Analysis of Poly(D,L-Lactic-Co-Glycolic Acid) Nanosphere Uptake by Human Dendritic Cells and Macrophages *In Vitro*

M. E. Christine Lutsiak,[1] Deborah R. Robinson,[1] Conrad Coester,[1] Glen S. Kwon,[1,2] and John Samuel[1,3]

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Purpose. The purpose of this study was to demonstrate and characterize phagocytosis of poly(D,L-lactic-co-glycolic acid) (PLGA) nanospheres by human dendritic cells (DCs).

Methods. Parallel cultures of DCs and macrophages (Mϕ) were established from peripheral blood leukocytes using media supplemented with granulocyte-macrophage colony stimulator factor and interleukin-4 (for DC) or granulocyte-macrophage colony stimulator factor alone (for Mϕ). PLGA nanospheres containing tetramethylrhodamine-labeled dextran with or without an adjuvant, monophosphoryl lipid A, were prepared using a water/oil/water solvent evaporation technique. Cells were incubated with the nanospheres for 24 h. Confocal laser scanning microscopy was used to determine the intracellular location of the nanospheres and flow cytometry to measure the fraction of phagocytic cells in the culture and the amount of uptake per cell. After phagocytosis, cells were stained for MHC class II molecules, CD14, CD80, and CD86 to identify the phagocytic population.

Results. DCs phagocytosed PLGA nanospheres as efficiently as Mϕ. Cell-surface marker expression conclusively established that the phagocytic cells were DC.

Conclusions. DCs can take up PLGA nanospheres. Because DCs are the key professional antigen-presenting cells capable of stimulating naive T cells, our data suggest that PLGA nanospheres can be used as an efficient delivery system for vaccines designed to activate T cell–mediated immune responses.

KEY WORDS: PLGA nanospheres; dendritic cells; antigen-presenting cells; phagocytosis.

INTRODUCTION

Dendritic cells (DCs) are the key antigen-presenting cells (APCs) responsible for the stimulation of naive T lymphocytes (1). This ability to invoke a primary response from T cells is a result of, in part, their constitutive expression of major histocompatibility complex class II molecules (MHC II) and the costimulatory molecule CD86. DCs are also aided in the stimulation of naive T cells by their ability to rapidly upregulate expression of these cell surface markers along with the novel expression of another costimulatory molecule, CD80 (2–5). In addition, the production of interleukin-12 (IL-12) by mature DCs is a major factor in the induction of type 1 T helper (Th1) responses (6,7). Antigen acquisition by DCs is important in determining the outcome in viral infections and cancer, where Th1 responses are thought to be protective. Therefore, therapeutic vaccines for cancer and viral infections should be designed to deliver antigens to DCs. DCs loaded with cancer antigens *ex vivo* are now under investigation as therapeutic cancer vaccines and show encouraging results in cancer patients (8,9). More efficient delivery of antigens to DCs *ex vivo* and delivery of antigens to DCs *in vivo* are both important goals for research into therapeutic vaccines for cancer and infectious disease.

When DCs were first characterized as APCs, they were thought to be nonphagocytic (10). More recently, they have been shown to phagocytose a variety of materials, including microorganisms and latex beads (11,12). However, DCs were reported to be much less efficient at phagocytosing microorganisms than macrophages (Mϕ; 11). The ability of DCs to phagocytose and process antigen is highly dependent upon the stage of DC differentiation (11,13). Immature cells residing in the tissue are highly phagocytic and are less efficient at stimulating T lymphocytes. After maturation, DCs cease phagocytosis and evolve into efficient APCs that express high levels of costimulatory molecules (11,13). Despite these studies, phagocytosis of clinically relevant vaccine delivery systems by DCs has not been systematically characterized.

Poly(D,L-lactic-co-glycolic acid) (PLGA) nanospheres have several features that make them an attractive vaccine delivery system. PLGA is a biodegradable polymer currently approved for use in humans and is suitable for the formulation of recombinant proteins (14,15), synthetic peptides (16,17), and plasmid DNA (18). Delivery of antigen in PLGA nanospheres can induce humoral (15,16), T helper (15,16,18), and cytotoxic T lymphocyte (19–22) responses *in vivo*. Finally, formulation characteristics can be tailored for optimal phagocytosis and antigen release kinetics. Therefore, information obtained about the phagocytosis of PLGA nanospheres by DCs is of major interest to researchers in the area of vaccine design. PLGA nanospheres also act as an excellent model system for comparing the phagocytosis of antigen delivery systems by various APCs. In this study, we report the first unambiguous evidence of the phagocytosis of PLGA nanospheres by human DCs. In addition, this phagocytosis was compared with particle uptake by Mϕ and characterization of the phagocytosis of PLGA nanospheres was begun.

