Biomaterials 61 (2015) 33–40

Contents lists available at ScienceDirect

Biomaterials

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

Polymeric microparticles for sustained and local delivery of antiCD40 and antiCTLA-4 in immunotherapy of cancer

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ARTICLE INFO

Article history:
Received 15 April 2015
Accepted 30 April 2015
Available online 17 May 2015

Keywords:
pLHMGA
Immunomodulatory antibody
CTLA4-blocking antibody
AntiCD40
Cancer immunotherapy
Sustained release
Local delivery
Incomplete Freund's adjuvant (IFA)

ABSTRACT

This study investigated the feasibility of the use of polymeric microparticles for sustained and local delivery of immunomodulatory antibodies in immunotherapy of cancer. Local delivery of potent immunomodulatory antibodies avoids unwanted systemic side effects while retaining their anti-tumor effects. Microparticles based on poly(lactic-co-hydroxymethyl-glycolic acid) (pLHMGA) and loaded with two distinct types of immunomodulatory antibodies (CTLA-4 antibody blocking inhibitory receptors on T cells or CD40 agonistic antibody stimulating dendritic cells) were prepared by double emulsion solvent evaporation technique. The obtained particles had a diameter of 12–15 μm to avoid engulfment by phagocytes and were slightly porous as shown by SEM analysis. The loading efficiency of the antibodies in the microparticles was >85%. The *in vitro* release profile of antiCD40 and antiCTLA-4 from microparticles showed a burst release of about 20% followed by a sustained release of the content up to 80% of the loading in around 30 days. The therapeutic efficacy of the microparticulate formulations was studied in colon carcinoma tumor model (MC-38). Mice bearing subcutaneous MC-38 tumors were treated with the same dose of immunomodulatory antibodies formulated either in incomplete Freund's adjuvant (IFA) or in microparticles. The antibody-loaded microparticles showed comparable therapeutic efficacy to the IFA formulation with no local adverse effects. The biodegradable microparticles were fully resorbed *in vivo* and no remnants of inflammatory depots as observed with IFA were present in the cured mice. Moreover the microparticles exhibited lower antibody serum levels in comparison with IFA formulations which lowers the probability of systemic adverse effects. In conclusion, pLHMGA microparticles are excellent delivery systems in providing long-lasting and non-toxic antibody therapy for immunotherapy of cancer.

1. Introduction

Immunotherapy has been established as a groundbreaking approach to treat cancer [1]. As opposed to conventional cancer treatment strategies which employ methods to eliminate all rapidly proliferating tumor cells, immunotherapy aims to use the immune system to attack the target of interest with high specificity and low toxicity [2–4]. Immunotherapy of cancer embraces several strategies, including application of immunomodulatory antibodies as monotherapy in the treatment of malignancies [5,6]. These antibodies do not directly target cancer cells but instead aim to induce and enhance immune responses against the tumor, particularly by CD8+ T cells which are crucial for tumor eradication [7]. The mode of action of such indirectly acting or immunomodulatory antibodies can be inhibitory or stimulatory, depending on the role of their target in the anti-tumor immune response. Check-point blocking antibodies such as antagonistic antiCTLA-4 have been developed to block inhibitory receptors expressed on T cells [8–10]. Other antibodies such as agonistic antiCD40 function at an earlier phase of the immune response by activating antigen presenting cells (APCs) including dendritic cells (DCs) which are responsible for the activation of tumor-specific CD8+ T cells by cross-presentation of tumor antigens [11].

The cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is one

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http://dx.doi.org/10.1016/j.biomaterials.2015.04.043
0142-9612/

of the key inhibitory receptors expressed by activated CD4+ and CD8+T cells as well as by memory and regulatory T cells [12], and is responsible for "pushing the brake" of the immune system [13,14]. CTLA-4 has a high affinity for CD80 and CD86 on APCs and competes with CD28, a major co-stimulatory signal required for T cell activation, for binding to these ligands. Binding of CTLA-4 to its natural ligands, CD80 and CD86 on APCs, results in decreased cytokine production and T cell proliferation [15]. The inhibitory role of CTLA-4 is crucial to maintain the balance of the immune system and to prevent autoimmunity, whereas cancer immunotherapy aims to reverse the CD8+ T cell inactivation [16]. To overcome the effect of inhibitory immune regulators, CTLA-4 blocking antibodies have been developed as potential anticancer agents [9,17–20].

