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Research Paper

Phenotype and functional analysis of human monocyte-derived dendritic cells loaded with biodegradable poly(lactide-*co*-glycolide) microspheres for immunotherapy

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Abstract

Dendritic cells (DC) are increasingly explored as cellular vaccines for tumor immunotherapy. In most reported DC-based cancer vaccine trials, DC have been pulsed with soluble tumor antigen-derived peptide ligands of MHC molecules. Considering that the half-life of peptide/MHC complexes on the cell surface is relatively short and that soluble exogenous protein antigens cannot be efficiently processed via the MHC class I-processing pathway, the current vaccination procedure is not optimal for the induction of strong T cell responses aiming at tumor rejection. Recently, we have shown that antigen presentation can be prolonged when synthetic peptides were encapsulated in biodegradable poly(D,L-lactide-co-glycolide) (PLGA) microspheres (MS) for uptake by DC. In the present study, we investigated the phenotypic and functional consequences of MS uptake by human monocyte-derived dendritic cells (MoDC) in vitro. We found that immature MoDC that were prepared in serum free media suitable for clinical application were able to phagocytose high numbers of MS, while matured MoDC showed a reduced capacity for phagocytosis of MS. The ingestion of MS did not change the cell surface expression of CD80, CD83, CD86 and HLA-DR of immature and mature DC, suggesting that MS uptake did not induce DC maturation but that maturation by cytokines or LPS was unaltered in the presence of MS. Furthermore, MS-loaded mature MoDC expressed normal levels of the chemokine receptor CCR7 and migrated as efficiently towards CCL19 or CCL21 as unloaded MoDC. DC viability and the secretion of TNF-α and IL-12 was not significantly changed by MS loading. Taken together, our data indicate that PLGA-MS loading has no negative effects on the pivotal properties of MoDC in vitro. It

Abbreviations: CCD, cytochalasin D; CFSE, 5- and 6-carboxyfluorescein diacetate succinimidyl ester; CTL, cytotoxic T lymphocytes; DC, dendritic cells; DMSO, dimethyl sulphoxide; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; LPS, lipopolysaccharide; 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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should therefore be feasible to further develop this antigen loading strategy for clinical use in immunotherapy against viral infections and tumors.

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

1. Introduction

Dendritic cells (DC) are professional antigen presenting cells which have been referred to as ''nature's adjuvants'' because of their great potency in stimulating cytotoxic T cells (CTL) and T helper cells (Banchereau and Steinman, 1998). DC reside in peripheral tissues in an immature differentiation state in which they actively acquire soluble and particulate material through pinocytosis and receptor mediated phagocytosis. After the encounter of prototypic pathogen derived molecules such as lipopolysaccharides (LPS) or proinflammatory cytokines such as TNF-α, IL-1β, or IL-6, immature DC differentiate into mature DC, a process which reduces their endocytotic uptake and leads to the upregulation of T cell stimulatory molecules like CD80, CD83, or CD86 as well as MHC class I and II molecules (Sallusto et al., 1995). DC differentiation also causes a change in the expression of chemokine receptors (Sallusto et al., 1998; Dieu et al., 1998; Sozzani et al., 1998) which renders them unresponsive to inflammatory chemokines. Instead, they upregulate the chemokine receptor CCR7 and become responsive to the lymph node derived chemokines CCL19 and CCL21 (Sallusto et al., 1999). This change in chemokine receptor expression results in DC migration to the T cell areas of draining lymph nodes where naïve T cells can be efficiently activated. The sum of these properties most likely accounts for the key role of DC in initiating cellular immune responses.

The immunostimulatory potential of DC has led to their exploration as cellular vaccines against tumors and viral infections (Hsu et al., 1996; Nestle et al., 1998; Holtl et al., 1998; Thurner et al., 1999). Most of the recent clinical trials have employed autologous human monocyte derived DC (MoDC) which can be generated under clinical conditions in great numbers and with appropriate stimulatory properties (Romani et al., 1996). The loading of MoDC with antigen was achieved in most trials by incubation of MoDC with MHC class I restricted synthetic T cell epitopes from validated tumor antigens together with immunogenic but tumor-unrelated proteins for nonspecific T cell help. This approach is suboptimal because T cell help should preferably be specific for a tumor antigen (Ossendorp et al., 1998) and because the peptide epitopes have to be adjusted to each patient's MHC alleles. Moreover, the short half-life of class I/peptide complexes formed on the cell surface after exogenous loading has been shown to limit the immunogenicity in vivo (Ludewig et al., 2001).

An interesting alternative to exogenous peptide loading is the administration of antigen in the form of microparticulate material. Both mouse bone marrow derived DC and human MoDC are able to process endocytosed protein and peptide antigens for presentation on MHC class I and class II molecules when the antigen is bound to microparticles of a size of 1–10 μm (Shen et al., 1997; Ikuta et al., 2002). Cross presentation, which is a special property of DC, is about 1000 fold more effective if the protein antigen is supplied in a microparticulate form as compared to soluble antigen. This phenomenon seems to be related to particle size rather than the chemical composition of the microparticles since it has been demonstrated for polysaccharides (Ikuta et al., 2002), iron oxide beads (Kovacsovics-Bankowski et al., 1993), latex beads (Shen et al., 1997), and poly(D,L-lactide-co-glycolide) microspheres (PLGA-MS) (Men et al., 1999).

Over recent years we have investigated biodegradable PLGA-MS as an antigen delivery system for direct immunization of mice. PLGA polymers have been widely used for the production of biodegradable surgical sutures and for the sustained delivery of drugs into humans (Johansen et al., 2000). PLGAs are polyesters made of lactic and glycolic acids at molar ratios ranging between 50:50 and 100:0. Copolymer composition and molecular weight both determine the hydrophobicity and biodegradation of the PLGA-MS as well as the release kinetics of entrapped materials

from the microparticles. In this study we exclusively applied PLGA-MS prepared from a low molecular weight (14 kDa) PLGA 50:50 carrying a free carboxyl end group. We focussed on this relatively hydrophilic PLGA type, because we have demonstrated previously that the derived PLGA-MS were well taken up by macrophages and DC and because peptides, proteins, and DNA can be microencapsulated into PLGA 50:50 with generally satisfactory efficiency (Thomasin et al., 1996; Walter et al., 2001). Typically, material embedded in PLGA-MS is released slowly with bursts of release occuring after about 1 and 30 days, depending on the molecular mass and chemical composition of the polymer (Thomasin et al., 1996). When protein antigens were incorporated in PLGA-MS for the immunization of mice, the induction of strong IgG antibody responses was observed as well as the stimulation of CTL and T helper cells (Men et al., 1995; Men et al., 1997). Consistently, protein antigens can be presented both on MHC class I and class II molecules when the antigen is administered to macrophages or DC in the PLGA-MS encapsulated form in vitro (Men et al., 1999; Audran et al., 2003).

Recently, we have shown that human MoDC present an HLA-A0201 restricted peptide ligand over a markedly prolonged time period of up to 7 days when the MoDC were loaded with the peptide encapsulated in PLGA-MS (Audran et al., 2003). Since a prolongation of class I restricted presentation could considerably improve the outcome of DC-based tumor immunotherapy, we decided to assess the impact of PLGA-MS ingestion on several functional properties of MoDC, which have been generated according to a protocol suited for clinical application. We report here that PLGA-MS treated DC are fully functional with respect to migration, cytokine secretion, viability, and T cell allostimulation and should therefore have great potential as cellular vaccines for tumor immunotherapy.