MATERIALS AND METHODS

Monoclonal Antibodies (mAbs)

Mouse mAbs specific for human CD14 (clone M5E2), CD80 (clone BB1), CD86 (clone 2331), MHC II (HLA-DR, DP, DQ) (clone TU39), and fluorescein-5-isothiocyanate (FITC)-labeled anti-mouse IgG1 (clone A85-1), IgG2a (clone R19-15), and IgM (clone DS-1) were purchased from Pharmingen (Mississauga, Ontario, Canada). A negative control antibody B80.3R4 (mouse IgG1) specific for prostate specific antigen was provided by Biomira Inc. (Edmonton, Alberta, Canada).

[1] Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, AB, T6G 2N8, Canada.

[2] School of Pharmacy, University of Wisconsin, Madison, Wisconsin 53706-1515.

[3] To whom correspondence should be addressed. (e-mail jsamuel@pharmacy.ualberta.ca)

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Isolation of Mononuclear Cells from Peripheral Blood Leukocytes

Human peripheral blood mononuclear cells were isolated from standard peripheral blood leukocyte preparations collected from normal blood donors by Canadian Blood Services (Edmonton, Alberta, Canada) by density gradient separation over Ficoll-Paque Plus (Pharmacia Biotech, Uppsala, Sweden). To remove residual platelets, the cells were washed with Hank's Balanced Salt Solution (HBSS; BioWhittaker, Walkersville, MD, USA). Peripheral blood mononuclear cells were depleted of T lymphocytes by rosetting with neuraminidase (Sigma Chemical Co., St. Louis, MO, USA)-treated sheep red blood cells (RAM Media, Calgary, Alberta, Canada). The T lymphocyte depleted (>90%) mononuclear cells were isolated by density gradient centrifugation, washed in HBSS, and resuspended in RPMI-10HS [RPMI 1640 supplemented with 200 mM L-glutamine, 50 μg/mL gentamicin solution (Gibco-BRL, Burlington, Ontario, Canada), and 10% human serum (blood group AB; ICN, Costa Mesa, CA, USA)].

Culture of DCs and Mϕ

T cell-depleted mononuclear cells were plated in 6-well tissue culture plates (Corning 25815, Fisher Scientific, Nepean, Ontario, Canada) at 2×10^6 cells/well in RPMI-10HS. After incubation for 1 h at 37°C/5% CO_2, nonadherent cells were removed by gentle washing and adherent cells were cultured with RPMI-10HS enriched with 500 U/mL granulocyte macrophage-colony stimulating factor (GM-CSF; Pharmingen) and 8 ng/mL IL-4 (Pharmingen) to generate DCs (23–25). For the generation of Mϕ, the adherent cells were cultured without IL-4. Culture media was replaced every 3 days.

Mixed Lymphocyte Reaction (MLR)

After 3 days in culture, DC and Mϕ were collected and washed three times with RPMI media before being resuspended to 1×10^5 cells/mL in RPMI-10HS media. T cells from an allogeneic donor were removed from –70°C storage, thawed, washed with RPMI media, and resuspended at 1×10^6 cells/mL in RPMI-10HS. The MLR was set up at three different stimulator (S): effector (E) ratios: 1:5, 1:20, and 1:50 in triplicate in a 96-well plate. After 3 days at 37°C, 1 μci of ^{3}H-thymidine was added to each well in 50 μL media. After 24 h the cells were harvested onto a filtermat using a Mach III Harvester 96 and the filter was read in a Microbeta Trilux reader for radioactivity (Wallac, Turku, Finland).

Monophosphoryl Lipid A (MPLA)

Synthetic MPLA was supplied by Biomira Inc.

Preparation of PLGA Nanospheres

PLGA nanospheres were prepared using a water/oil/water solvent evaporation technique (26). Briefly, 100 μL of a 10 mg/mL solution of tetramethylrhodamine (TMR)-conjugated dextran (Molecular Probes, Eugene, OR, USA) was emulsified in 500 μL of chloroform containing 100 mg of PLGA (BPI, Birmingham, AL, USA) polymer (Mw = 50,000) with no adjuvant or with 200 μL of a 2 mg/mL solution of MPLA using a microtip sonicator (Model XL2010, Heat Systems INC., Farmingdale, NY, USA). The resulting primary emulsion was added into 2 mL of 9% w/v polyvinyl alcohol (PVA; 87–89% hydrolyzed, MW 31–50,000; Aldrich Chemical Co. Inc., Milwaukee, WI, USA) and was further sonicated to form the secondary emulsion. This emulsion was added dropwise into 8 mL of 9% w/v PVA and stirred for 3 h to evaporate the chloroform. The nanospheres were then collected by ultracentrifugation (40,000*g* for 10 min at 20°C), washed twice with distilled water (20 mL) to remove residual PVA, resuspended in 5 mL of distilled water, and freeze-dried for 3 days. Nanospheres were stored at –20°C. Before their addition to culture, nanospheres were resuspended in RPMI at a concentration of 5 mg/mL.