CD40 is a receptor on APCs as well as on several other cells and binds to its ligand (CD154-also called CD40L) on activated CD4+ Th cells [21]. The CD40–CD40L interaction is essential for maturation of DCs (up-regulation of co-stimulatory molecules, increased secretion of cytokines) and consequently for CD8+ T cell priming and induction of CD8+ T cell response [22]. Earlier studies have shown that the CD40L signal from CD4+ Th cells can also be provided by agonistic antiCD40, encouraging their use for the induction of a robust T cell response [23]. Despite the promising results obtained with clinical trials using immunomodulatory antibodies in advanced stage cancer patients [18,24], after systemic administration, immune related adverse effects such as autoimmune and inflammatory reactions and cytokine release syndrome have been observed [20,25–30]. To minimize these adverse effects, Fransen et al. used Montanide ISA 51 to prepare a sustained-release water-in-oil emulsion for local delivery of an agonistic CD40 antibody in a preclinical mouse model. Unlike systemic antibody administration, this allowed local treatment with a lower dose of antibody, abrogating systemic toxicity while remaining effective in activating T cells [31,32]. In a study the effect of different administration methods on anti-tumor efficacy and toxicity of antiCD40 was evaluated in adenovirus protein E1A-expressing tumor-bearing mice. It was shown that the antitumor efficacy of 30 μg antiCD40 administered locally either in saline or Montanide was comparable to 3 consecutive intravenous injections of 100 μg antiCD40 (survival 70–80%) while single intravenous injection of 30 μg antiCD40 showed minimal tumor growth reduction (survival 30%). In addition, local treatment with low dose of antiCD40 resulted in lower toxicity than high dose intravenous treatment and sustained release formulation of antiCD40 in Montanide caused the lowest adverse effects, which was characterized by organ histology and liver enzymes in the blood [32]. Montanide ISA 51 is a commercially available mixture of light mineral oils (similar to incomplete Freund's adjuvant (IFA)) with mannide monooleate (as surfactant) and has been used extensively in clinical trials [30,33]. Nevertheless, administration of Montanide ISA 51–based emulsion and similar formulations has been associated with several side effects such as inflammation and swelling, painful granulomas at the injection site, fever, cysts and sterile abscesses [34]. In order to provide a safe formulation for local delivery of immunomodulatory antibodies, microparticulate formulations loaded with CTLA-4 blocking antibody and CD40 agonistic antibody were developed in this study using the biodegradable polymer (poly(d,l lactic-co-hydroxymethylglycolic acid) (pLHMGA)). Although similar in backbone to pLGA, pLHMGA possesses pendant hydroxyl groups which increase the hydrophilicity of the polymer. This results in less acidification inside the particles upon degradation and protects the protein/peptide from chemical modification [35,36]. As a result pLHMGA and similar hydrophilic polymers have shown better protein/peptide compatibility and complete release of encapsulated proteins/peptides as compared to pLGA [36–38]. Moreover, these polymers have been successfully used locally as antigen or drug delivery systems *in vivo* without showing toxicity [39,40]. In the present study, first, pLHMGA microparticles were optimized using – for economic reasons – polyclonal human IgG, to obtain a formulation with the desired particle size and antibody release profile. Because these particles were intended for local and sustained release of the antibody and not to be taken up by e.g. macrophages, the desired particle size should be larger than 10 μm [41]. Next, based on experience with the IgG formulations, antiCD40 and antiCTLA-4 loaded microparticles were prepared and characterized. The anti-tumor efficacy of the obtained microparticles was compared with that of IFA formulations in tumor-bearing mice. Antibody serum levels were monitored during treatment for potential systemic toxicity and the site of injection was studied for local reactions.

2. Materials and methods

2.1. Materials

Poly(lactic-co-hydroxymethyl glycolic acid) with a copolymer ratio of 50/50 was synthesized and characterized as described previously [40,42] (Supplementary data, Fig. S1 and Table S1). IRDye680RD N-hydroxysuccinimide ester (NHS ester) was obtained from LI-COR Biosciences, USA. Polyclonal human IgG (50 mg/mL in glucose 5%) was a gift from Sanquin, the Netherlands. Polyvinyl alcohol (PVA; Mw 30,000–70,000; 88% hydrolyzed) and dimethyl sulfoxide (DMSO) were obtained from Sigma–Aldrich, USA. Sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) were obtained from Merck, Germany. Dichloromethane (DCM) was purchased from Biosolve, the Netherlands. Sodium azide (NaN_3, 99%), sodium hydroxide (NaOH), dipotassium hydrogen phosphate (K_2HPO_4) and sodium dodecyl sulfate 20% (SDS) were purchased from Fluka, the Netherlands. Bicinchoninic acid assay (MicroBCA) reagents were obtained from Thermo Fisher Scientific, USA. Phosphate buffered saline (1.8 mM NaH_2PO_4, 8.7 mM Na_2HPO_4, 163.9 mM Na^+, 140.3 mM Cl^-, pH 7.4) (PBS) was obtained from B Braun, Germany. Pyrogen-free water was obtained from Carl Roth, Germany. Polyclonal anti-rat antibody (BD biosciences, USA) was used for analysis of antiCD40 by ELISA and antiCTLA-4 was analyzed by biotin-labeled mouse anti-hamster antibody (clone 192-1) (BD biosciences, USA). Chemicals were used as received without further purification, unless otherwise stated.