2. Materials and methods

2.1. Materials and cell culture reagents

The poly(lactide-*co*-glycolide) used was a 14 kDa PLGA 50:50 with uncapped end-groups (Resomer RG502H) from Boehringer-Ingelheim (Ingelheim, Germany). Rhodamine B and Coumarin 6 were purchased from Fluka (Buchs, Switzerland). All other chemicals used in this study were of analytical grade (from Fluka, Buchs, Switzerland) unless otherwise specified. AIM V medium used for in vitro DC culture was purchased from Invitrogen (Groningen, The Netherlands). Ficoll-Paque® was obtained from Pharmacia (Uppsala, Sweden). Human recombinant cytokines IL-4, TNF-α, IL-1β, and IL-6 were purchased from Strathmann (Hamburg, Germany). Human GM-CSF was obtained from Novartis (Leukomax, Basel, Switzerland). LPS (from *Salmonella abortus equi*) was purchased from Sigma (Buchs, Switzerland). Prostaglandin E2 (PGE2, Prostin E2) was bought from Pharmacia & Upjohn (Dübendorf, Switzerland). Human chemokines CCL19 and CCL21 were obtained from R&D Systems (Wiesbaden-Nordenstadt, Germany).

2.2. Preparation of microspheres

PLGA-MS were prepared by the spray-drying method described elsewhere (Thomasin et al., 1997) with some modification. Briefly, to prepare Rhodamine B-containing MS (MS-RB), a Rhodamine B solution (5 μg/ml in water) was dispersed in a solution of 10% (w/w) PLGA in ethyl formate (w/w) by ultrasonication (Vibra-cell®, Sonics and Materials, Danbury, CT, USA). The water-in-oil dispersion formed was then spray-dried with a Mini Spray-Dryer 190 (Büchi, Flawil, Switzerland). Coumarin 6-containing MS (MS-C6) were prepared by directly adding Coumarin 6 to a 10% PLGA in ethyl formate solution (final concentration 5 μg/ml) for spray-drying. MS without any contents (empty MS, MS-E) were prepared by directly spray-drying 10% PLGA in ethyl formate solution. The MS obtained were washed and dried under vacuum for 24 h.

2.3. Generation of MoDC

MoDC were generated from human peripheral blood mononuclear cells (PBMC) as previously described (Scandella et al., 2002). In brief, PBMC were separated by standard density gradient centrifugation on Ficoll-Paque and resuspended at 6×10^6 cells/ml in AIM V medium. The cells were seeded to tissue culture flasks (TPP, Switzerland) for adhering

on the plastic surface for 2 h at 37°C. Non-adherent cells were then removed by three times washing with PBS. Tightly adhered monocytes were further cultured in DC-medium (AIM V medium containing 50 ng/ml of GM-CSF and 800 U/ml of IL-4). Alternatively, monocytes were isolated by anti-CD14 conjugated magnetic microbeads (Miltenyi, Bergisch Gladbach, Germany) and cultured at 1×10^6 cells/ml in DC-medium in cell culture flasks. On day 3, the same volume of fresh DC-medium was added to the cell cultures. On day 5 or day 6, non-or loosely adherent cells were harvested. These cells showed a typical immature DC phenotype as $CD14^-$, $CD16^-$, $CD19^-$, $CD3^-$, $CD80^+$, $CD83^{low}$, $CD86^+$ and MHC class I and II molecules positive (see Results) and were hence called immature monocyte-derived dendritic cells (MoDC). The purity of MoDC obtained under our experimental conditions was >90% by flow cytometric analysis (data not shown). For MoDC maturation, immature MoDC were stimulated with the indicated maturation stimuli for an additional 48 h. The DC maturation stimuli used in this study were either 5 μg/ml of LPS or a proinflammatory cytokine cocktail (20 ng/ml TNF-α, 10 ng/ml IL-1β and 1000 U/ml IL-6 dissolved in fresh DC-medium). Where indicated, PGE2 (final concentration 1 μg/ml) was added during the stimulation.

2.4. Determination of MoDC phagocytosis

Fluorescent MS (MS-C6 or MS-RB) were dispersed in DC culture medium and subsequently added to immature or matured MoDC at a concentration of 0.2 mg of particles per 1×10^6 cells per well in 24-well cell culture plates (Cellstar, Greiner Bio-One, Frickenhausen, Germany). In some cases, immature MoDC were co-incubated with MS and matured at the same time. Incubation was performed at 37°C for the indicated time periods. Cells were then collected and washed three times with PBS. The MS uptake of MoDC was examined with a Leica DMR fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Phase contrast and fluorescence pictures were taken through a digital camera and were analysed with Leica IM 1000 Image Manager software. In some experiments, cells were resuspended in PBS containing 2% FCS and the uptake of fluorescent MS was analysed on a FACScan® flow cytometer (Becton Dickinson). To inhibit phagocytosis, 20 μM cytochalasin D (Sigma) was added to the MoDC for 3 h at 37 °C prior to the addition of MS and remained in the medium during MoDC/MS co-incubation.

In order to remove MS adherent to the MoDC surface after MoDC/MS co-incubation (Fig. 2), MoDC were collected in 15 ml centrifuge tubes and spun down to remove the medium. One hundred microlitres of pure DMSO (Sigma D-2650, Switzerland) were added to cell pellets, mixed for 3 s to dissolve PLGA particles and immediately diluted with 15 ml PBS. Cells were then washed another 3 times, resuspended in DC medium for incubation overnight at 37 °C. After DMSO treatment and overnight incubation, about 80% of MoDC remain viable as determined by trypan blue staining (data not shown).

2.5. FACS analysis of MoDC phenotype

MoDC were analysed on a FACScan® flow cytometer (Becton Dickinson) after staining with the following mAbs according to the manufacturer's protocols: FITC-labeled anti-CD83 (Immunotech, Berlin, Germany), FITC-labeled anti-CD86, PE-labeled anti-CD80, FITC-labeled anti-HLA-DR, and FITC-labeled anti-CD95 (all from Pharmingen, Basel, Switzerland). The respective isotype controls were FITC-labeled mouse IgG1, IgG2a, IgG2b and PE-labeled IgG1 (Pharmingen). Rat anti human-CCR7 mAb (Förster et al., 1993) was kindly provided by Dr. Reinhold Förster (University Erlangen-Nürnberg, Germany). FITC-conjugated goat-anti-rat IgG (Jackson Immunoresearch, La Roche, Switzerland) was used as the secondary antibody for anti-CCR7 staining.

