i.p.* immunization [16]. Despite the large influx of neutrophils taking up vaccine into the SOI, no vaccine<sup>+</sup> neutrophils were observed in the dLNs, indicating that the neutrophils at the SOI do not migrate to the dLNs, and hence are not involved in the lymphatic targeting.

The hypothesis of the study was that PEGylation of CAF09 reduces depot formation at the SOI and enhances the drainage to the dLNs. It was shown that grafting of CAF09 with DSPE-PEG<sub>2000</sub> resulted in drainage of a larger dose fraction to the dLNs, as compared to non-PEGylated CAF09. However, it was not possible in the present study to confirm that this was indeed due to a reduction of depot formation at the SOI. Hence, the results rather indicate that a vaccine depot will form with these adjuvants, but drainage to the dLNs can be achieved by specifically designing the adjuvants for the purpose, *e.g.* by PEGylation.

## 5. Conclusion

Grafting of CAF09 with PEG-lipid and PEG-peptide showed a tendency, though statistically not significant, toward induction of stronger CD8<sup>+</sup> T-cell responses in the blood upon *s.c.* immunization than found for non-PEGylated CAF09. CAF09-PEG-lipid induced significantly higher CD8<sup>+</sup> T-cell responses in the dLNs as compared to the other adjuvants. The biodistribution studies correlated with these findings, as significantly larger numbers of cDCs in the dLNs were vaccine<sup>+</sup> following *i.m.* immunization with OVA/CAF09-PEG-lipid, as compared to OVA/CAF09. From the present studies, CAF09 grafted with 10 mol% DSPE-PEG<sub>2000</sub> appears as the most promising of the tested adjuvants because it displays drainage of the largest dose fraction to the dLNs and induces the strongest local immune response. *I.m.* immunization with OVA/CAF09-PEG-peptides resulted in increased stimulation of pro-inflammatory cytokines, which resulted in an increased influx of neutrophils into the SOI, as compared to that observed when immunizing with OVA/CAF09. The strategy of PEG grafting CAF09 for the induction of CD8<sup>+</sup> T-cell responses upon *s.c.* or *i.m.* administration is promising, which may accomplish significant CD8<sup>+</sup> T-cell responses through further optimization of the design of the PEG-peptides and the liposomal formulations.

## Declaration of Competing Interest

KSK, PA and DC are employed by Statens Serum Institut, a nonprofit government research facility, which holds patents on the cationic liposomal adjuvants (CAF). All other authors report no potential conflicts.

## Acknowledgements

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejpb.2019.04.020>.

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