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# PLGA microspheres for improved antigen delivery to dendritic cells as cellular vaccines

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**Abstract**

Dendritic cells (DC) are currently employed as cellular vaccines in clinical trials of tumor immunotherapy. In most trials, peptide epitopes derived from tumor antigens are being exogenously loaded onto human DC for binding to MHC class I molecules. While this is a convenient method, it suffers from the drawback that the persistence of class I/peptide complexes on the cell surface is in the order of a few hours. This drawback limits the success of vaccination. We have investigated biodegradable poly(D,L-lactide-co-glycolide) microspheres (PLGA-MS) as delivery tools for antigen loading of human monocyte-derived DC (hMoDC). Immature hMoDC readily take up PLGA-MS and present epitopes from encapsulated proteins or peptides both on MHC class I and class II. Interestingly, antigen presentation by hMoDC was markedly prolonged when hMoDC were charged with PLGA-MS-encapsulated as opposed to soluble antigens. The properties of hMoDC with respect to migration, cytokine secretion, survival and allostimulation were not adversely affected by the uptake of PLGA-MS. In this article, we will review the properties of PLGA-MS as an adjuvant and summarize recent data on their potential for antigen delivery to dendritic cells.

*Keywords:* Dendritic cells; Microspheres; Poly(lactide-co-glycolide); Immunotherapy; Antigen processing; Vaccine delivery

**Contents**



*Abbreviations:* ctl, cytotoxic T lymphocytes; DC, dendritic cells; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; LPS, lipopolysaccharides; mAb, monoclonal antibody; MoDC, monocyte-derived dendritic cell; MS, microspheres; PLGA; Poly(D,L-lactide-co-glycolide); PBMC, peripheral blood mononuclear cells.

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## 1. Introduction

Dendritic cells (DC) have recently made a leap in their career from being 'Nature's adjuvant' to becoming 'The Oncologist's adjuvant'. The elimination of tumor cells by cytotoxic T lymphocytes (CTL) requires a very strong stimulation of CTL, which is vigorous and enduring enough to kill tumor cells despite an antiinflammatory environment created by many tumors and despite the partial down-regulation of MHC class I molecules. Mouse DC appear to be essential for cross priming and the initiation of a CTL response in vivo [1,2]. A comparison of different methods available for CTL induction in mice revealed that mouse DC are superior in inducing protective immune responses [3]. However, the potency of DC-based vaccination is limited by the short persistence of trimeric MHC class I/peptide/β2-microglobulin complexes on the cell surface, in particular when soluble synthetic class I peptide ligands are bound from the outside [4]. Moreover, confining immunotherapy to patients with the appropriate restriction elements puts a limit on patient accrual and requires laborious MHC subtyping by PCR.

Throughout the past three years, we have worked on a method of antigen loading of human monocyte-derived DC (hMoDC), which has the potential to solve all the aforementioned problems of conventional peptide delivery. This method is based on the inclusion of peptides or proteins in biodegradable poly(D,L-lactide-co-glycolide) microspheres (PLGA-MS). PLGA-MS of appropriate size are efficiently taken up by mouse and human DC, and they are slowly hydrolyzed so that prolonged antigen presentation ensues. Remarkably, PLGA-MS-encapsulated proteins reach the processing pathways for MHC class I and class II molecules, so that the stimulation of antigen specific CTL and T helper cells occurs. In this review, we will summarize our experience and the current state of knowledge about PLGA-MS as adjuvant and antigen delivery system for DC as cellular vaccines.