2.6. MoDC and BM-DC migration assays

In vitro migration of MoDC was measured by a chemotaxis assay as previously described (Scandella et al., 2002). Briefly, 600 μl of DC-medium containing either 250 ng/ml CCL19, 250 ng/ml CCL21 or DC-medium alone were added to the bottom chamber of 24-well transwell plates with polycarbonate filters of 5 μm pore size (Corning Costar, Cambridge, MA). A number of 10^5 MoDC obtained from designated

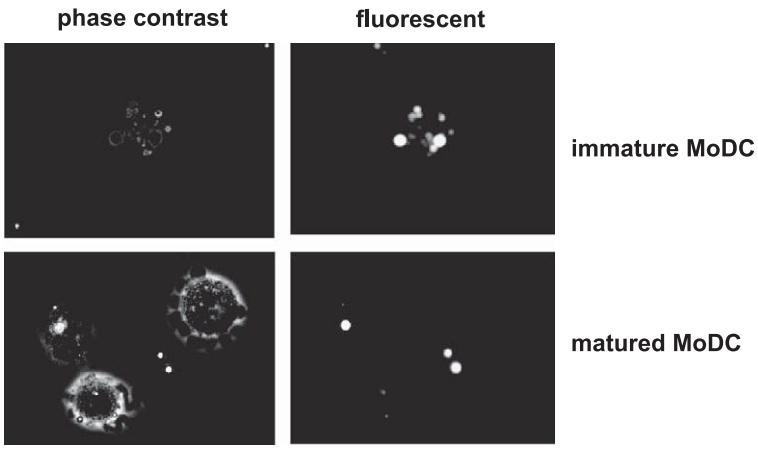

Fig. 1. Phagocytosis of fluorescent microspheres (MS) by MoDC. Immature (upper panels) or LPS matured (bottom panels) MoDC were incubated with Coumarin 6 containing MS (0.2 mg per MS 1×10^6 cells) for 18 h at 37 °C. Cells were then collected and examined by both phase contrast (left panels) and fluorescence (right panels) microscopy of the same field. Magnification: 40×.

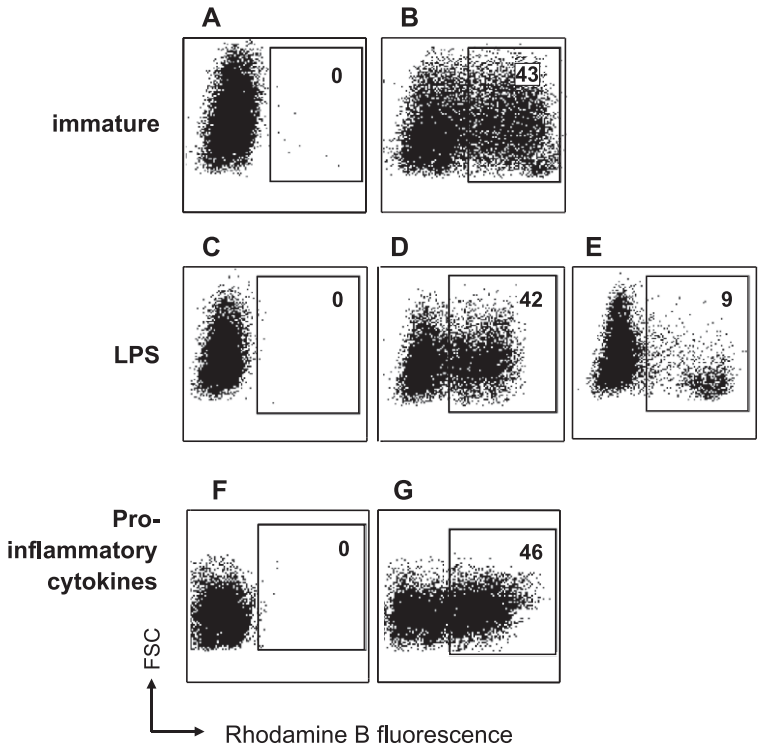

Fig. 2. Quantification of MS uptake by MoDC with fluorescent MS. Immature MoDC alone (panel A), immature MoDC loaded with fluorescent MS-RB (0.2 mg MS for 1×10^6 cells, panel B), LPS matured MoDC alone (panel C), MoDC matured with LPS in the presence of MS-RB (panel D), proinflammatory cytokines (TNF-α, IL-1β, IL-6) matured MoDC alone (panel F), or MoDC matured with proinflammatory cytokines in the presence of MS-RB (panel G) were incubated for 18 h at 37°C. In panel E, MoDC were first matured with LPS for 48 h and then loaded with MS-RB for 18 h at 37 °C. After washing, cells were analyzed by flow cytometry. The numbers within the dot plots represent percentages of the positive cells from the whole forward/side scatter gated MoDC population. One representative experiment out of three is shown.

experimental conditions were added to the upper chamber in a total volume of 100 µl of DC-medium. The plates were then incubated for 3 h at 37 °C. After careful removal of the upper chamber, a 500-µl aliquot of cells (migrated cells) in the bottom chamber was collected to count for 60 s by flow cytometry using CellQuest software (Becton Dickinson). Each experiment was performed in duplicate. The percentage of migrated cells was calculated as follows: % Migrated DC=(number of migrated DC/total DC number)× 100%. Values are given as the mean percentage of migrated cells±SEM.

For in vivo migration of murine bone-marrow derived dendritic cells (BM-DC), femurs and tibiae were removed from 6- to 7-week-old C57BL/6 mice. The two ends of the bones were cut and bone marrow

