



Research paper

Encapsulation of antigen in poly(D,L-lactide-co-glycolide) microspheres protects from harmful effects of γ -irradiation as assessed in miceDeepa Mohanan^a, Bruno Gander^b, Thomas M. Kündig^a, Pål Johansen^{a,*}^a Department of Dermatology, University Hospital of Zurich, Zurich, Switzerland^b Institute of Pharmaceutical Science, ETH Zurich, Zurich, Switzerland

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ABSTRACT

During the last two decades, synthetic polymers such as poly(lactide-co-glycolide) (PLGA) have been investigated for the development of nano- or microparticles as adjuvants or antigen vehicles. To enable transfer of this technology to human settings, the issue of sterilisation is of central importance. Since most polymers are heat-sensitive, sterilisation of polymeric microspheres for parenteral administration is assured either by costly and laborious aseptic preparation or the more preferred γ -irradiation. Many studies have investigated the effect of γ -irradiation on various physicochemical properties of the microspheres, but investigations on immunological effects are rare. We prepared poly(lactide-co-glycolide) (PLGA) microspheres containing ovalbumin (OVA) and tested the effect of γ -irradiation on the various immunological properties in mice. For reference, OVA was γ -irradiated and tested equivalently. The ability of encapsulated or non-encapsulated OVA to trigger activation of dendritic cells (DCs) was not affected by irradiation. However, while γ -irradiation of free OVA strongly influenced the antigen presentation, encapsulated OVA was not affected by irradiation. γ -Irradiation of OVA also reduced the immunogenicity in mice with regard to OVA-specific IgG1 production. In contrast, the antibody and the T-cell responses in mice immunised with PLGA-encapsulated OVA were similar irrespective of the γ -irradiation status. Hence, encapsulation of antigen into PLGA microspheres protects antigen from the potential detrimental effect of γ -irradiation leading to inactivation or altered immunogenicity. Sterilisation by γ -irradiation therefore enables a cost-effective production of PLGA-based antigen-delivery systems as compared to the more laborious and expensive aseptic production of such vaccines.

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1. Introduction

Micro- or nanoparticles fabricated from polymer poly(lactide-co-glycolide) (PLGA) have been ascribed adjuvant properties with potential application in vaccination and immunotherapy [1–5]. PLGA is widely used in pharmaceutical products and medical devices and has an excellent safety record, and particles thereof are stable at ambient temperature and can be chemically and physically modified for particular applications [6–8]. PLGA particles effectively target antigen-presenting cells due to their particulate nature as well as their ability to provide a depot effect or sustained antigen delivery because of the slow degradation of the polymer [9]. The erosion or the degradation time of the polymer can be controlled by choosing a polymer of appropriate physico-chemical

properties such as hydrophobicity and molecular weight, by which antigen release can be sustained and thereby mimic multiple doses of conventional vaccines based on aluminium-adsorbed antigens. However, the clinical transfer of the PLGA-vaccine technology is hampered by the difficulties in assuring sterile samples for human testing and use. As the preparations are heat-sensitive, moist heat sterilisation (autoclaving) is not possible, and PLGA particles are typically too large to be sterile-filtered through 0.22- μ m filters. Sterility by aseptic preparation is possible, but adds significant complexity and costs to the production. Hence, sterilisation by γ -irradiation would be a more convenient and cost-effective method to achieve sterility.

Sterilisation by γ -irradiation has been shown to affect various physico-chemical properties of polymer-based delivery systems. Collectively, these studies suggest that γ -irradiation may alter the release kinetics of encapsulated drugs [10–12], the distribution of the drug within the polymer matrix [13], and the stability of both polymer and embedded protein [14–18]. Although γ -irradiation is well established and regularly used for end-sterilisation of polymeric dosage forms and implants, degradation products of

Abbreviations: Irr, irradiated; Non-irr, non-irradiated; OVA, ovalbumin; MS, microspheres; Ab, Antibody.

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both carrier and content due to the high-energy ionisation may pose an important challenge for the development and quality assurance of such products. Hence, while regulatory organisations such as FDA and EMA may provide recommendation in terms of sterility assurance, measures must be balanced against risk of causing formation of new products due to ionisation of the formulation as well as the risk of changing fundamental formulation properties such as the targeted biological effects. With respect to polymer-based vaccines, only a few studies have investigated the effect of γ -irradiation on the immunological properties of antigen-loaded microspheres *in vivo* [18,19]. One approach to overcome the potential detrimental effects of γ -irradiation on the immunogenicity could be to irradiate empty PLGA microspheres, and in a second step, to adsorb sterile antigens on the particle surface [20]. However, to simplify production, protect the antigen immunogenicity, and to provide sustained or delayed antigen release, it is preferable to encapsulate the antigen within the particles. The aim of the current study was therefore to investigate the effect of γ -irradiation of antigen-containing PLGA microspheres on important adjuvant and vaccine properties *in vitro* and in mice. The study revealed that γ -irradiation indeed impaired the immunogenicity of non-encapsulated OVA, but it did not cause changes in antigen presentation (*in vitro*) and immunogenicity (*in vivo*) of the antigen when encapsulated within PLGA microspheres.