## 2. Properties of PLGA-MS

Poly(D,L-lactide-co-glycolide) (abbreviated either PLG or PLGA) is a polymeric ester of the two α-hydroxyacids lactic and glycolic acids. The hydrolysis of the polymer in aqueous solution leads to the release of the two acids, which can be metabolized via the citric acid cycle. This material has hence been used for many years for the production of biodegradable surgical sutures and for the encapsulation of drugs for protracted release in humans. Thus, the material is well characterized and approved for application in humans, which is an obvious advantage for antigen delivery [5]. PLGA polymers can be purchased with different stoichiometric ratios of lactic and glycolic acids and molecular mass, two parameters that are of determining influence on the hydrophobicity and release kinetics of the polymer. Importantly, PLGA polymers can be converted into insoluble microspheres of a defined size of 1.5–7 μm by spray drying or 10–100 μm by a coacervation technique. The manufacture of PLGA-MS by spray drying has the advantage that the particle size is ideal for the uptake by macrophages and DC [6,7] and that this process is a continuous process suited for industrial production, whereas coacervation or solvent extraction are batch methods often used in laboratories, as no special instrument is required.

The choice of PLGA polymer for antigen microencapsulation depends on the purpose of the immunization strategy. For earlier studies of developing "single-dose" vaccines, PLGA-MS were prepared with polymers with fast or slow degradation rates (e.g., RG502, RG752 and RG206) by either spray drying or coacervation, to yield small and big particles

with fast or slow antigen release profiles [7–10]. The idea behind these studies was that PLGA-MS may serve as single-injection vaccines because they release their content with one burst, occurring at about day 1, and several delayed release pulses after subcutaneous injection, which could obviate the requirement for booster vaccinations (also discussed below).

To load PLGA-MS into the DC to serve as cellular vaccine for immunotherapy, in our experiments, we have mainly focused on Resomer® RG502H, which has a molecular mass of about 14 kDa and is composed of about 50% glycolate and 50% lactate (http://www.boehringer-ingelheim.com/finechem/products/Resomer/RG+502+H.pdf). This fairly hydrophilic polymer is almost completely degraded within 30 days in water. The spray-drying technique was chosen for the purpose of loading MS into the DC because this technique yields MS sizes suitable for DC phagocytosis (Fig. 1). It has been shown that enclosed proteins are continuously released from spray-dried MS at a low level but there is a peak of MS hydrolysis and release of encapsulated material during the first day of MS incubation, as well as on the occasion of complete desintegration after about 30 days [7]. This release kinetic is well compatible with the limited survival period of hMoDC and the requirement of immediate antigen delivery to activate specific T cells.

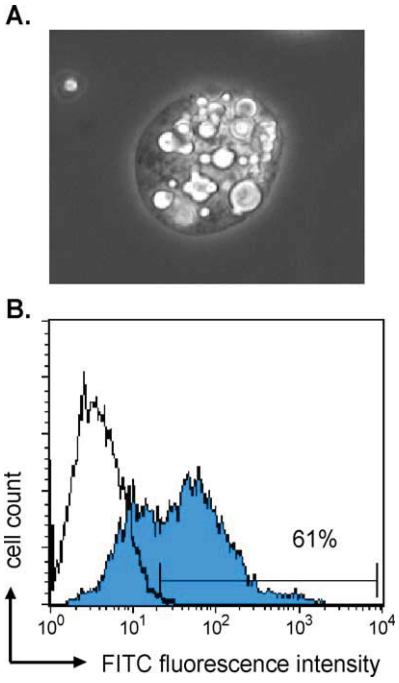


Fig. 1. Phagocytosis of fluorescent microspheres (MS) by human MoDC. Immature hMoDC were incubated with FITC containing MS for 18 h at 37 °C. Cells were then collected, washed and examined by phase contrast microscopy. (A) One immature hMoDC containing many MS is shown at magnification 40×. (B) Quantitative uptake of fluorescent MS by immature hMoDC is examined by flow cytometry. The number within the histogram plot represents the percentage of MS-FITC fluorescent hMoDC based on the whole pregated hMoDC population.