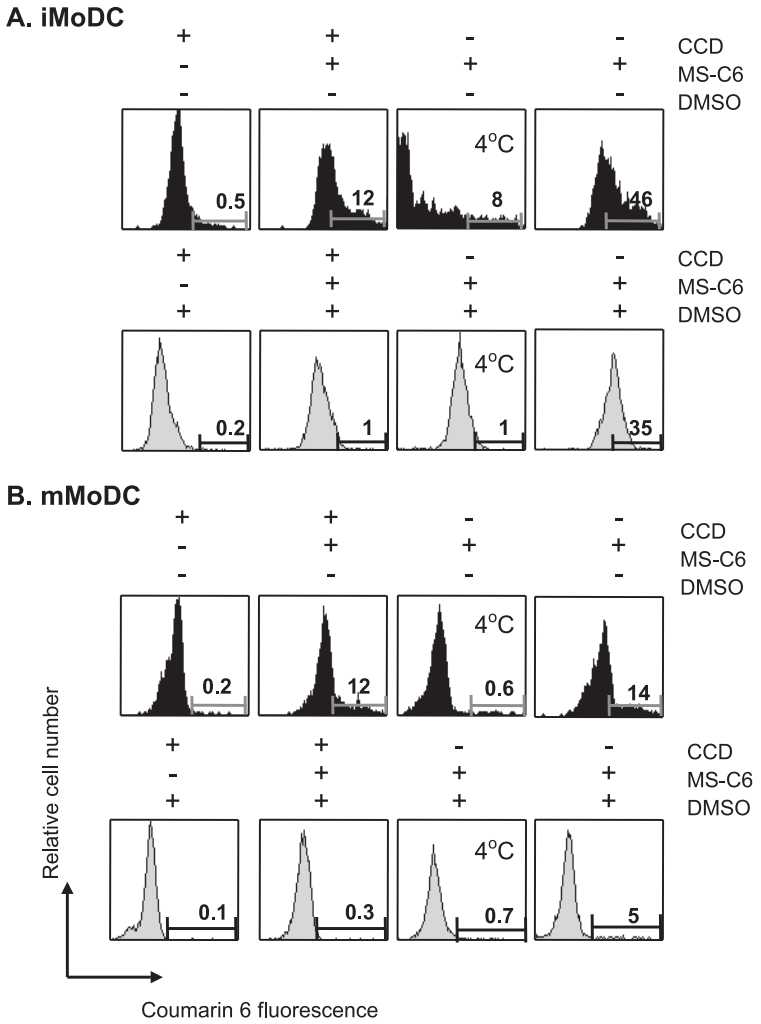

Fig. 3. DMSO treatment to remove PLGA-MS from the surface of MoDC. Immature MoDC (panel A) or MoDC matured with LPS for 48 h (panel B) were pre-treated with 20 µM CCD for 3 h at 37 °C or left untreated. MoDC were then incubated with fluorescent MS-C6 for 18 h in the presence or absence of CCD at 37 °C, or at 4 °C as indicated. After washing, MoDC cultures obtained from each experimental condition were analysed for MS-C6 uptake with flow cytometry (black histogram plots). Where indicated, DMSO treatment was applied to remove externally attached MS (for details see Materials and methods). DMSO treated MoDC were then incubated overnight at 37 °C prior to flow cytometric analysis for MS-C6 uptake (grey histogram plots). The numbers within the histogram plots represent percentages of MS-C6 fluorescent MoDC based on the whole gated MoDC population. One representative experiment out of three is shown.

was flushed out and washed with PBS. NH_4Cl (1.66%) was added to lyse erythrocytes. The bone marrow cells then were washed, counted and resuspended at 1×10^6 cells/ml in RPMI medium supplemented with 5% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol and 1000 U/ml murine GM-CSF (kindly provided by Novartis, Vienna) and cultured in 6-well cell culture plates (Cellstar, Greiner Bio-One, Frickenhausen, Germany). On day 5, fluorescent MS-C6 were added to BM-DC at a concentration of 0.3 mg of particles per 1×10^6 cells. Four hours later, 1 μg/ml of LPS was then added to the BM-DC culture. After overnight co-incubation, BM-DC were collected and purified with anti-murine CD11c conjugated magnetic microbeads (Miltenyi). The purity of CD11c positive BM-DC was >95% by flow cytometric analysis (data not shown). 5×10^5 MS-C6 loaded BM-DC were then injected subcutaneously into the hind footpads of C57BL/6 mice. As a positive control, the same number of BM-DC were labeled with CFSE (5- and 6-carboxyfluorescein diacetate succinimidyl ester) (Molecular Probes, OR, USA) and purified with CD11c specific MACS beads, and injected. The draining lymph nodes (LN) from the injected mice were removed 22 h later to prepare single LN cell suspensions using 400 U/ml Collagenese D (Roche, Indianapolis, USA). Cells were then stained with PE-labeled anti-mouse CD11c (Pharmingen) and resuspended in 500 μl FACS buffer for flow cytometric analysis. Green fluorescence of MS-C6 or CFSE labeled cells was determined in the gated $CD11c^+$ population.

2.7. Cell viability and apoptosis assay

Cell viability was determined by Annexin V staining which detects apoptotic cells according to the manufacturer's protocol (Pharmingen). In brief, cells were labeled with Annexin V-FITC and propidium iodide (PI) to identify apoptotic cells by flow cytom-

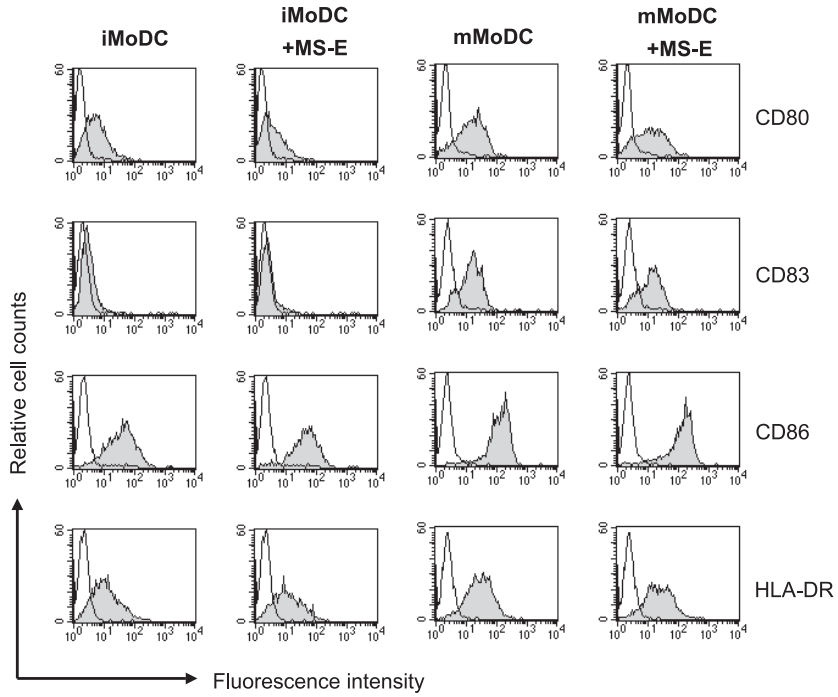

Fig. 4. Comparison of phenotype and maturation of MoDC with or without MS uptake. Immature MoDC (iMoDC), immature MoDC loaded with empty MS (0.2 mg MS for 1×10^6 cells for 48 h, iMoDC+MS-E), MoDC matured with LPS for 48 h (mMoDC), or MoDC loaded with empty MS and matured with LPS for 48 h (mMoDC+MS-E) were analysed by flow cytometry for the surface expression of CD80, CD83, CD86, and HLA-DR molecules (filled gray lines). Open black histograms represent staining with isotype matched control mAbs. Data represent one out of four independent experiments which all gave similar results.

etry analysis. Live cells would remain Annexin V and PI negative. Where indicated, 1 μg/ml of human anti Fas mAb clone 7C11 (CD95, Immunotech, Marseille, France) was added for an 18 h pre-incubation to trigger apoptosis.

2.8. Mixed lymphocyte response

Naïve $CD4^+CD45RA^+$T cells were purified by negative selection using the $CD4^+/CD45RO^-$Multi Sort Kit (Miltenyi). This method yielded purified (>95%) $CD4^+/CD45RA^+/CD45RO^-$T cells as assessed by flow cytometry (data not shown). For mixed lymphocyte response experiments immature or matured allogeneic MoDC were extensively washed, irradiated (30 Gray) and cultured at different cell numbers in duplicates with 1×10^5 $CD4^+CD45RA^+$T cells in 96-well flat bottom plates. On day 4, ^{3}H-thymidine (1 μCi/well) was added and incorporation was determined after 16–18 h.