2. Materials and methods

2.1. Animals

Female BALB/c mice were purchased from Harlan (Horst, The Netherlands). T-cell transgenic mice C.Cg-Tg(DO11.10)10Dlo/J recognising the ovalbumin peptide OVA_{323–339} on a 2H^d MHC-class II context in a BALB/c background [21] were originally purchased from Jackson Laboratories (stock number 003303; Bar Harbor, ME) and further bred in our facility. All mice were maintained in specific-pathogen-free conditions according to the guidelines of the veterinary authorities of the canton of Zurich. The mice typically entered studies *in vivo* at and age of 6–10 weeks.

2.2. Preparation of PLGA microspheres

Poly(lactide-co-glycolide) (PLGA) microspheres were prepared by spray-drying as described elsewhere [22]. Briefly, 50 mg OVA (CalbioChem) was dissolved in 0.5 ml water, and this solution was mixed with 1 g uncapped PLGA 50:50 (Resomer RG503H; Boehringer-Ingelheim, Ingelheim, Germany) dissolved in 20 ml dichloromethane (Fluka, Buchs, Switzerland). The aqueous and organic phases were homogenised under ultrasonication (Hielscher, Teltow, Germany, UP200 H, Amplitude 40%) for 10 s on ice, and the obtained w/o-dispersion was spray-dried (Mini Spray-Dryer B-191, Büchi, Flawil, Switzerland) at a flow rate of 2 ml/min and inlet/outlet temperatures of 40/37 °C. The obtained microspheres were rinsed from the collection vessels with an aqueous 0.05% poloxamer 188 (Synperonic®F68, Serva Electrophoresis, Heidelberg, Germany) solution, collected on a cellulose acetate membrane filter, dried at 20 mbar and room temperature for 18 h, and stored in air-tight glass containers at 4 °C. Particle characteristics such as size, zeta potential and antigen loading were measured as described [23]. The amount of OVA within PLGA microspheres was determined by incubating 10 mg microspheres in a 3-ml solution consisting of 0.1 N sodium hydroxide and 5% sodium dodecylsulfate at room temperature overnight. The protein content within the solution was then measured using a BCA kit (Thermo Fisher 109 Scientific, Lausanne, Switzerland). The size and the zeta poten-

tial of the PLGA microspheres were determined using Mastersizer 2000 (Malvern Instruments, Herrenberg, Germany) and Delsa™ Nano (Beckman Coulter, Krefeld, Germany), respectively. Burst release was determined by incubating 20 mg of PLGA microspheres in 4 ml of 67 mM PBS (pH 7.4) containing 0.02% sodium azide and 0.01% polysorbate 20 at 37 °C under mild rotational movement (3 rpm.). Supernatant (1 ml) was collected at regular intervals, replaced by fresh buffer, and the antigen content determined fluorimetrically ($\lambda_{\text{ex}}/\lambda_{\text{em}}$: 338/280 nm; Cary Eclipse, Varian, Zug, Switzerland). All analyses were done in triplicates.

2.3. Irradiation and microbiological testing of native and microencapsulated OVA

Samples of pure crystalline OVA and OVA-loaded PLGA microspheres were weighed into 1.5 ml Eppendorf tubes, and residual humidity was removed under reduced pressure in a vacuum desiccator over phosphor pentoxide at 4 °C. After 2 h of drying, the desiccator was carefully vented with dry nitrogen, and the tubes were sealed. The samples were then placed on ice and irradiated with 12.5 or 25 kGy using an in-house ⁶⁰Co-source. All samples were kept sealed at 4 °C until further use.

The presence of viable microbes in irradiated and non-irradiated samples was determined by aseptically re-suspending 1 mg of either pure OVA or OVA-loaded PLGA microspheres in 1 ml of Luria-Bertani (LB) broth, and the sealed tubes were incubated under agitation at 37 °C for 48 h. The turbidity of the broth was then determined by photometry as a measure for microbiological growth, and dilutions of the aliquots were plated on solid agar plates for another 48 h to test for colony formation. As a microbiological control, *Candida* spores resuspended in PBS were either irradiated or non-irradiated under similar conditions as that of free and encapsulated OVA, and aliquots of these suspensions were plated on Sabouraud's agar at room temperature for 24 h.

2.4. Generation of bone-marrow-derived dendritic cells

BALB/c mice were euthanised, and the femurs and tibia removed from mice were flushed with complete medium [RPMI 1640 medium supplemented with foetal calf serum (FCS; 10%) L-glutamine (2 mM), streptomycin (100 ng/ml), penicillin (100 U/ml), sodium pyruvate (1 mM), and 2-mercaptoethanol (5 μ M)], using 2 ml syringes equipped with 25G needles; all medium supplements were from Invitrogen Life Technologies (purchased from LuBioScience, Lucerne, Switzerland). Erythrocytes were removed using red blood cell lysing buffer (Sigma-Aldrich, Buchs, Switzerland), and the remaining bone-marrow cells were seeded at a concentration of 1×10^6 cells/ml in complete medium supplemented further with GM-CSF (10% of supernatant from GM-CSF-transfected murine myeloma X-63 cell line; a gift from A. Rolink, Basel). Ten million cells were plated in 10-cm polystyrene petri dishes, and every second day, 75% of the cell supernatant was carefully removed and replaced with fresh GM-CSF-supplemented medium. After 7 days, the loosely adherent dendritic cells (DCs) were harvested by flushing with medium. The collected DCs were washed once and re-suspended in complete medium to a final concentration of 1×10^6 cells/ml for further use.