2.2. Labeling IgG with NIR fluorescent dyes

Given the limited availability of immunomodulatory antibodies, human IgG was used as a model antibody to optimize the pLHMGA microparticle formulations. In order to accurately characterize the release kinetics of the formulations, IgG was labeled with IRDye680RD (IR680) by coupling the NHS ester of the dye to the protein. In a typical procedure, the medium in which the IgG was provided (50 mg/mL in glucose 5%) was exchanged to PBS (B Braun, Germany, pH 7.4) using a Zeba™ spin desalting column (7 kDa, Thermo Fisher Scientific, USA). Next, the pH of the antibody solution was adjusted to 8.5 by adding 0.1 mL of K_2HPO_4 1 M pH 9.0 to 1 mL of IgG in PBS. The IRDye680RD NHS ester was dissolved in DMSO (4 mg/mL) and 0.67 mL of this solution (2.7 mg of the dye) was added to the IgG solution yielding 2:1 molar ratio of dye/IgG. The reaction was carried out at room temperature for two hours. The unreacted dye was subsequently removed using Zeba™ spin desalting columns (7 kDa) equilibrated with HEPES buffer 50 mM pH 7.0 in two consecutive steps and IR680-IgG was collected in HEPES buffer and kept at 4 °C. IR680-IgG was characterized by gel permeation chromatography (GPC) as described previously [40].

2.3. Preparation of the microparticles

IR680-IgG loaded microparticles were prepared using a double emulsion solvent evaporation method [37,43]. One hundred and twenty five μL of 5 mg/mL IR-IgG in HEPES 50 mM pH 7.0 was emulsified in 0.5 ml of solution of pLHMGA (10%, 15%, 20% and 30%) in DCM by homogenization (IKA® T10 basic ULTRA-TURRAX, Germany) at 20,000 rpm for 45 s. This primary emulsion was subsequently emulsified in 1 mL aqueous solution of PVA 1% (20,000 rpm for 45 s) and transferred into 5 mL of PVA 0.5% in 0.9% NaCl in water. After evaporation of DCM (3 h, RT), the particle suspension was centrifuged at 3000 g for 3 min and the pellet was washed twice with pyrogen-free water and freeze-dried overnight. Immunomodulatory antibody loaded microparticles were prepared by the same method using 125 μL antiCD40 (5 mg/mL in HEPES 50 mM pH 7.0) or antiCTLA-4 (3 mg/mL in HEPES 50 mM pH 7.0) and polymer concentration of 15%. Empty microparticles were prepared using polymer concentration of 15% and by replacing the antibody solution with pyrogen-free water.

2.4. Characterization of the microparticles

2.4.1. Size and morphology of the microparticles

The average size of the microparticles dispersed in water was measured using a light obscuration particle counter (Accusizer 780, USA).

The morphology of the microparticles was studied by scanning electron microscopy (SEM) (Phenom, FEI, the Netherlands). Freeze-dried microparticles were transferred onto 12-mm diameter aluminum specimen stubs (Agar Scientific Ltd., England) using double-sided adhesive tape, and prior to analysis were coated with a 6 nm platinum layer using sputter coater.

2.4.2. Antibody loading in the microparticles

The loading efficiency of antibodies in the microparticles was determined by measuring the antibody content of digested microparticles [44]. In brief, around 5 mg of microparticles (accurately weighed) was dissolved in 0.5 mL DMSO. After complete dissolution, 2.5 mL of 50 mM NaOH containing 0.5% (w/v) SDS was added and the samples were incubated at 37 °C overnight to accelerate the degradation process. For IR680-IgG loaded microparticles, the amount of antibody in the resulting solution was determined based on the IR680 label using an Odyssey™ scanner (LI-COR Biosciences, USA) at the 700 nm channel for IR680 and calibration was done using IR680-IgG (0.4–12.5 μg/mL) in DMSO:NaOH 50 mM/SDS 0.5% (1:5). The amount of antiCD40 and antiCTLA-4 in microparticles was quantified by MicroBCA protein assay kit and calibration was done using 2–40 μg/mL of antibody solution in DMSO:NaOH 50 mM/SDS 0.5% (1:5).

2.4.3. In vitro release of antibodies from microparticles

Ten to twenty mg of freeze-dried antibody-loaded microparticles was accurately weighed and suspended in 1.5 mL of phosphate buffered saline pH 7.4 (49 mM NaH_2PO_4, 99 mM Na_2HPO_4, 6 mM NaCl and 0.05% (w/v) NaN_3). Samples were incubated at 37 °C under mild agitation. At different time points, samples were centrifuged (2000 g for 2 min) and 0.75 mL of the supernatant was replaced by 0.75 mL of buffer. The supernatants were kept at 4 °C before quantification. The protein content in the supernatant of samples containing IR680-IgG loaded MPs was measured with an Odyssey™ scanner using the 700 nm channel to detect IR680-IgG. Calibration was done using IR680-IgG in PBS (0.19–25 μg/mL). Quantification of antiCD40 and antiCTLA-4 was done using the intrinsic fluorescence of the antibody (excitation 280 nm and emission 345 nm) and calibration was done with the corresponding antibody in PBS (1.5–50 μg/mL).