Nanosphere Size Determination

Nanosphere size was determined by dynamic light scattering (model BI-90 Particle Sizer, Brookhaven Instruments Corp., Holtsville, NY, USA). The sample was diluted 100× with phospate-buffered saline (PBS) and size was measured at 25°C. TMR-dextran nanospheres measured 500 nm ± 118 nm (mean ± SD; n = 5).

Phagocytosis

On day 6, 7, or 8 of culture, 500 μg of PLGA nanospheres containing TMR-dextran (5 μg) with no adjuvant or with MPLA (2 μg) in 100 μL were added to wells. Control wells were pretreated with 5 μg/mL of a phagocytosis inhibitor, cytochalasin B (27). Twenty-four hours later cells were harvested, washed, and resuspended in staining buffer. Phagocytosis was measured by flow cytometry.

Confocal Microscopy

On day 3 in culture, DCs were transferred into Lab-Tek II 8 well chamber slides (Nalge Nunc Int., IL, USA) at a concentration of 2×10^5 cells/300 μL. Control wells were pretreated for 10 min with 5 μg/mL cytochalasin B (Sigma-Aldrich Canada Ltd., Ontario, Canada). Each chamber received 100 μg of TMR-dextran containing nanospheres in 100 μL. After 24 h the supernatant was removed and the cells were washed three times with 500 μL of PBS buffer. To label the cell membranes, the cells were incubated with 100 μL of 0.0005% FITC-concanvalin A (Molecular Probes, OR, USA) in PBS buffer for 2 min. Cells were washed three times with PBS and then were fixed with 100 μL of 4% paraformaldehyde in PBS for 10 min. After a final wash with PBS, slides were prepared with a solution of 2.5% 1,4-Diazabicyclo [2.2.2.]octane (Aldrich Chemical Company Ltd., WI, USA) and 7.5% gelatin in 50:50 PBS:glycerol. Slides were then examined by confocal microscopy using a Zeiss 510 LSM NLO (Carl Zeiss Microscope Systems, Zena, Germany).

Fluorescence-Activated Cell Sorting

After 24 h of incubation with TMR-dextran–containing nanospheres, DCs or Mϕ were harvested by scraping and washed. Aliquots of 2×10^5 cells were added into microcentrifuge tubes in 19 μL of cold wash buffer (PBS containing 10% fetal calf serum and 0.05% sodium azide). mAbs specific for CD14 (0.5 mg/mL), CD80 (1 mg/mL), and MHC II (0.5

mg/mL) were diluted 5-fold with buffer. Anti-CD86 was used without dilution at a concentration of 0.25 mg/mL. Two microliters of mAb solution was added to each tube. After 30 min of incubation at 4°C, the cells were washed three times with wash buffer. Pellets were resuspended in 100 μL of wash buffer, and 2 μL of FITC-conjugated secondary Ab (0.5 mg/mL; either FITC-IgG1, FITC-IgG2a, or FITC-IgM) was added to each tube. After 30 min at 4°C in the dark, cells were washed with the wash buffer and transferred to Falcon tubes. Samples were analyzed by flow cytometry on a Becton-Dickinson FACScan (Franklin Lakes, NJ, USA).

The fluorescence threshold for cells described as positive for TMR (TMR^+) or FITC ($FITC^+$) was established as follows. Cells that were not treated with TMR nanospheres were used to set the threshold for "red" fluorescence. Cells treated with negative control FITC-antibody (isotype matched) were used to set the threshold for "green" fluorescence. The baseline threshold was set so that greater than 98% of the negative cells fell below that value. There were slight day-to-day variations, but the threshold was always set just above 10^1 on the log scale measuring fluorescence intensity. Cells above this threshold level were considered to be positive for TMR or FITC.

RESULTS

Characterization of Human DCs and Mϕ

Parallel cultures of DC and Mϕ were generated using monocyte precursors from the same donor using GM-CSF and IL-4 (for DCs) or GM-CSF alone (for Mϕ). This is a well-established and widely used methodology for *in vitro* generation of DCs and Mϕ (23–25). The identity of DCs and Mϕ in cultures was confirmed by the presence of cell surface markers CD14, MHC II, CD80, and CD86. On day 8 of culture, DCs were $CD14^{-/low}$, MHC II^+, $CD80^{low}$, and $CD86^{hi}$, and Mϕ were $CD14^{hi}$, MHC II^+, $CD80^{-/low}$, and $CD86^{-/low}$ (Fig. 1). It is also possible to verify the identity of the cells as DC through a functional assay, the MLR (28). The MLR studies demonstrated that the DC culture was more efficient at stimulating the T cell response to allogeneic cells than the Mϕ culture (Fig. 2). This functional assay confirms the identify of cells in the DC culture as DCs.