2.5. Analysis of DC activation by flow cytometry

OVA dissolved in FCS or OVA-loaded PLGA microspheres re-suspended in FCS were incubated with bone-marrow DCs for 24 h. The cells were then harvested and washed with FACS buffer (PBS with 1% FCS and 1 mM EDTA) and analysed for expression of CD11c, CD80, and CD86 using fluorescently labelled antibodies against from eBioscience (Bender MedSystems, Vienna, Austria) and flow

cytometry (FACSCanto, BD Biosciences, San Jose, CA). Data were analysed using FlowJo 8.5.2 software (Tree Star, Ashland, OR).

2.6. Antigen presentation studies with OVA-specific T cells

CD4 T cells were purified from splenocytes of the TCR transgenic DO11.10 mice using MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Equal numbers (1×10^5) of DO11.10 CD4 T cells, and syngeneic DCs were co-cultured with varying amounts of irradiated or non-irradiated OVA or OVA-loaded PLGA microspheres in FCS. After 24 h, the supernatants were collected and assayed by enzyme-linked immunosorbent assay (ELISA) for interleukin (IL)-2 (kit from eBioscience) and interferon (IFN)- γ (kit from BioLegend, Fell, Germany), according to the providers' protocol. The cells were harvested and analysed for lymphocyte activation by flow cytometry as described above after staining with fluorescently labelled antibodies against CD3, CD4, CD25, and CD69 (eBioscience).

2.7. Immunisation of mice

BALB/c mice were immunised subcutaneously (s.c.) with a single dose of either irradiated or non-irradiated OVA-containing PLGA microspheres at doses of 200 and 40 μg of OVA. For injection, 25 mg (high dose) or 5 mg (low dose) of PLGA microspheres was suspended in 1.2 ml of PBS containing 0.025% polysorbate 20 (Serva Electrophoresis, Heidelberg, Germany) and 2.5% lecithin (Epikuron 200, Degussa, Hamburg, Germany). The suspensions were sonicated for 2 min in a TCP-40 ultrasonic compact cleaner (Telsonic, Bronschhofen, Switzerland). Then, 200 μl of the suspension was injected in the scruff of the neck using 24G needles. Vaccine preparations with irradiated or non-irradiated OVA adsorbed on aluminium hydroxide were used as controls and prepared by dissolving 11.20 mg (high dose) or 2.24 mg (low dose) OVA in 8.56 ml PBS and adding 2.64 ml of aluminium hydroxide (Alhydrogel 3%, Brenntag Biosector, Fredrikssund, Denmark). The OVA-Alhydrogel mixture was kept under mild agitation for 1 h before injection (200 μl per mouse). Mice were bled at various intervals, and the collected sera frozen at -20°C for later analysis of OVA-specific antibodies.

2.8. *In vitro* re-stimulation of splenocytes for analysis of T-cell responses

On day 70 post-immunisation, mice were euthanised, spleens harvested, and single-cell suspension of erythrocyte-free splenocytes prepared. Then, 2×10^5 splenocytes were re-stimulated with 10 $\mu\text{g}/\text{ml}$ of OVA in 96-well, flat-bottom culture plates. After 24 h, supernatants were collected and stored at -20°C until analysed by ELISA for IL-2 as a measure for T-cell proliferation.

2.9. Analysis of OVA-specific antibodies by ELISA

For detection of OVA-specific antibodies, Immuno 96 MicroWell Maxisorb plates (Nunc, Wiesbaden, Germany) were coated with either 50 μl of 10 $\mu\text{g}/\text{ml}$ OVA for IgG1 quantification or 100 μl of 5 $\mu\text{g}/\text{ml}$ OVA for IgG2a quantification. The OVA was dissolved in carbonate buffer of pH 9.6, and plates were kept at 4°C overnight. All subsequent manipulations were performed at ambient temperatures, and the incubation steps were intercepted by washing the plates four times with PBS containing 0.05% Tween 20 (PBST). The plates were blocked for 1 h with 2.5% skimmed dry milk in PBST (PBSTM). For analysis of IgG1, 1000-fold and 10,000-fold dilutions of serum samples as well as a monoclonal anti-OVA antibody standard (OVA-14 from Sigma) in PBSTM were added and incubated for 2 h. The plates were then incubated with 0.5 $\mu\text{g}/\text{ml}$ bi-