2.9. Cytokine ELISA

The supernatants of immature MoDC or MoDC stimulated under the indicated experimental conditions in the presence or absence of MS were col-

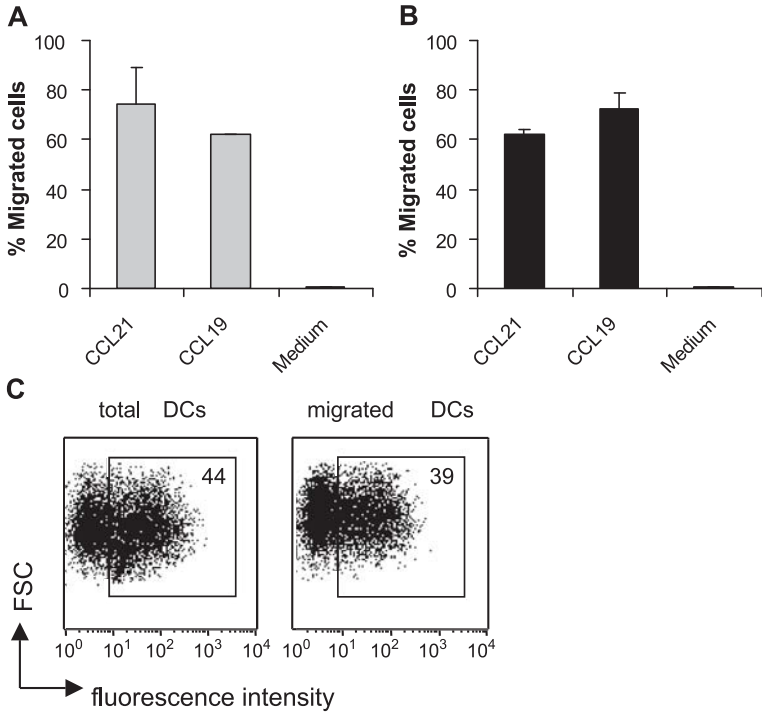

Fig. 5. Strong migration capacity of MS-bearing MoDC towards CCL19 and CCL21. MoDC matured with TNF-α, IL-1β, IL-6, and PGE2 were tested for CCL19-and CCL21-triggered migration with chemotaxis assays either alone (panel A) or in the presence of MS-RB (0.2 mg MS-RB per 1×10^6 cells, panel B). Medium without chemokines was used for spontaneous cell migration. The percentage of migrated cells was calculated as the mean of duplicate measurements±SEM (for details, see Materials and methods). The migrated cells or the total cells from MS-RB treated matured MoDC (panel B) were collected and evaluated for the MS-RB uptake by flow cytometry (panel C). MoDC were gated in forward/side scatter and analysed for Rhodamine B fluorescence intensity. Numbers within the dot plots represent percentages of the positive cells related to the whole forward/side scatter gated MoDC population. One representative experiment out of four is shown. (D) In vivo migration capacity of BM-DC loaded with MS-C6. BM-DC prepared from C57BL/6 mice were loaded with MS-C6 (top panels), labeled with CFSE (middle panels) or left unlabeled (bottom panels) and were purified with CD11c-MACS beads (injected BM-DC, left panels). C57BL/6 mice (two per groups) were then injected subcutaneously with 5×10^5 of BM-DC. After 22 h, draining lymph nodes from mice were removed and LN cells were stained with CD11c-PE mAb for flow cytometric analysis. The gated $CD11c^+$ BM-DC were measured for green fluorescence of MS-C6 and CFSE in the FL1-H channel (recovered BM-DC, right panels). The experiment was repeated twice and gave similar results.

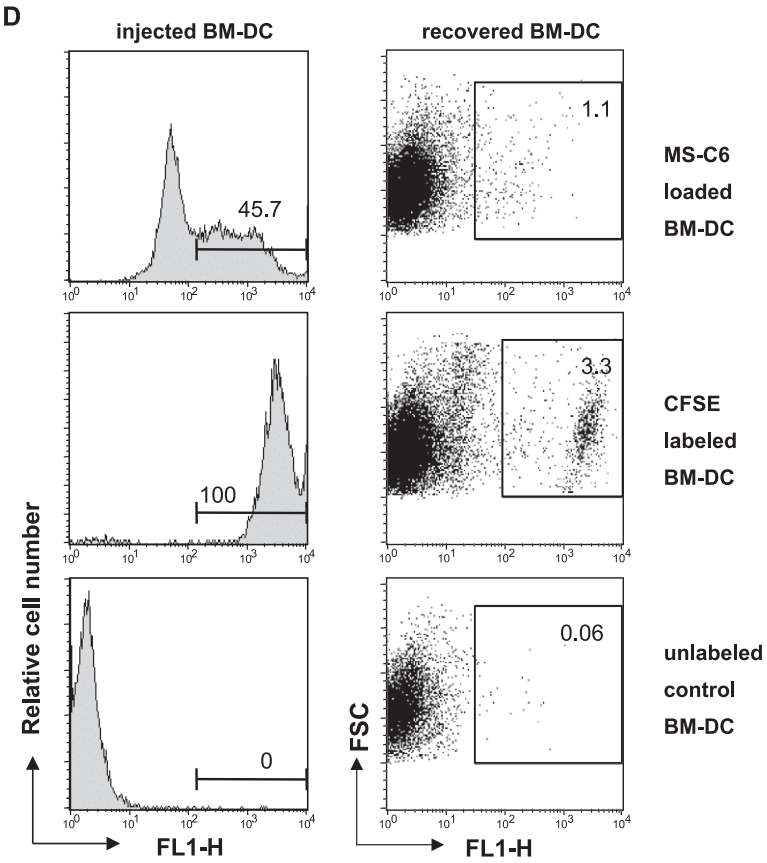

Fig. 5 (*continued*).

lected for evaluating the cytokine production pattern using commercially available cytokine ELISA kits such as TNF-α, IL-10 (Pharmingen) and IL-12p70 (Endogen, Woburn, MA), according to the manufacturer's protocols.

3. Results

3.1. Phagocytosis of PLGA-MS by MoDC

Initially, we determined whether MoDC were capable of efficiently taking up PLGA-MS when they were prepared in serum free medium under conditions suitable for application in clinical trials. To facilitate the quantification of MS uptake by flow cytometry and fluorescence microscopy, fluorescent MS containing either Coumarin 6 (MS-C6) or Rhodamine B (MS-RB) were prepared (see Materials and methods). The fluorescent microspheres obtained had an approximate mean diameter of 3–4 μm (data not shown), which should be appropriate for DC uptake. Phase contrast and fluorescence microscopy clearly demonstrated that immature MoDC efficiently internalized large numbers of MS (Fig. 1, upper panels) in accordance with earlier studies (Walter et al., 2001; Pietzonka et al., 2002). By contrast, MoDC that had been matured with LPS ingested only marginally MS particles (Fig. 1, lower panels).

The results obtained by fluorescence microscopy were further confirmed by flow cytometric analysis. As shown in Fig. 2B, a co-incubation of immature MoDC and Rhodamine B microspheres (MS-RB) for 4 h resulted in more than 40% of fluorescing cells suggesting the uptake of MS-RB. Interestingly, when MoDC were first matured and subsequently incubated

for the same time with the same amount of MS-RB, the fluorescent population was reduced to less than 10% (Fig. 2E). This result is in accordance with the previously documented low phagocytic activity of mature DC (Sallusto et al., 1995, see also Fig. 3 below). When immature MoDC were incubated with MS-RB for 48 h and simultaneously matured with LPS or the proinflammatory cytokines TNF-α, IL-1β, and IL-6, the same number of MS-RB positive cells was detected as in immature MoDC (Fig. 2D and G, respectively). Even prolonged exposure of immature MoDC to MS-RB did not lead to a proportion of fluorescent cells higher than about 40% although the uptake of FITC-Dextran was homogeneous (data not shown). At present we cannot explain why only about 40% of MoDC ingested PLGA-MS while the residual cells remained unloaded.