2.5. Experimental animals and cell lines

The experiments were approved by the Animal Experimental Committee of the University of Leiden. C57BL/6 mice were purchased from The Jackson Laboratory, USA. The FGK-45 hybridoma cells producing antiCD40 (a rat IgG2a provided by A. Rolink, Basel Institute for Immunology, Basel, Switzerland) [45] and hybridoma cells producing antiCTLA-4 (a Syrian hamster IgG, clone 9H10) [13] were cultured in Protein-Free Hybridoma Medium (Gibco), and antibodies were purified using a Protein G column. Antibody purity was checked by SDS-PAGE.

2.6. Detection of IgG in sera of mice following administration of IR680-IgG loaded microparticles

IR680-IgG loaded microparticles (Formulation 2) were administered subcutaneously to non-tumor bearing mice and at several time points after injection, blood samples were drawn from mice and IgG was detected by ELISA using Protein A coating (Sigma–Aldrich, USA) and HRP conjugated goat-anti Human IgG (Southern Biotech, USA).

2.7. Tumor experiments with immunostimulatory loaded microparticles and serum analysis

MC-38 tumor cells (murine colon carcinoma cell line) [46] were cultured in Iscove's Modified Dulbecco's Medium (IMDM; BioWhittaker) supplemented with 4% fetal calf serum (FCS), 50 mM 2-mercaptoethanol, and 100 IU/mL penicillin/streptomycin. MC-38 tumor cells (10^5 dispersed in 100 μL PBS) were injected subcutaneously into the right flank of 8 to 12-week-old female mice. Treatment was started when the tumors were palpable (6–10 days after tumor inoculation; tumor size 0.5–3 mm^3), the tumor size was measured with calipers in three dimensions and mice were sacrificed when tumors size exceeded 1 cm^3. Mice (14–18 per group) were either left untreated or were injected subcutaneously close to the tumor with 30 μg antiCD40 or 50 μg antiCTLA-4 in IFA (Dibco, USA) or encapsulated in microparticles. A group of 5 mice was treated with empty microparticles. IFA formulations were prepared by mixing the antibody in PBS at a concentration of 0.3 mg/mL for antiCD40 and 0.5 mg/mL for antiCTLA-4 with IFA (1:1), and vortexing for 30 min to form a water in oil emulsion. The injected volume was 200 μL [32]. At several time points after administration of the formulations, blood samples were drawn from mice and antiCD40 and antiCTLA-4 levels in serum were analyzed by ELISA.

2.8. Statistical analyses

All data were analyzed using GraphPad Prism 5.02 software. For tumor experiments Kaplan–Meier survival curves were applied and the differences between survival curves were analyzed by log-rank test. Antibody serum levels in different groups of mice were compared using two-tailed unpaired Student's t test and $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Characterization of IR680-IgG

To ensure accurate and sensitive detection of released antibody in *in vitro* studies, human IgG was labeled with IR680. IR680-IgG was obtained by coupling IR680 to IgG and was analyzed by GPC.

Overlapping peaks corresponding to IgG (excitation 280 and emission 340 nm) and IR680 (excitation 672 and emission 694 nm) in the GPC chromatogram (see supplementary data Fig. S2) of the purified IR680-IgG confirmed that the IR680 was indeed conjugated to IgG. No additional peaks (fluorescence detection) were observed, indicating that the free dye was completely removed by purification. Conjugation efficiency calculated by UV measurements showed an average molar dye/protein ratio of 1.4.

3.2. Optimization of pLHMGA microparticulate formulations

IR680-IgG loaded microparticles were prepared using a double emulsion solvent evaporation process as described in Section 2.3. In the current study, these antibody-loaded particles were designed for sustained delivery. Because the antibody must be released in the extracellular matrix and not inside the cells where it can be degraded in the lysosomes, the particles were designed to be large enough to prevent uptake by the mononuclear phagocyte system, which is able to uptake particles ranging from 0.5 to 10 μm, with its most efficient uptake of around 2–3 μm [41,47]. Besides particle size, sustained and complete release of the antibody is essential in the development of an optimal formulation [48,49]. High burst release might result in high local and systemic concentrations which in turn might cause toxicity [50,51]. The particle preparation was therefore optimized to obtain particles that are larger than 10 μm and provide sustained release of the antibody. Among several parameters involved in particle preparation, polymer concentration is a critical one which affects multiple characteristics of the microparticles, such as size, burst release, loading efficiency and duration of release [52]. The optimal formulation, with an appropriate particle size and fast release profile, was selected from 4 different IR680-IgG loaded pLHMGA microparticles, prepared by varying the polymer concentration from 10% to 30% w/v. The characteristics of these IR680-IgG loaded microparticles are summarized in Table 1. For all formulations, volume-average particle size was >10 μm. Further, when the polymer concentration in the DCM solution was increased from 10% to 30%, the average particle size increased from 12 to 25 μm. Particle size determinations obtained by light obscuration (Table 1) were confirmed through SEM analysis (Fig. 1), which also revealed that microparticles were spherical and slightly porous. IR680-IgG was encapsulated in the microparticles with high loading efficiency (>80%). The loading efficiency increased with increasing polymer concentration in the oil phase. Formulation 1, prepared with the lowest polymer concentration (10%), showed a burst IR680-IgG release of around 35% followed by sustained release of antibody up to 90% by day 7. Formulations 2 and 3 exhibited a burst release (12% and 8% respectively) and a sustained release of IR680-IgG up to 75% of the loading in 35 days. Formulation 4 prepared with the highest polymer concentration (30%), showed very low burst release (1%) and at day 35, 50% of the loaded IR680-IgG was released (Fig. 2). These observations are in agreement with earlier studies which showed that an increasing polymer concentration in the DCM solution and consequently a high viscosity of polymer solution resulted in an increase in both the particle size and the loading efficiency as well as retardation in release kinetics [52,53]. The burst release of IgG from the pLHMGA microparticles can be ascribed to the porosity of the microparticles. The burst release decreased with increasing polymer concentration (Fig. 2). The high burst release of particles of Formulation 1, can be attributed to the high surface to volume ratio of these particles and low polymer density inside the microparticles which result in fast hydration of the particles and consequently rapid diffusion of protein from water-filled pores [53]. Nevertheless all formulations showed sustained release of the IR680-IgG due to polymer degradation [37]. The characteristics of the obtained microparticles are presented in Table 1. Formulation 2 was chosen for further characterization because of its low burst as well as sustained release (up to 75% for 35 days *in vitro*) and relatively higher loading percentage comparing to formulation 3 which showed similar release kinetics.