otinylated rat anti-mouse IgG1 (MCA336B, Serotec, Düsseldorf, Germany) in PBSTM for 2 h. Finally, the plates were incubated with a 1:1000 dilution of streptavidin-conjugated horseradish peroxidase (BD Pharmingen, Basel, Switzerland) for 1 h and developed with the substrate 3,3',5,5'-tetramethylbenzidine (BD Pharmingen) for 15 min before reaction was stopped with 2 N sulphuric acid. The endpoint optical density was measured at 450 nm using a microplate reader (Model 550, BioRad, Hercules, CA, USA). For IgG2a measurements, serial dilutions of individual sera were used. OVA-specific antibodies were then detected with 1 $\mu\text{g}/\text{ml}$ biotinylated rat anti-mouse IgG2a (BD Pharmingen) and streptavidin-conjugated horseradish peroxidase. The plates were developed with 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulphonic acid) diammonium salt (Sigma-Aldrich, Buchs, Switzerland) in 1 M sodium dihydrogenphosphate and measured at 405 nm as above. The antibody titres were defined as the inverse of the highest dilution reaching an absorbance equal or higher than that of a negative serum plus two standard deviations, and the titres were expressed as means \pm standard deviation ($n = 5$).

2.10. Statistical analyses

All *in vivo* experiments were performed twice and within each experiment, there were five animals per group. *In vitro* experiments were performed at least thrice. Mean values and standard deviations were calculated for each data, and representative experiments are shown in the figures. *In vivo* data were analysed using a two-way ANOVA with Bonferroni's post-test. The significance level was set at 5%. Statistical analysis was performed using GraphPad Prism v.5.

3. Results

3.1. Particle characteristics

The spray-drying of OVA/PLGA produced spherical microspheres with a mean particle size of approximately 880–900 nm. The particles carried a negative charge with a zeta potential of -29 ± 1 mV. The content of OVA was 43 ± 6 $\mu\text{g}/\text{mg}$ microspheres, and the burst release of OVA was $32 \pm 3\%$ of the total dose, as measured after 24 h incubation in PBS and at 37°C . None of these properties significantly changed after irradiation of the particles. The microbial testing in LB broth and on agar plates revealed that sterility was obtained at irradiation doses of both 25 and 12.5 kGy (data not shown).

3.2. γ -Irradiation did not alter the capacity of OVA-loaded PLGA microspheres to induce DC maturation

To investigate whether γ -irradiation of native and microencapsulated OVA altered the capacity to induce maturation of antigen-presenting cells, bone-marrow-derived DCs were cultured for 24 h with OVA-containing PLGA microspheres or soluble OVA. The maturation of DCs was determined by the expression of the co-stimulatory molecules CD80 and CD86. OVA-loaded PLGA microspheres caused up-regulation of both CD80 and CD86 co-stimulatory antigens on DC at levels significantly above the levels measured with non-stimulated DC, and the maturation level was not affected by the irradiation of the particles (Fig. 1). Soluble OVA also stimulated CD80 and CD86 expression on DCs irrespective of irradiation status. Of note, the expression of these maturation markers was lower in DC cultures with soluble OVA than in cultures with OVA-loaded PLGA microspheres, clearly demonstrating intrinsic adjuvant properties of the particles.

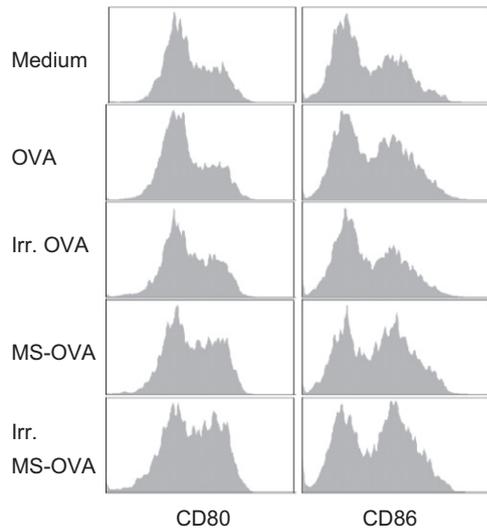


Fig. 1. BALB/c bone-marrow DCs were incubated with 10 $\mu\text{g/ml}$ ovalbumin (OVA) either entrapped in PLGA microspheres (MS-OVA) or dissolved in PBS (OVA). Non-stimulated DCs were used as controls. After 24 h, the expression of CD11c, CD80, and CD86 on DCs was analysed by flow cytometry. The histograms illustrate representatively the expression of CD80 (A) and CD86 (B) on CD11c-positive cells from one out of three experiments.