In order to ascertain that the fluorescence of cells was not merely due to an external adhesion of MS to MoDC, we developed a procedure to remove externally bound PLGA-MS from MoDC by a 3-s incubation of cells in pure DMSO followed by immediate dilution in growth medium. This procedure dissolved externally adhering PLGA-MS but did not affect the viability or integrity of the cells. As shown in Fig. 3A, without DMSO treatment, 8–12% of MS-C6 positive cells were detected in CCD pre-treated iMODC, or when iMoDC were incubated with MS-C6 at 4 °C. However, this population of fluorescent cells was reduced to <1% after DMSO treatment, indicating that MS were only tightly attached to the MoDC surface rather than internalized. Immature MoDC that had taken up MSC6 remained fluorescent after the DMSO treatment thus indicating that MSC6 had been ingested and were protected inside the cells. The inhibition of MS uptake by CCD suggests that a reorganization of the actin cytoskeleton was required for their internalisation. In the case of matured MoDC (Fig. 3B), only 5% mMoDC were MS-C6 positive after DMSO treatment, further confirming that MS uptake capacity was significantly decreased in matured MoDC.

3.2. The effect of MS uptake on the phenotype and maturation of MoDC

Next, we investigated whether the MS uptake by MoDC would influence their phenotype and ability to mature in vitro. For this purpose, we compared the expression of the DC surface markers CD80, CD83, CD86, and HLA-DR on immature and matured MoDC loaded with or without MS. As shown in Fig. 4, immature MoDC bearing empty MS exhibited a similar DC phenotype to those without MS loading, which was CD80 low, CD83 negative, and CD86 and HLA-DR positive. MS-bearing MoDC could be readily matured by different stimuli, such as LPS and the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. The addition of LPS or other maturation stimuli to MS-bearing MoDC induced significant upregulation of CD80, CD83, CD86, and HLA-DR which was comparable to that observed without MS loading (Fig. 4). These results indicate that MS uptake did not influence MoDC phenotype and their ability to mature. Moreover, under the present experimental conditions, the uptake of PLGA-MS did not by itself induce phenotypic maturation of MoDC.

3.3. Migration capacity of MS-bearing MoDC in vitro and BM-DC in vivo

For DC-based immunotherapy, intradermally injected DCs should be able to migrate from the site of injection to the secondary lymphoid organs where they can present the acquired antigens to T cells. We therefore investigated whether the uptake of MS by MoDC would affect their migratory capacity. Since it is known that CCR7 expression is essential for DC migration (Förster et al., 1999), we first determined CCR7 expression on MoDC prepared under our experimental conditions. As expected, CCR7 expression was negligible on immature MoDC before or after MS uptake and both DC populations were unable to migrate towards the CCR7 ligands CCL19 and CCL21 in transwell chemotaxis assays in vitro (data not shown). The addition of maturation stimuli resulted in a significant upregulation of CCR7 expression which was the same for MS-bearing or control MoDC (data not shown). The matured MoDC migrated efficiently towards CCL19 and CCL21 in the transwell chemotaxis assay irrespective of whether they had engulfed MS-RB (Fig. 5B) or not (Fig. 5A). Since not all MoDC were MS-carrying cells (see Figs. 2 and 3), one could argue that only MoDC devoid of MS were able to migrate through the transwell filter. To exclude this possibility, MoDC

that were collected from the bottom chamber of transwells were examined for their MS-RB loading status by flow cytometry. As shown in Fig. 5C, 39% of the migrated MoDC contained MS-RB which was similar to the loading status of the input population (44%) thus ruling out the possibility that MS uptake negatively affected their migratory capacity.

In order to confirm the migration capacity of DC loaded with PLGA-MS in vivo, murine BM-DC were prepared from C57BL/6 mice and loaded with MS-C6 or labeled with CFSE. The $CD11c^{+}$ BM-DC were then injected subcutaneously into the footpad of mice followed by recovery from draining lymph nodes 22 h later. As shown in Fig. 5D, both MS-C6 loaded and

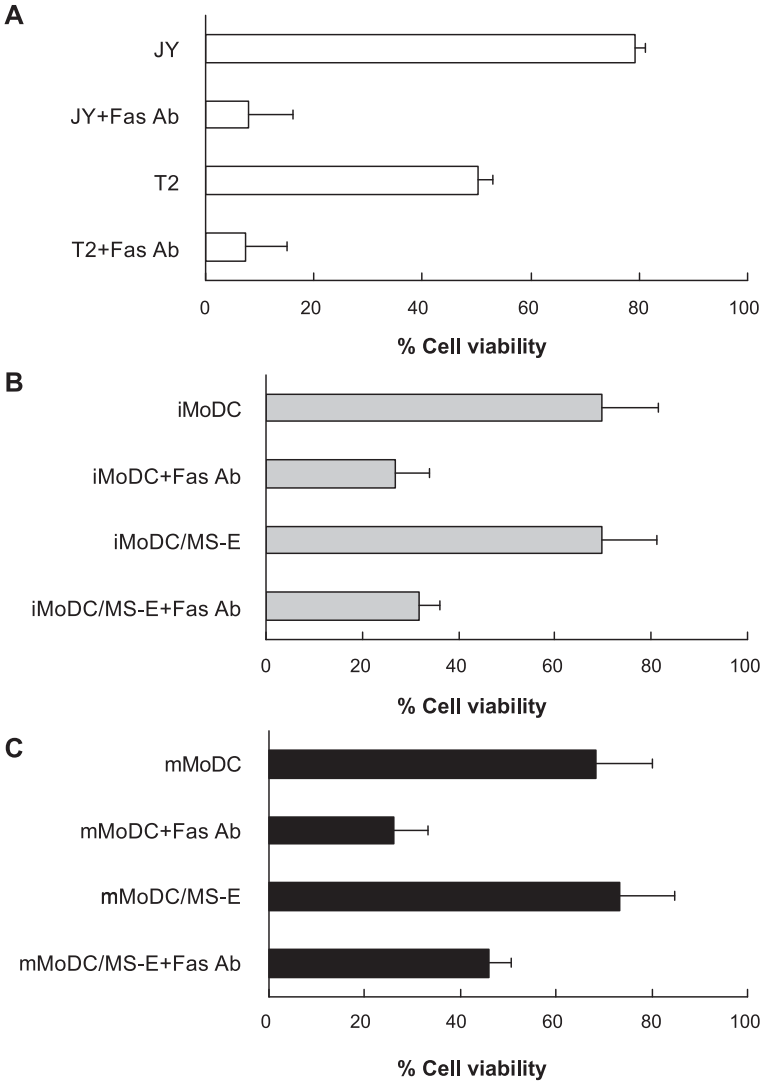

Fig. 6. MoDC viability and survival after Fas mAb treatment. Fas-sensitive cell lines JY and T2 (panel A), immature MoDC alone or loaded with MS-E (0.2 mg MS per 1×10^{6} cells) for 18 h at 37 °C (panel B) or MoDC stimulated with 5 μg/ml LPS alone or co-incubated with MS-E (0.2 mg MS per 1×10^{6} cells) during maturation (panel C) were treated with anti-Fas mAb 7C11. Cells that did not receive Fas mAb treatment were included for determining spontaneous apoptosis. After 18 h incubation, Fas-triggered apoptosis was evaluated by flow cytometric analysis. Cells were first gated in forward/side scatter and then analysed for Annexin V-FITC/PI double staining. The data collected were obtained from five independent experiments and are expressed as mean percentage of cell viability±SEM.