## 3. Direct vaccination of mice with PLGA-MS

Before we explored PLGA-MS as antigen delivery systems for DC in vitro, PLGA-MS were extensively evaluated as an antigen controlled-release formulation for direct vaccination of mice. Tetanus toxoid (TT) was encapsulated in different types of PLGA-MS, and the antibody titers were recorded for a period of 45 weeks [8]. A single injection of 20 μg of TT in PLGA-MS yielded the same TT titers as did three injections of the same amount of TT formulated in Alumn-3 with booster injections given after 5 and 14 weeks. The antibody isotypes IgG1, IgG2a and IgG2b were induced at similar titers as achieved with Alum, thus indicating that the isotype switch occurred readily after the PLGA-MS-mediated vaccination. In particular, no prominent switch towards IgE was observed after PLGA-MS administration, which can occur when Alum preparations are used as adjuvant. Although the role of the humoral response to tumors is currently not so intensively investigated, the encouraging clinical results from tumor immunotherapy with monoclonal antibodies emphasize the importance of antibodies. The ability to induce antibodies is therefore a major advantage of PLGA-MS.

The T cell proliferative responses to TT were similar after immunization with PLGA-MS and Alum, thus indicating that specific T cells were activated. Similar results with respect to antibody titers, antibody isotypes and T cell proliferation were obtained when PLGA-MS were compared with incomplete Freund's adjuvant (IFA) using polypeptides from *Plasmodium falciparum* as model antigens [9]. Because IFA still serves as the gold standard for the potency of an adjuvant, the direct subcutaneous

injection of protein containing PLGA-MS by itself appears to be a useful approach.

A crucial issue, however, is the induction of CTL in vivo, as it has been shown in many tumor models that tumor eradication relies on $CD8^+$ T cells. The induction of CTL responses after s.c. inoculation of mice with either IFA or PLGA-MS was compared using the CTL epitope $Pb_{252–260}$ from the circumsporozoite protein of *Plasmodium berghei* as a model peptide antigen. Specific CTLs to $Pb_{252–260}$ could be detected in cytolytic assays after a single round of restimulation in vitro, and the lysis was similar for IFA and PLGA-MS [10]. Given that mouse DC appear to be essential for the initiation of CTL responses, these results already suggested that PLGA-MS can be taken up by professional antigen presenting cells in vivo for the stimulation of specific CTL. It should be pointed out, however, that the CTL response was still moderate and probably not as vigorous as achieved by DC-based vaccination [3]. Nevertheless, the joint induction of antibodies, T helper cells and CTL through direct immunization with PLGA-MS holds potential for tumor immunotherapy, especially if we succeed in formulating PLGA-MS in a way that leads to a better uptake by DC followed by DC maturation.

## 4. Antigen delivery to DC via PLGA-MS leads to prolonged antigen presentation in vitro

For the presentation of MHC class I restricted T cell epitopes from PLGA-MS-encapsulated protein antigens, the involved antigen presenting cells must be able to cross present; that is, protein antigens must be released from endocytosed PLGA-MS and find their way into either the classical proteasome and TAP-dependent pathway or an alternative proteasome and/or TAP-independent pathway of antigen processing for presentation on MHC class I molecules. Macrophages and DC, as well as endothelial cells, have been shown to be competent for cross presentation [11–13]. Cross presentation of soluble proteins by DC can occur, but it is extremely inefficient, as it usually requires the incubation with protein antigens in the lower millimolar range. Remarkably, the incorporation or attachment of proteins to microparticles in a size range from 0.1 to 10 μm allows an about 1000-fold increase in the efficiency of cross presentation [12], thus allowing potent CTL stimulation in vitro with protein concentrations in the upper nanomolar range. This effect appears to be independent of the chemical nature of the microparticles, as it has been observed for iron oxide beads [11], latex beads [12], polysaccharides [14] or PLGA-MS [6]. For PLGA-MS, it was initially shown that mouse macrophages were lysed by a TT-specific CTL line when they were pulsed with TT protein encapsulated in PLGA-MS. The lysis depended on proteasome activity and was sensitive to brefeldin A, suggesting that TT processing occurred according to the classical pathway. Moreover, presentation was abrogated by cytochalasin D, indicating that the function of actin microfilaments was required for the phagocytosis of PLGA-MS [6]. Subsequently, the analysis was extended to human monocyte-derived dendritic cells (hMoDC), where we could show that a human CTL line specific for the HLA-A0201 restricted M1 epitope from influenza virus matrix protein recognized the hMoDC that had been loaded with PLGA-MS containing recombinant matrix protein (unpublished data).