3.3. γ -Irradiation of OVA-loaded microspheres did not affect antigen presentation

The generation of adaptive immune responses requires efficient antigen processing and presentation of antigen epitopes to T cells. We therefore tested whether irradiation of PLGA microspheres altered the presentation of OVA by DCs to T cells. Briefly, OVA-loaded PLGA microspheres were incubated with DCs for 24 h, and the cultures were then added CD4 T cells from T-cell receptor transgenic DO11.10 mice. After further incubation for 24 h, the mixed cultures were harvested and the cells stained for the T-cell markers CD3 (all T cells) and CD4 (T helper cells) and the expression of the early activation marker CD69 and the IL-2 receptor subunit alpha CD25, which also serves as an activation and proliferation marker of T cells. PLGA microspheres clearly facilitated antigen presentation to T cells when compared to soluble non-irradiated OVA, because the frequency of both CD69- and CD25-expressing CD4 T cells was notably higher in cultures incubated with microspheres than in cultures with soluble non-irradiated OVA (Fig. 2). Importantly, γ -irradiation of the microspheres exerted no effect on the T-cell activation. In contrast, γ -irradiation of non-encapsulated OVA strongly affected the presentation of the OVA_{323–339} epitope. Increased CD69 and CD25 expression were observed in T cells incubated with irradiated OVA as compared to those exposed to non-irradiated OVA.

Further analysis of the effect of γ -irradiation of OVA-containing PLGA microspheres on antigen presentation was done by measuring secretion of IFN- γ and IL-2 into culture supernatants. A dose-dependent secretion of IFN- γ was observed from T cells incubated with PLGA microspheres at doses corresponding to 0.1, 1, 10 and 100 μg OVA (Fig. 3A, left panel), while the IL-2 secretion reached a maximum equilibrium value at a dose of 1 μg OVA (Fig. 3B, left panel). γ -Irradiation of the PLGA microspheres did not affect IFN- γ and IL-2 secretion. In contrast, γ -irradiation of non-encapsulated OVA strongly affected the cytokine secretion. In agreement with the increased CD69 and CD25 expression of the T cells, the secretion of both IFN- γ and IL-2 was highly increased in cultures stimulated with irradiated OVA as compared to cultures stimulated with non-irradiated OVA (Fig. 3A and B, right panel). Of note,

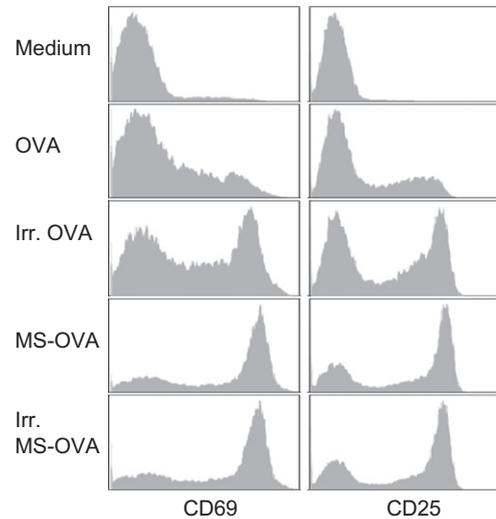


Fig. 2. BALB/c bone-marrow DCs were co-cultured with purified CD4-positive and OVA-specific T cells from DO11.10 mice. The cultures were incubated with 10 $\mu\text{g/ml}$ OVA either entrapped in PLGA microspheres (MS-OVA) or dissolved in PBS (OVA) or were left un-stimulated. After 24 h, the expression of CD3, CD4, CD69 and CD25 on the T cells was analysed by flow cytometry. The histograms illustrate representatively the expression of CD69 (A) and CD25 (B) on CD3 and CD4 double-positive cells from one out of three experiments.

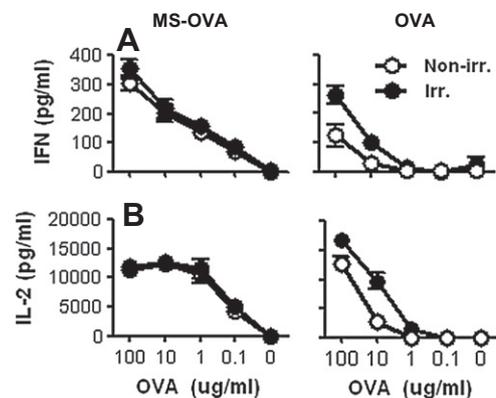


Fig. 3. BALB/c bone-marrow DCs were co-cultured for 24 h with purified CD4-positive and OVA-specific T cells from DO11.10 mice. Dissolved or micro-encapsulated OVA was added at different concentrations. Supernatants were analysed for IFN- γ (A) or IL-2 (B). The graphs are representative of three individual *in vitro* experiments and show mean \pm S.D. ($n = 3–4$).

OVA in PLGA microspheres mediated stronger cytokine secretion than did non-encapsulated OVA, because 10–100-fold higher doses of free OVA were required for induction of cytokine secretion that was comparable to that mediated by OVA-containing PLGA microspheres.