CFSE labeled BM-DC were detected in draining lymph nodes, indicating the in vivo migration capacity of these BM-DC. A higher portion of fluorescent cells was recovered from mice injected with CFSE labeled (3.3%) as compared to MS-C6 loaded BM-DC (1.1%). However, given that only 45.7% of BM-DC had ingested MS-C6 whereas 100% of cells were stained with CFSE, the migratory capacity of MS-C6 loaded BM-DC was only slightly lower (by 28%) than that of CFSE labeled BM-DC. We conclude that the loading of DC with PLGA-MS has only a very minor negative effect on their migratory capacity in vivo.

3.4. Viability and survival of MoDC treated with Fas mAb

Another important issue in DC-vaccination is the DC survival period. The injected DC should not only be able to migrate, but also need to remain viable long enough to fully activate T cells after their arrival in the draining lymph nodes. Therefore, another pivotal point in this study was to examine whether particle loading would shorten MoDC half-life and/or trigger DC apoptosis. Fig. 6 summarizes the results on MoDC viability and survival obtained with Annexin V staining. Two Fas-sensitive human cell lines JY and T2 were included in this study as positive controls for which the respective viabilities of 80% and 60% declined to less than 10% after 18 h of treatment with an activating anti-Fas mAb (Fig. 6A). In the case of immature MoDC (Fig. 6B), MS loading did not influence the level of viability (about 65%). The cellular survival of immature MoDC after Fas mAb treatment was about 30% and their viability was not affected by MS ingestion. Also for cytokine matured MoDC, MS loading did not influence cellular viability. Interestingly, the survival of mature MoDC after treatment with the Fas specific mAb was consistently improved by about 50% through MS uptake when they were matured with LPS (Fig. 6C) but not when they were matured with the cocktail of TNF-α, IL1β, IL-6, and PGE2. The improved survival observed in LPS matured MoDC was not due to Fas down-regulation, because similar levels of Fas expression were detected on all tested cells (data not shown). We monitored the viability of immature and mature MoDC which had ingested PLGA-MS over a time period of four days but no significant difference in survival was observed when compared with untreated MoDC (data not shown).

3.5. In vitro stimulation of T lymphocytes by MoDC

Next, we investigated the capacity of MoDC to stimulate T cells in a mixed T lymphocyte reaction in vitro. Titrated numbers of immature and mature MoDC, which were either left untreated or loaded with empty PLGA-MS, were incubated with allogeneic naïve $CD4^+CD45RA^+$T helper cells for 4 days. Subsequently, T cell proliferation was measured by ^{3}H-thymidine incorporation. As shown in Fig. 7, immature MoDC with or without MS uptake were poor T cell stimulators. After maturation with LPS, MoDC with or without MS induced strong T cell proliferation under our experimental conditions. These results show that MS uptake does not alter the capacity of DC to stimulate allogeneic T helper cells and that a

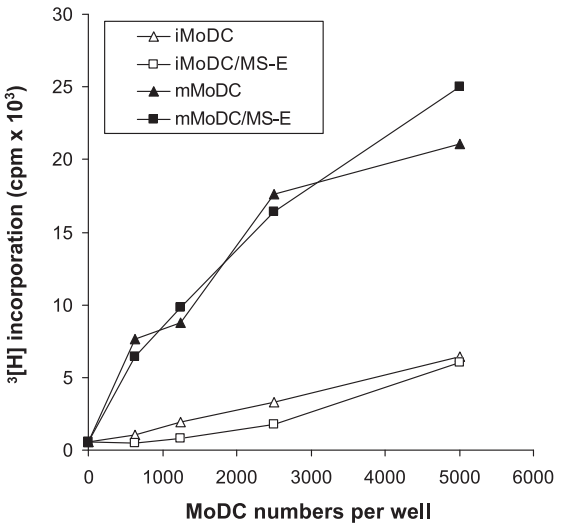

Fig. 7. T cell stimulatory capacity of MoDC determined with mixed T lymphocyte reaction. Immature MoDC alone, immature MoDC loaded with empty MS (MS-E, 0.2 mg MS for 1×10^6 cells for 18 h), LPS matured MoDC alone, or MoDC matured with LPS in the presence of MS-E were incubated with purified allogeneic naïve $CD4^+CD45RA^+$ T cells (1×10^5 T cells/well). After 4 days of co-culture, T cell proliferation was measured by ^{3}H-thymidine incorporation. The data are shown as the means of duplicate cultures±SEM and represent one out of three independent experiments.

potential external adherence of PLGA-MS to MoDC does not interfere with T cell stimulation in vitro.

3.6. Cytokine secretion patterns of MoDC loaded with MS

For the function of DC and their potential to govern T helper cell differentiation the secretion of IL-10 and the functional p35/p40 heterodimer of IL-12 is pivotal. Hence, we examined the secretion of these two interleukins as well as TNF-α into the growth medium by ELISA. For all cytokines the secretion was strongly upregulated when cells were matured with LPS (Fig. 8). The uptake of MS did not significantly change the secretion profile of immature MoDC and also the secretion of TNF-α by mature DC was not changed by the incorporation of MS. The secretion of IL-10 by mature MoDC was significantly lower when the DC had been charged with MS, and the production of IL-12 was slightly