3.3. IR680-IgG serum levels in mice

After subcutaneous injection of equal doses of IgG whether in PBS, emulsified in IFA or encapsulated in microparticles (Formulation 2), sera of mice were collected at several time points and the antibody levels were quantified by ELISA. As depicted in Fig. 3, subcutaneous injection of IgG dissolved in PBS resulted in high levels of serum IgG. Administration of IFA formulation resulted initially in lower concentrations than the PBS formulation but increased in time, resulting in comparable levels at day 6 post injection. Administration of IgG in microparticles was associated with at least 10 times lower serum concentrations than observed after administration of the soluble protein. This is likely due to the antibody being released locally in a controlled and sustained manner from the microparticles, reducing the risk of systemic toxicity. In the present study we were able to analyze the serum levels only for a limited time because on day 6 the levels were close to the detection limit of the ELISA assay (0.05 μg of human IgG/mL). It is therefore possible that the antibody was still released from the microparticles but it was below the detection limit.

Possible formed mice anti-human antibodies were not analyzed since Fig. 2 shows that the most of the loaded human IgG is released within the first two weeks which is too short for development of anti-human antibodies.

3.4. Preparation and characterization of antiCD40 and antiCTLA-4 loaded microparticles

Based on the results obtained with the microparticles using the IR680-IgG as model antibody (Section 3.3), immunomodulatory antibodies were encapsulated in microparticles using polymer concentration of 15% (Formulation 2, Table 1). This formulation was chosen because of its low burst release, high encapsulation efficiency and sustained release of the cargo in 21 days. The characteristics of the prepared microparticles are summarized in Table 2. AntiCD40 and antiCTLA-4 loaded microparticles were respectively around 12 and 15 μm in diameter, as measured by light obscuration. As shown by SEM, they were spherical and slightly porous (Fig. 4). The loading efficiency of antiCD40 was 86% and of antiCTLA-4, 89%. As Fig. 5 indicates, both microparticle formulations showed a 20% burst release (0.5 h) followed by sustained release, up to 80% of the

Table 1
Characteristics of the IR680-IgG loaded pLHMGA MPs.

Formulation	Polymer concentration (%)	Volume average particles size (μm)	Loading efficiency (%)	Loading %
1	10	12	86	1.09
2	15	15	74	0.62
3	20	18	86	0.54
4	30	25	100	0.42

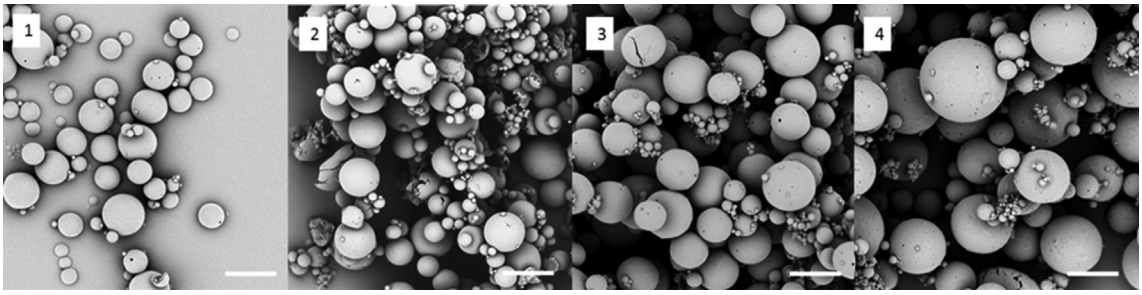

Fig. 1. SEM images of the IR680-IgG MPs. Scale bar represents 10 μm.