Once we had confirmed that immature hMoDC can take up PLGA-MS and present CTL epitopes derived from MS-encapsulated proteins, we investigated how long the hMoDC that were charged with PLGA-MS are able to sustain MHC class I restricted antigen presentation. Initially, the external loading of hMoDC with soluble peptides was compared with peptide delivery via PLGA-MS [15]. When hMoDC were incubated with 10 μM influenza matrix M1 peptide and then washed, the peptide loaded hMoDC were able to stimulate HLA-A0201/M1-specific CTL for only 4 days. In contrast, when hMoDC were loaded with an equivalent amount of peptides formulated in PLGA-MS, CTL activation was still observed 7 or 9 days after antigen loading. The prolongation of antigen presentation was similarly observed when recombinant matrix protein was encapsulated into PLGA-MS for delivery to hMoDC. We assume that this extension of antigen presentation is due to the slow hydrolysis of PLGA-MS in the endosomes or cytoplasm of hMoDC, which provides a continuous supply of peptide ligands for newly synthesized MHC class I molecules. Peptide/class I complexes that disappear from the cell surface because of endocytosis

or disintegration will hence be replaced. How PLGA-MS-encapsulated proteins or peptides become accessible to the proteasome in the cytoplasm is still not clear. Electron microscopic images of hMoDC that have taken up PLGA nanoparticles suggest that they remain surrounded by an endosomal membrane [7], while rapid release of endocytosed PLGA-MS into the cytoplasm was reported for arterial smooth muscle cells [16]. It was suggested that the gradual acidification of endosomes would lead to the protonation of the PLGA polymer, resulting in enhanced hydrophobicity and attachment and rupture of the endosomal membrane [16]. However, this mechanism cannot explain why microparticles composed of inert materials, like latex, also promote cross presentation, suggesting that, so far, undefined release mechanisms contribute to the phenomenon as well.

Unlike MHC class I restricted cross presentation, the presentation on MHC class II molecules occurs efficiently when soluble proteins are taken up by hMoDC through pinocytosis. When hMoDC were charged with soluble TT protein in the growth medium, a concentration of 100 ng/ml was sufficient to stimulate the proliferation of a T helper cell line specific for the HLA-DR5-restricted T helper epitope tt30. Remarkably, when the same TT protein was offered in an PLGA-MS-encapsulated form, a 10-fold lower concentration of TT (10 ng/ml) was sufficient to obtain the same level of T cell proliferation (Y. Waeckerle-Men et al., unpublished data). When we determined the kinetics of antigen presentation with hMoDC loaded with a limiting concentration of TT protein (10 ng/ml), we observed that hMoDC loaded with soluble TT ceased to stimulate specific T cells 6 days after antigen loading whereas PLGA-MS-charged DC stimulated these T cells even 10 days after PLGA-MS uptake. Our concern that the improvement in cross presentation may be paralleled by a loss in classical MHC class II restricted antigen presentation turned out to be unjustified. On the contrary, MHC class II restricted presentation also was more sensitive and longer lasting when antigen delivery occurred via PLGA-MS. Taken together, it appears that this method of antigen delivery to DC leads to a prolongation of antigen presentation by MHC class I and II molecules in vitro, and hence, may lead to better immune stimulation in immunotherapy.