3.4. γ -Irradiation of non-encapsulated OVA but not of PLGA-OVA impaired antibody responses in mice

To assess whether γ -irradiation of microencapsulated OVA altered the immunogenicity *in vivo*, both irradiated and non-irradiated formulations were administered by a single subcutaneous injection at doses corresponding to 40 μg or 200 μg OVA. In a first experiment, PLGA microspheres irradiated at 25 kGy, 12.5 kGy or left non-irradiated were subcutaneously administered to BALB/c mice at a dose of 200 μg OVA. Immunisation triggered significant titres of both OVA-specific IgG1 and IgG2a antibodies. The titres measured in serum after 31 days did not vary as a function of

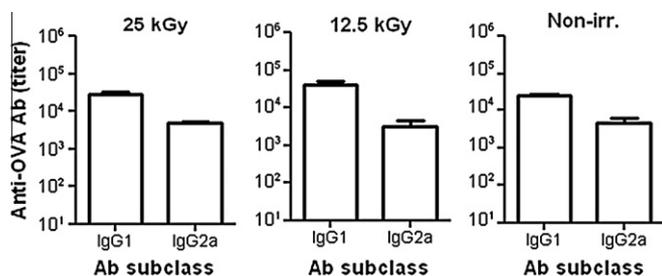


Fig. 4. PLGA microspheres containing OVA were γ -irradiated at 25 kGy (left panel), 12.5 kGy (centre panel) or not (right panel). Groups of five BALB/c mice were then immunised subcutaneously with irradiated or non-irradiated microspheres corresponding to 40 μ g OVA. On day 31 after immunisation, the animals were bled, and ovalbumin-specific IgG1 and IgG2a in sera were analysed by ELISA. The results are illustrated as antibody titres. Analysis of sera collected on days 17 and 61 showed similar results.

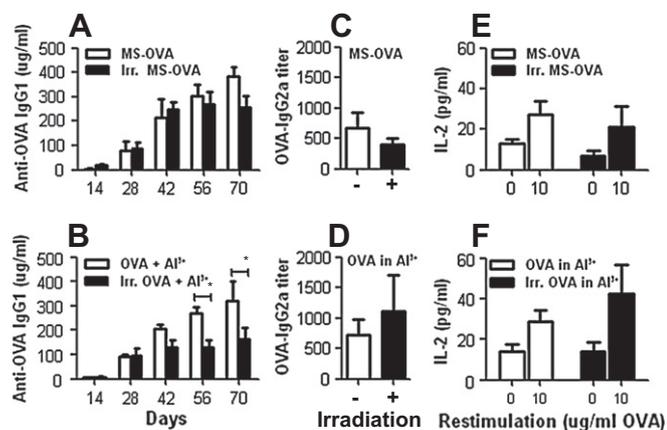


Fig. 5. Groups of five BALB/c mice were immunised subcutaneously with 200 μ g OVA entrapped in PLGA microspheres (upper panel) or adsorbed on aluminium hydroxide (lower panel). OVA-specific IgG1 (A–B) and IgG2a (C–D) were analysed by ELISA. On day 70, the animals were euthanised and the splenocytes re-stimulated with 10 μ g/ml OVA or left un-stimulated for analysis of secretion of IL-2 after 24 h (E–F). Open bars illustrate non-irradiated MS-OVA (A, C, E) or OVA (B, D, F), while filled bars illustrate irradiated MS-OVA or OVA. The data are representative of two individual *in vivo* experiments.

irradiation dose (Fig. 4), and the titres were stable for at least 61 days (not shown).

In a second experiment, irradiated and non-irradiated PLGA-based formulations were compared with irradiated and non-irradiated control preparations of OVA that were adsorbed on aluminium hydroxide. Sero-conversion of the major immunoglobulin allotype IgG1 was observed after 28 days with both the PLGA- and aluminium-based formulations (Fig. 5A and B). Immunisation with 40 μ g OVA produced similar anti-IgG1 kinetics (data not shown), but the endpoint concentrations of the antibodies were only one tenth of that achieved after immunisation with 200 μ g OVA. No significant affect of γ -irradiation on serum IgG1 levels was observed in mice immunised with non-irradiated or irradiated PLGA microspheres (Fig. 5A). In contrast, γ -irradiated OVA in the aluminium-based vaccine caused significantly reduced IgG1 antibody responses (Fig. 5B; $p < 0.05$ by 2-way ANOVA with Bonferroni correction). No significant differences were observed in the stimulation of IgG2a antibody responses when comparing γ -irradiated and non-irradiated preparations of PLGA microspheres or aluminium hydroxide (Fig. 5C and D).

On day 70 post-immunisation, the mice were euthanised, and the harvested splenocytes were re-stimulated *ex vivo* with OVA for analysis of IL-2 secretion in the supernatants. At an immunisa-

tion dose of 200 μ g OVA, irradiated and non-irradiated PLGA microspheres evoked similar levels of antigen-specific IL-2 production (Fig. 5E). Similarly, aluminium hydroxide-based OVA also facilitated antigen-specific IL-2 secretion in overnight splenocyte cultures (Fig. 5F), which was surprisingly not affected by the γ -irradiation of the formulations.