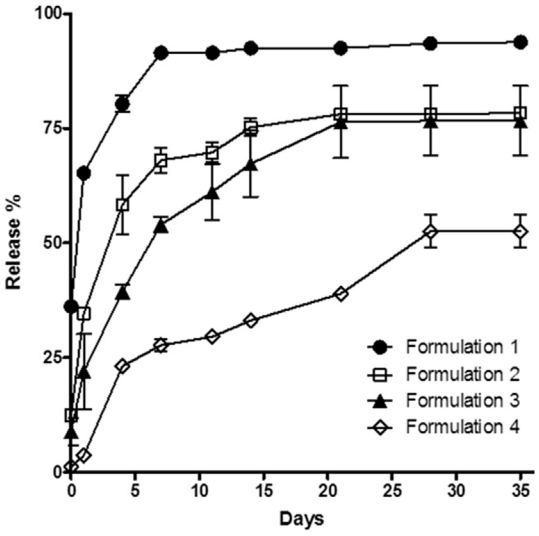

Fig. 2. *In vitro* release of IR680-IgG from microparticle formulations. The characteristics of the formulations are given in Table 1. Values shown are the average + SD of three measurements.

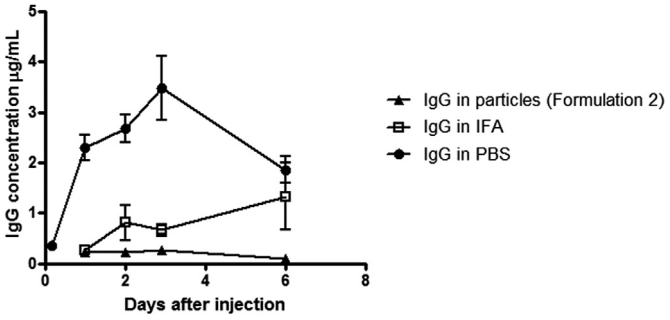

Fig. 3. Antibody levels in serum after s.c. administration of 75 μg of IgG in various formulations in non-tumor-bearing mice. Samples were taken at regular intervals and the antibody levels in mice were measured by ELISA, N = 3 mice per group.

loading in around 30 days. Though high burst release might be unfavorable in some sustained release formulations, the burst release observed with these formulations may be favorable in our study, achieving optimal therapeutic efficacy by providing a minimum therapeutic antibody level promptly after administration.

3.5. Serum levels in tumor-bearing mice after treatment with antiCD40 and antiCTLA-4 formulations

Sera of mice were collected at certain time points up to day 8 after administration of the immunomodulatory antibody formulations and quantified by ELISA. Following injection of antiCD40-loaded microparticles, a peak was reached in serum at day 3. Nevertheless, at all time points the level of antiCD40 antibody in the serum was lower than the antibody level detected following administration of IFA formulations. In case of antiCTLA-4 microparticles, no peak was detected and antibody concentrations in blood were significantly lower (5–10 times) than the levels detected after injection of antiCTLA-4 IFA formulation up to day 6 (Fig. 6). This is in agreement with the results obtained from administration of IgG microparticles in serum (Fig. 3), indicating that subcutaneous injection of microparticles causes low antibody serum levels and thus likely prevents systemic adverse effects. To test the hypothesis that biodegradable sustained-release formulations can be used as an alternative to IFA, we assessed the injection site of pLHMGA microparticles and IFA emulsions at the end of the experiment in mice cured of their tumor. Sixty days after administration of the different antibody formulations, mice treated with IFA had a palpable lump at the injection site, which was confirmed by post-mortem examination (Fig. 7a). In contrast, cured mice treated with pLHMGA microparticles showed no apparent remainder of the protein formulation (Fig. 7b).

As mentioned in Section 3.4, these microparticles were completely degraded after 30 days *in vitro*; thus it is reasonable to assume that the same would be true 60 days following subcutaneous injection, and indeed no residues were found. These findings are in agreement with previous studies, which have shown that the microparticles based on pLHMGA with similar characteristics degrade in 30–60 days both *in vitro* [43,54] and *in vivo* [39].

3.6. Anti-tumor efficacy of antiCD40 and antiCTLA-4 formulations

To evaluate the therapeutic efficacy of the microparticulate formulations, mice were inoculated with MC-38 tumor cells and the antibody treatment was started when the tumors were palpable (0.5–3 mm^3) and tumor outgrowth was monitored for

Table 2
Characteristics of empty, antiCD40 and antiCTLA-4 loaded pLHMGA microparticles. Data shown is the result of several (8–15) pooled batches.