## 5. The influence of PLGA-MS uptake on the properties of monocyte derived DC

A sensitive and long-lasting presentation of CTL and T helper epitopes is very helpful, provided that other pivotal parameters for the function of hMoDC are not negatively affected. The hydrolysis of PLGA-MS leads to the liberation of lactic and glycolic acids. One could expect that the resulting acidification could negatively affect the cellular functions of hMoDC. We hence characterized the consequences of PLGA-MS uptake on crucial functions of hMoDC which, were prepared in serum-free media, according to a protocol [17] that we are currently applying in a DC-based phase I trial of immunotherapy against prostate carcinoma. First, we determined whether an incubation of immature hMoDC with PLGA-MS would lead to the phenotypic maturation of DC, but none of the tested surface markers (CD80, CD83, CD86 and HLA-DR) was altered, indicating that PLGA-MS per se did not induce DC maturation [18]. On the other hand, immature hMoDC, which had taken up PLGA-MS, could be fully matured with either lipopolysaccharides (LPS) or a cocktail of proinflammatory cytokines [TNF-α, interleukin (IL)-1β, IL-6, PGE2; Fig. 2]. Because DC have been shown to down-regulate their endocytotic activity after maturation [19], it was not unexpected that mature hMoDC were virtually unable to internalize PLGA-MS. The procedure of choice will therefore be that PLGA-MS are loaded onto immature hMoDC, followed by treatment with maturation stimuli. The functional hallmarks of hMoDC maturation were also not changed through PLGA-MS uptake. PLGA-MS-treated and, subsequently, matured hMoDC displayed the same strong enhancement in their capacity to stimulate naive allogeneic T helper cells and secreted the same amounts of the cytokines IL-12, IL-10 and TNF-α as did untreated control cells [18]. We also tested their ability to migrate towards the lymph-node-derived chemokines CCL19 and CCL21 in Transwell® assays, which was not affected by the ingestion of PLGA-MS. The latter finding is almost surprising, given that the particle size of the largest PLGA-MS is similar to the pore size of Transwell® filters (5 μm in diameter). We also tested the in vivo migration of mouse bone-marrow-derived DC after PLGA-MS uptake. DC that had engulfed fluorescent PLGA-MS

migrated from the subcutaneous injection site to the draining lymph nodes as efficiently as did the control DC, which had been labeled with the dye carboxyl fluorescein succinimidyl ester (CFSE) [18]. In summary, we can say that immature hMoDC that are prepared under serum-free conditions take up a number of 10–30 PLGA-MS particles per cell within a time period of 1–4 h and that the ingestion of PLGA-MS does not adversely affect pivotal functional parameters required for their application as cellular vaccines.