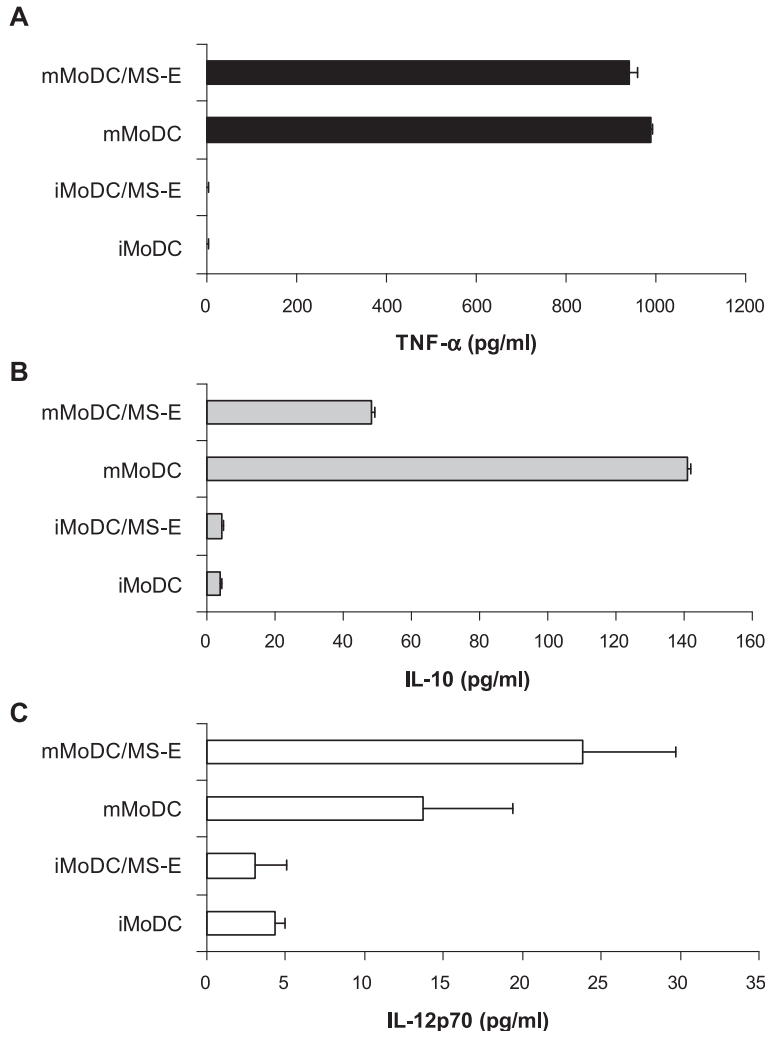

Fig. 8. Cytokine secretion by PLGA-MS treated and untreated MoDC. Supernatants of 1×10^6 of immature MoDC alone (iMoDC), immature MoDC loaded with 0.2 mg MS-E (iMoDC/MS-E), MoDC matured with LPS for 18 h (mMoDC), or MoDC loaded with MS-E and matured with LPS for 18 h (mMoDC/MS-E) were collected and analysed for the levels of cytokines TNF-α (panel A), IL-10 (panel B) or IL-12 (panel C) by ELISA. The data are shown as the means of duplicate measurements±SEM.

upregulated. Such a shift in cytokine production could promote the differentiation of Th1 cells in vivo and hence would be of interest. However, these differences in cytokine production were much less prominent when PLGA-MS loaded MoDC were matured with TNF-α, IL-1β, and IL-6 (data not shown) and, therefore, we did not further investigate this phenomenon.

4. Discussion

The main aim of this study was to determine how the uptake of PLGA-MS affects key functional properties of MoDC that have been prepared under serum free conditions appropriate for clinical application. These properties encompassed: (1) the ability of immature MoDC to mature; (2) the recovery and survival rate of immature and mature DC; (3) migration after stimulation with the chemokines CCL19 and CCL21; (4) the ability to stimulate naïve T cells and (5) the capacity to secrete proinflammatory cytokines. Taken together, we found that none of these functional features of MoDC in vitro were adversely affected by the uptake of PLGA-MS. These findings therefore pave the way for the further investigation and development of PLGA-MS as antigen delivery devices for MoDC. Given that we recently demonstrated a prolongation of MHC class I restricted antigen presentation of peptides by MoDC (Audran et al., 2003), this approach may lead to a considerable improvement in DC-based tumor immunotherapy.

A critical issue for the success of PLGA-MS mediated antigen loading is the number of particles that are engulfed by MoDC. Since PLGA-MS tend to adhere to the surface of MoDC it was important to discriminate between endocytosed and cell surface attached PLGA-MS. We first addressed this issue by comparing the number of MS within immature and mature MoDC by fluorescence microscopy. We consistently observed that about 40–50% of immature MoDC had taken up between 5 and 30 MS whereas hardly any MS were found within mature MoDC that had been incubated with the same concentration of MS for 18 h (Fig. 1). Secondly, the uptake of fluorescent PLGA-MS was monitored by flow cytometry. This technique per se cannot discriminate between external adherence and intracellular localization of PLGA-MS, but the three- to four-fold enhanced fluorescence of immature DC as compared to mature DC argues that external adherence contributed only marginally to the fluorescent population, because it is well known that immature MoDC lose their ability to phagocytose within two days after maturation is initiated in vitro (Sallusto et al., 1995). In order to further corroborate the intracellular localization of PLGA-MS we removed externally adhering PLGA-MS from immature MoDC by a very short DMSO treatment. Thirty-five percent of immature MoDC treated in this way remained fluorescent, indicating that the fluorescent MS were protected inside the cells (Fig. 3A). Using this approach, we found that uptake of PLGA-MS was maximal after 4 h (data not shown), which is in agreement with a recent report (Walter et al., 2001).

The maturation of MoDC is a prerequisite for their use as immunostimulatory cellular vaccines, because immature MoDC inhibit whereas mature MoDC activate CTL (Dhodapkar et al., 2001; Jonuleit et al., 2001). Furthermore, maturation of MoDC is required for their migration to draining lymph nodes. As judged by cell surface staining for the maturation markers CD80, CD83, CD86, and HLA-DR, we observed the same upregulation of these markers for untreated and PLGA-MS treated MoDC (Fig. 4). Moreover, functional parameters of MoDC maturation such as migration towards CCL19 and CCL21 (Fig. 5), the marked upregulation of their potential to stimulate naïve $CD4^+$ T helper cells (Fig. 7), and a striking enhancement in the secretion of TNF-α and IL-12 (Fig. 8) were all fully preserved when immature DC were matured either with LPS or a proinflammatory cytokine cocktail of TNF-α, IL-1β, IL-6, and PGE2, as used by ourselves and others for MoDC maturation in clinical trials (Jonuleit et al., 1997; Scandella et al., 2002). A significant difference between maturation with this cytokine cocktail and LPS was that PLGA-MS uptake consistently conferred a partial resistance of LPS-matured MoDC to Fas-triggered apoptosis (Fig. 5) which was not evident when MoDC were matured with the cytokine mix (data not shown). Another significant effect of PLGA-MS uptake was a reduction in the secretion of IL-10 by LPS matured MoDC (Fig. 8), but not by MoDC matured with the cytokine cocktail. A particular concern for us was whether the

fairly bulky MS would impede MoDC migration because some of the MS are of a similar size as the pores of the trans well filters that are 5 μm in diameter. However, no negative effect on migration was evident since the portion of PLGA-MS containing cells was the same before and after migration in vitro. Though a slightly lower in vivo migratory capacity of BM-DC loaded with PLGA MS has been observed in mice (Fig. 5D), equally strong CTL responses were induced by immunization of mice with BM-DC charged with a H-K^b CTL epitope in soluble form or encapsulated in PLGA MS (Ying Waeckerle-Men, unpublished data), suggesting this minor "negative" effect on migration of MS loaded BM-DC does not impact on their antigen presentation capacity in vivo.

A very important question we are currently addressing is whether the prolonged presentation of MHC class I epitopes achieved by protein antigen loading via PLGA-MS in vitro translates into strong and more sustained CTL responses in vivo. We have started to address this issue in the mouse model and indeed obtained about 5% specific CTL in the blood of mice on day 6 after immunization when a protein antigen was loaded onto DC in a PLGA-MS encapsulated form, whereas soluble protein loading yielded no response (Ying Waeckerle-Men, unpublished data). Similarly encouraging data in the mouse model were recently reported by Ikuta et al., who used hydrophobized polysaccharide microspheres for antigen loading of mouse bone marrow derived DC. These were clearly superior in the induction of CTL and for tumor immunotherapy as compared to DC that had been charged with soluble peptide (Ikuta et al., 2002). The present demonstration that clinically approved PLGA-MS have no adverse effect on the key functions of MoDC in vitro is an important prerequisite for testing this method of antigen loading in DC-based immunotherapy.

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