Loading	Volume average particle size (μm)	Loading efficiency (%)	Loading %
AntiCTLA-4	15 ± 3	89	0.50
AntiCD40	13 ± 1	86	0.83
Non	11 ± 4	–	–

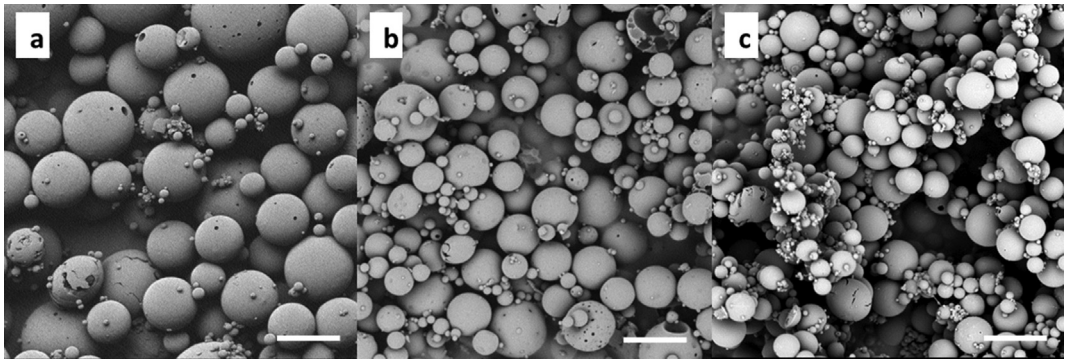

Fig. 4. SEM images of the microparticles a) antiCTLA-4, b) antiCD40 c) empty. Scale bar represents 10 μm.

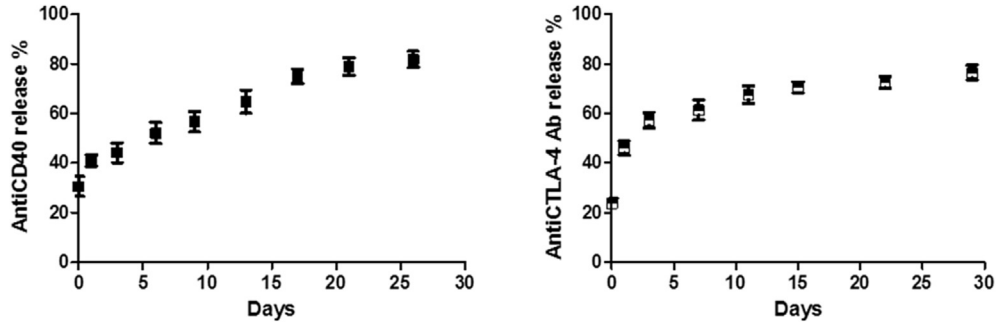

Fig. 5. Sustained *in vitro* release of antibody from antiCD40 microparticles and antiCTLA-4 microparticles. Antibody-loaded microparticles were dispersed in PBS and incubated at 37 °C while agitated. At various time points, samples were taken and the released antibody was measured by intrinsic fluorescence of the antibody. Mean + SD of three measurements is presented.

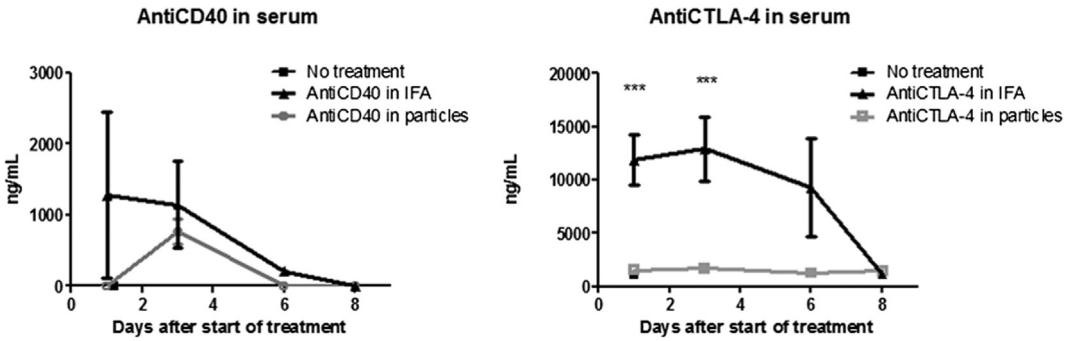

Fig. 6. Antibody levels in serum after s.c. administration of various formulations in tumor-bearing mice. Samples were taken at regular intervals after treatment and the antibody levels in mice were detected by ELISA (***$p < 0.001$), N = 3 mice per group. Values shown are the average + SD of three measurements.

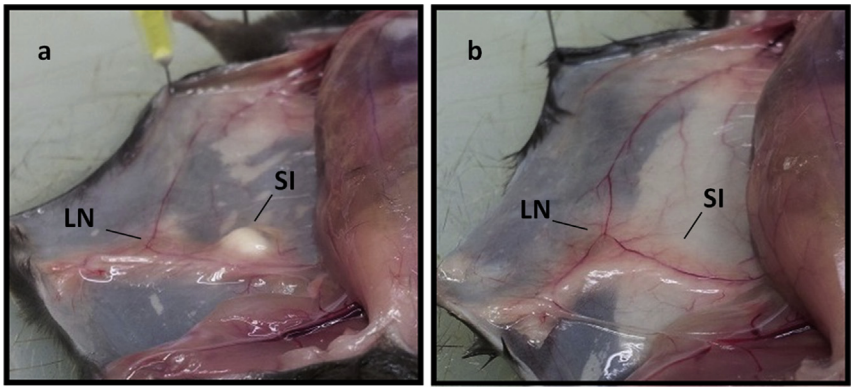

Fig. 7. Post mortem examination of mice two months after treatment with immunomodulatory antibodies formulated in a) IFA or b) microparticles. LN: inguinal lymph node. SI: site of injection, Representative images of N = 4 mice per group injections.