4. Discussion

The use of biodegradable PLGA polymers in parenteral antigen-delivery systems requires product sterility. If aseptic processing is not readily feasible, sterility is most commonly warranted by γ -irradiation of the end product. Several reports have documented detrimental effects of γ -irradiation on the physical and chemical properties of PLGA-based antigen-delivery systems [24–28], only one study addressed questions regarding how γ -irradiation affected the antibody responses against encapsulated antigens [19], and no study investigated the influence of γ -irradiation on adjuvanticity, i.e., innate immune responses and antigen presentation. The primary objective of the current study was thus to test to what extent γ -irradiation of a vaccine based on PLGA microspheres affected the immunological performance *in vitro* as well as *in vivo*. While γ -irradiation affected antigen presentation *in vitro* and immunogenicity *in vivo*, of the model antigen OVA, the encapsulation of OVA within PLGA microspheres protected the antigen from detectable alterations in the stimulation of the innate and adaptive immune responses. Importantly, γ -irradiation produced sterile vaccines.

In a simplified model, the generation of immune responses is dependent on four signals: signal 0 is represented by danger or non-self; signal 1 is the stimulation of T-cell receptors by MHC-bound antigens; signal 2 comprises the co-stimulation of CD28 or CTLA-4 by molecules such as B7.1 (CD80) or B7.2 (CD86); signal 3 is delivered by cytokines such as IL-6, TNF- α , TGF- β and IL-12 that control the differentiation of T cells into various types of effector cells [29,30]. In this respect, the use of particles in vaccines has several purposes. Firstly, particles possess adjuvant properties which enable the immune system to recognise the vaccine as foreign or danger (signal 0). This immunological recognition can be further facilitated by fabricating particles that also stimulate pathogen-recognising receptors, e.g., encapsulation of toll-like receptor ligands. Secondly, particles are utilised in order to protect antigen and epitopes that are sensitive to de-stabilising reactions during storage and administration which might result in decreased affinity to MHC molecules and T-cell receptors (signal 1). Finally, particles can trigger maturation of APCs with expression of the co-stimulating molecules such as CD80 and CD86 (signal 2), [31].

A thorough investigation of the effect of γ -irradiation on these different features of the immune response was never reported. The integrity of a synthetic anti-malaria antigen SPf66 [19] was maintained after γ -irradiation and irradiated PLGA particles produced similar antigen-specific titres of IgG in mice when compared to mice that were immunised with non-irradiated PLGA particles. However, other attributes of the immune response were not analysed, for which reason it is unknown whether the preparation also maintained its general adjuvant (signal 0) capability as well as its ability in stimulating secretion of different IgG-subclasses or evoking different T-cell responses. Moreover, the SPf66 antigen consists of 3–5 peptides, of approx. 5 kDa. These small antigens are less prone to conformational changes caused by irradiation-induced free radicals and oxidation of amino acid residues [32] than are larger antigens such as the 45 kDa OVA used in our study. The SPf66-containing PLGA particles were furthermore prepared by solvent evaporation with potential residuals of poly(vinyl alcohol) and 2-isopropanol, which may have protective effects on the protein

stability [33,34]. In our study, we looked at the effect of γ -irradiation on the various steps leading up to protective immune responses. Not only were the antigen-specific cellular and humoral responses unaffected by γ -irradiation, but also the adjuvant properties of PLGA particles remained unchanged after γ -irradiation. This was in contrast to results achieved from testing non-encapsulated antigen.

The potentially detrimental effects of γ -irradiation on polymers and antigens are typically due to the free radicals formed during irradiation [11]. Gamma irradiation is ionising by definition and causes formation of temporary free radicals, most of which will disproportionate or recombine at the end of irradiation. However, as much as 10% of the formed radicals may remain and cause damage to both the antigen and the polymer [35,36]. Surprisingly therefore, neither the strength nor the kinetics of the immune responses evoked by encapsulated antigen were notably affected by γ -irradiation in the present study. Moreover, the *in vitro* and *in vivo* data for PLGA microspheres were congruent inasmuch as the γ -irradiation did not exert any measurable effect on the immunological parameters tested. In contrast, whereas the irradiation improved the antigen presentation of non-encapsulated or free OVA as measured by T-cell activation and cytokine secretion *in vitro*, the antigen-specific IgG1 antibody response in mice was reduced. One possible explanation for the apparent contradiction in latter results is that γ -irradiation can cause denaturation and aggregation of the protein and that the formed aggregates were more readily available for phagocytosis than soluble protein [37]; soluble protein is generally taken up by antigen-presenting cells through pinocytosis, which is less efficient than phagocytosis [38,39]. This may also explain why IgG2a antibody responses did not decrease after irradiation as the IgG2a epitopes are often more hydrophobic and hidden in the core of large and folded proteins [40] and upon γ -irradiation, the antigen may have undergone denaturation and changes in the relative exposure of epitopes. The irradiation-induced stimulation of more or new T-cell epitopes may have compensated for the overall reduced immunogenicity as seen for IgG1. Another explanation for the increased *in vitro* antigen presentation and decreased *in vivo* antibody response of non-encapsulated antigen may be that the *in vitro* assay measured the reactivity against a single T-cell epitope, while the readout in the *in vivo* assay is a result of a polyclonal activation of both T and B cells against other antigenic determinants of OVA [41,42]. In compliance with this, *in vitro* re-stimulation of splenocytes from mice immunised with irradiated non-encapsulated OVA produced similar or even stronger T-cell responses than did re-stimulation of splenocytes from mice immunised with non-irradiated OVA. Hence, the γ -irradiation of non-encapsulated OVA might have helped to improve the processing and the presentation efficacy of the short OVA_{323–338} sequence, which is the CD4 T-cell epitope recognised by the transgenic T cells utilised, whereas the processing and presentation of the longer epitopes involved in the generation of the polyclonal antibody responses *in vivo* were impaired after γ -irradiation. The affinity of longer epitopes to T- and B-cell receptors is more sensitive to changes in the conformation as compared to shorter and more linear epitopes. However, it should be noted that our study was limited to a single protein antigen, namely OVA, and that the effect of γ -irradiation on the physico-chemical and antigenic integrity of proteins antigens may be amino-acid- sequence dependent. It has been shown that amino acids such as tryptophan, tyrosine, histidine, methionine and cysteine are more susceptible to free-radical-mediated oxidation than other amino acids [43,44]. Reactions in antigens containing cysteine typically involve the oxidation of the sulfhydryl groups and the formation of disulfide bridges between two cysteine residues within the protein or between two proteins [45–47]. If the antigen contains several cysteines, this can of course cause polymerisation of the