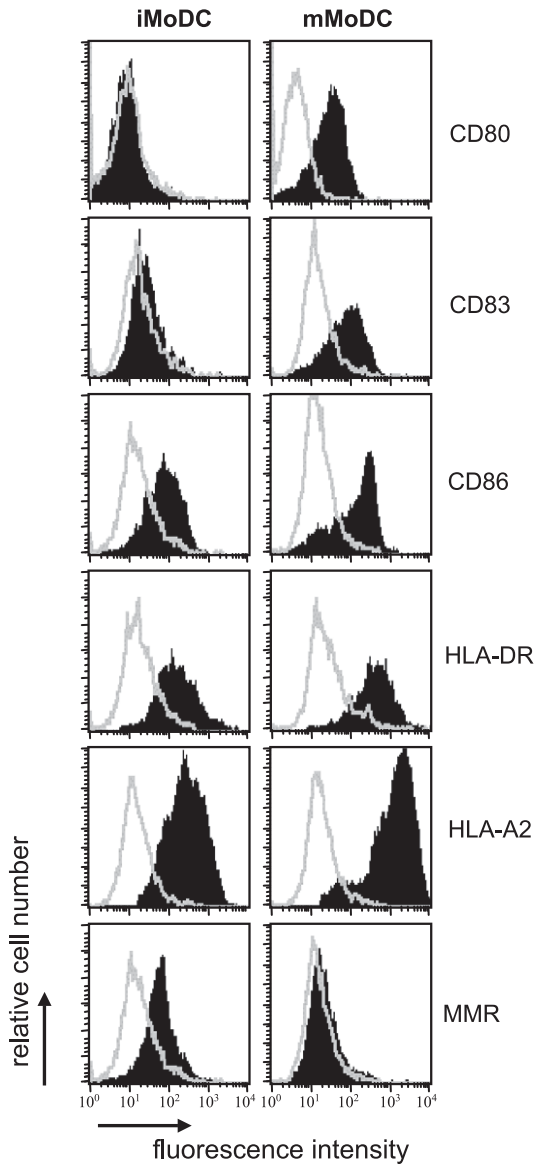


Fig. 2. Phenotype of immature and mature MoDC containing PLGA-MS. Immature hMoDC obtained from HLA-A2-positive donors were loaded with fluorescent Rhodamine B PLGA-MS (0.2 mg MS for $1\times10^6$ cells) in the absence (iMoDC) or presence of proinflammatory cytokines TNF-α, IL-1, IL-6 or prostaglandin E2 (mMoDC), as described [18]. After 18 h of incubation for immature hMoDC (iMoDC) and 48 h of incubation for mature hMoDC (mMoDC), cells were collected, washed and analysed by flow cytometry for the surface expression of CD80, CD83, CD86, HLA-DR, HLA-A2 and macrophage mannose receptor (MMR) on the pregated fluorescent MS containing hMoDC (filled black lines). Open grey line plots represent staining with isotype matched control monoclonal antibodies (mAbs).

## 6. Outlook

The DC-based immunotherapy of cancer, although effective in mouse models, has, so far, not fulfilled the expectations in the clinic. Only in a small number of patients have clinical responses been observed, and the expansion and activation of CTL in patients do not seem to suffice for the removal of substantial tumor masses in most cases [20–25]. The prolongation of antigen presentation by hMoDC awarded by antigen delivery via PLGA-MS may improve the efficacy of immunotherapy and certainly deserves to be tested in clinical trials. A so far unsolved problem along this avenue is the sterilization of antigen containing PLGA-MS because γ-irradiation has been shown to alter the release properties of PLGA-MS, and it may partially destroy encapsulated antigens [26,27]. Whether the γ-irradiation of PLGA-MS prior to hMoDC uptake interferes with antigen presentation to T cells remains to be tested. The only alternative to γ-irradiation is the production of PLGA-MS under aseptic GMP conditions, which is quite costly.

On the long run, the labor-intensive and expensive in vitro generation of autologous DC may not become an option for routine application in clinical immunotherapy. The short life span of mature hMoDC in the lymph node requires that a continuous flow of antigen-bearing DC into the lymph nodes is warranted to elicit a vigorous cytotoxic T cell response. Ideally, the antigen would be deposited in the dermis in a depot form, such that it is picked up by newly generated DC over a

certain time period. The intradermal injection of antigen-bearing PLGA-MS may provide such a depot, but at least, the PLGA-MS that we have investigated did not induce DC maturation. The maturation of DC, however, is an important parameter for their use as cellular vaccines, as it has been demonstrated that immature hMoDC suppress, rather than stimulate, a CTL response [28,29]. Hence, it should be tested whether surrogate DC maturation stimuli like polyI:C or CpG oligonucleotides can be coencapsulated with the antigen in PLGA-MS, such that their release in the tissue will induce DC maturation in situ. Another strategy that may render PLGA-MS as adjuvants more effective is the inclusion of chemoattractants, like the complement fragment C5a, the bacterial formyl peptide fMLP or the chemokines CCL2 (MCP-1) and CCL3 (MIP1α). The in vivo release of encapsulated chemoattractants from PLGA-MS may lead to the recruitment of DC precursors or immature DC into the vicinity of PLGA-MS. It will remain to be tested in vivo if the encapsulation of DC maturation stimuli or chemoattractants in PLGA-MS will lead to an even better performance of PLGA-MS as an adjuvant and if T cell responses can be obtained by this means, which are equal or even better than achieved by DC-based vaccination.

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