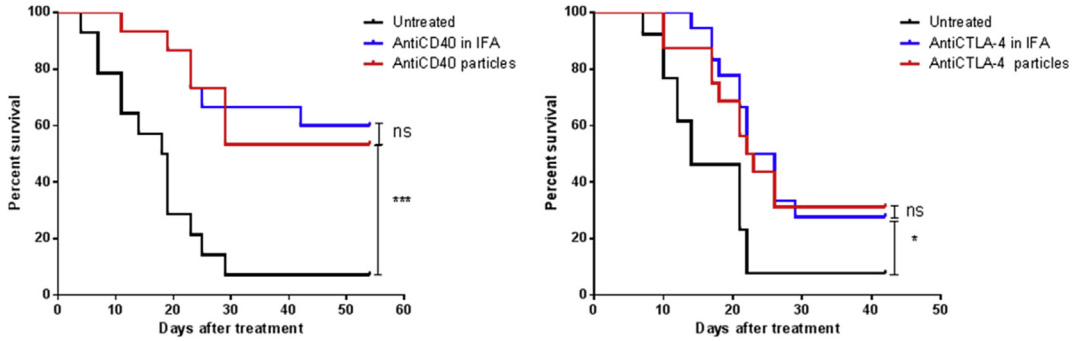

Fig. 8. Kaplan–Meier plot presenting the survival proportions of MC-38 tumor bearing-mice treated with different formulations containing antiCD40 or antiCTLA-4. Pooled data from two experiments (N = 14–18 mice) are shown.

42–54 days after initiation of the treatment. Survival proportions of mice that received the different formulations are given in Kaplan–Meier plots (Fig. 8). The majority (about 90%) of non-treated mice were sacrificed before day 30 after antibody treatment while in mice treated with antiCD40, 50% survival was observed at the end of the tumor experiment (day 54). The survival rate was comparable for both groups receiving antiCD40 encapsulated in microparticles or formulated in IFA as we have reported before [32]. A 40% survival rate was seen in mice treated with antiCTLA-4 microparticles, and 30% survival was observed in mice treated with antiCTLA-4 in IFA emulsion. Here again the therapeutic efficacy of microparticles and IFA formulation were comparable [31]. Treatment with empty microparticles showed no effect on tumor outgrowth and the survival of the mice treated with these microparticles was comparable with that of untreated mice (Supplementary Fig. S3). This supports earlier studies which have shown the advantages of local and sustained delivery of antiCD40 and antiCTLA-4 as well as other immunomodulatory molecules in cancer treatment in comparison with systemic administration [31,32,55]. It has been shown that low doses of immunomodulatory antibodies are capable of inducing CD8+ T cell immune responses that are as effective as systemic high doses without leading to the adverse effects associated with high antibody serum levels, such as autoimmune and inflammatory conditions. Therefore, local delivery of these antibodies is plausible and sustained-release formulations were developed to provide sustained delivery of the antibodies locally where they are needed [56]. IFA and similar vehicles have been widely used in preclinical studies as well as in clinical trials [57]. Though these vehicles differ in characteristics, they are used to formulate an emulsion (w/o in case of IFA and Montanide, or o/w in case of MF-59) containing the immunotherapeutic agent. This emulsion forms a depot and provides sustained release of the cargo. The challenges that emerge from using these formulations are the local and systemic adverse effects associated with the IFA formulations (as well as Montanide ISA 51) [58]. Alternatively, dextran microparticles containing antiCD40 have been used for this purpose in a preclinical study. These particles were successful in providing a sustained antibody release *in vitro* and *in vivo*, although they caused increased tumor outgrowth and local inflammation as well as ulcerating subcutaneous swelling [59]. Importantly, as the present study shows, pLHMGA microparticles loaded with antiCD40 or antiCTLA-4 can result in efficient antitumor efficacy in a therapeutic setting in tumor-bearing mice comparable to IFA formulation but without causing adverse effects. This was expected because microparticles showed low antibody serum levels at early and late time points following administration in mice.

4. Conclusion

This study shows that polymeric microparticles based on pLHMGA are capable of providing sustained delivery of encapsulated antibodies, and when administered locally and close to the tumor microenvironment, exhibit equal and efficient therapeutic efficacy as compared to IFA formulations. These biodegradable particles importantly display no local adverse effects. Moreover, low antibody serum levels at different time points suggest a strong limitation of systemic adverse effects. In conclusion, pLHMGA microparticles are attractive systems for local and sustained delivery of biotherapeutics.

Acknowledgements

This research was performed within the framework of the Cancer Vaccine Tracking project (#03O-302), Center for Translational Molecular Medicine (CTMM).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.04.043.

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