antigen. Another common radiation-induced reaction involves the change of chirality from L- to D [48,49]. While disulfide formation will have significant effects on the protein conformation and hence its affinity to B-cell receptors, altered chirality will also strongly affect the affinity of MHC-restricted antigen presentation. It is expected that a L-to-D conversion of one amino acid will be sufficient to cause sterical hindrance of the T-cell epitope inside the MHC groove. On the other hand, highly reactive amino acids can also act as radical scavengers, and when their position in an antigen is not crucial for the immunogenicity of the antigen, such amino acids may have a stabilising effect on the epitopes. Hence, depending on the abundance of these amino acids in the antigen epitope, the effect of γ -irradiation on antigenicity and immunogenicity is expected to vary.

Our study revealed that both DC activation and antigen presentation were higher with PLGA microspheres when compared to the non-encapsulated OVA – independent of the γ -irradiation status. This is in line with earlier studies, where the immunogenic properties of PLGA microspheres have been investigated and have been ascribed to preferential recognition and phagocytosis of the solid microspheres when compared to soluble antigen and which make PLGA particle attractive as adjuvant or as a carrier of antigens in vaccines [50,51]. This is one of the factors that make PLGA particle attractive as adjuvant or as carrier of antigens in vaccines.

For sterilisation of parenteral medicinal products, including vaccines, an irradiation dose of 25 kGy is recommended by different pharmacopoeias and by the European Medicines Agency (EMA). When lower irradiation doses are utilised, extensive microbiological testing has to be performed in order to document product sterility. The actual dose required to sterilise PLGA particles has been investigated in a few studies. Although no studies systematically investigated the effect of polymer type and molecular weight on sterilisation efficiency by γ -irradiation of polymeric microspheres, higher γ -irradiation doses seem to be required for particles fabricated from polymers of higher molecular weights [13,52]. In our study, 35 kDa uncapped PLGA was used to fabricate the microspheres, and 12.5 kGy was sufficient to achieve product sterility. Importantly, neither 12.5 kGy nor a dose of 25 kGy caused observable changes in the immunogenicity of encapsulated antigen. In contrast, the irradiation of non-encapsulated antigen caused alterations in the immunological performance of the antigen, both in its soluble form *in vitro* or *in vivo* after adsorption to the conventional adjuvant aluminium hydroxide. Since radical formation is a default consequence of high-energy ionisation by γ -irradiation, changes in the polymer and/or in the antigen will generally occur. Hence, one challenge in the development of PLGA-based vaccines is to balance the irradiation conditions that they provide sterility without entailing undue damage to the formulation with respect to the targeted effects, e.g., immunogenicity and safety, e.g., no toxicity of degradation product caused by the irradiation. A valid approach may be to reduce the irradiation dose to a validated minimum and to use antioxidants that scavenge the free radicals formed. Nonetheless, the effect of irradiation on the immunogenicity of encapsulated antigens is antigen-specific due to the different susceptibility of different amino acids to get involved in such radical reactions.

5. Conclusion

In this study, we investigated the impact of sterilisation of PLGA-based vaccines by γ -irradiation on the most crucial immunological properties. While γ -irradiation altered the capacity of non-encapsulated antigen to stimulate immune responses *in vitro* and *in vivo*, this method of sterilisation did not alter microencapsulated antigen as measured by *in vitro* antigen presentation and immune

responses in mice. Although the benefit of PLGA-based vaccines in humans still awaits demonstration the results of this study support our hypothesis that γ -irradiation of PLGA-based antigen-delivery systems may be a viable and cost-effective alternative to the more strenuous aseptic production of such vaccines. It should be noted that this study was limited to a single protein and that the radiation-related susceptibility of protein antigens may be amino-acid sequence dependent. However, if the presented data and our interpretation can be confirmed for other candidate antigens and PLGA-based formulations γ -irradiation may facilitate formulation development and clinical testing of vaccines based on the PLGA nano- and microparticle technology.

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