Phagocytosis of PLGA Nanospheres by Human DCs and Mϕ

PLGA nanospheres were visible inside DCs after a 24-h co-incubation (Fig. 3 B and D), but nanospheres were not visible inside the DCs when cells were pretreated with cytochalasin B, a phagocytosis inhibitor (Fig 3 A and C).

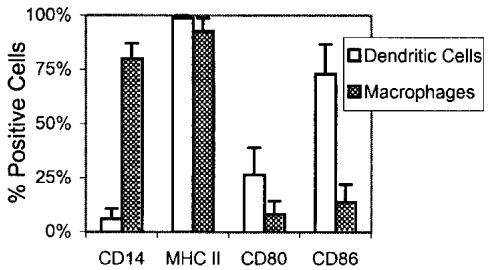

Fig. 1. Characterization of human dendritic cells and macrophages in culture. Percentage of cells expressing cell-surface markers, based on reactivity with specific antibodies. The data are presented as the mean ± SD (error bars of the values of samples from eight different donors.

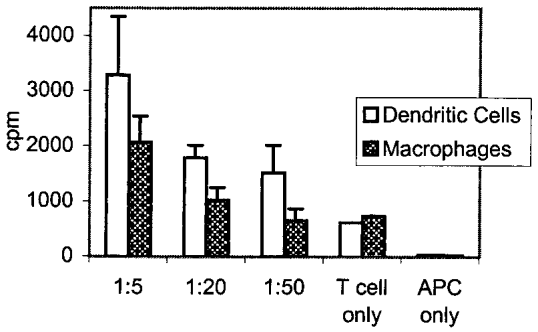

Fig. 2. Activation of allogeneic T lymphocytes by dendritic cells (DCs) and macrophages (Mϕ) in culture (mixed lymphocyte reaction). DCs or Mϕ were incubated with T cells from an unrelated donor at varying stimulator (DC or Mϕ) to effector (T cells) ratios. The proliferation of the T cells was measured by the incorporation of ^{3}H-thymidine. The experiment was performed in triplicate using DCs or Mϕ from one donor, and the results are shown as mean cpm ± SD (error bars).

It can be argued that phagocytosis in DC cultures is a result of contaminating Mϕ. Two-color staining with TMR-dextran–containing nanospheres and FITC-labeled antibodies to cell-surface markers was used to unambiguously identify the phagocytic populations in the DCs and Mϕ cell cultures. To identify the phagocytic subset of the cells, the cell-surface marker expression on all live cells in culture was compared with the expression on TMR^+ cells.

CD14 is a monocyte marker constitutively expressed at high levels on Mϕ (29,30). Some monocyte-derived DCs express low levels of CD14, and this expression may increase during activation, for example, after phagocytosis or exposure to immunostimulatory agents (29). The phagocytic population in day 8 DC cultures had a significantly lower number of $CD14^+$ cells than the Mϕ cultures (Fig. 4 A and B; $p < 0.005$). Mean fluorescence intensity of positive cells was 73 for Mϕ and 6 for DCs. This lack of high CD14 expression on the TMR^+ cells demonstrates that non-Mϕ cells in the DC culture are indeed phagocytic.

MHC Class II molecules are constitutively expressed by DCs and Mϕ (2–5). Virtually all DCs and Mϕ expressed some MHC II (data not shown). However, DC expressed higher levels of MHC II than Mϕ (Fig. 4C).

CD80 is a costimulatory molecule that is not constitutively expressed on DCs but is upregulated during maturation or under certain culture conditions. In our study, DC cultures had a higher proportion of cells expressing CD80 than Mϕ cultures (Fig. 4 A and B). The increased expression of CD80 on test cells as compared with control cells signifies that the marker was upregulated within the last 24 h in culture after the nanospheres were added. CD86 is another costimulatory molecule involved in the activation of T cells (2–5). There was a significant difference in the number of cells expressing CD86 between DC and Mϕ cultures (Fig. 4 A and B; $p < 0.0001$). Up to 90% of DC expressed CD86 whereas very few Mϕ expressed it.

DCs and Mϕ Phagocytose PLGA Nanospheres to the Same Extent

DCs and Mϕ were compared for their extent of phagocytosis of PLGA nanospheres. On day 8, TMR-dextran–containing nanospheres were added to DC and Mϕ cultures. After 24 h, both the number of phagocytic cells in culture

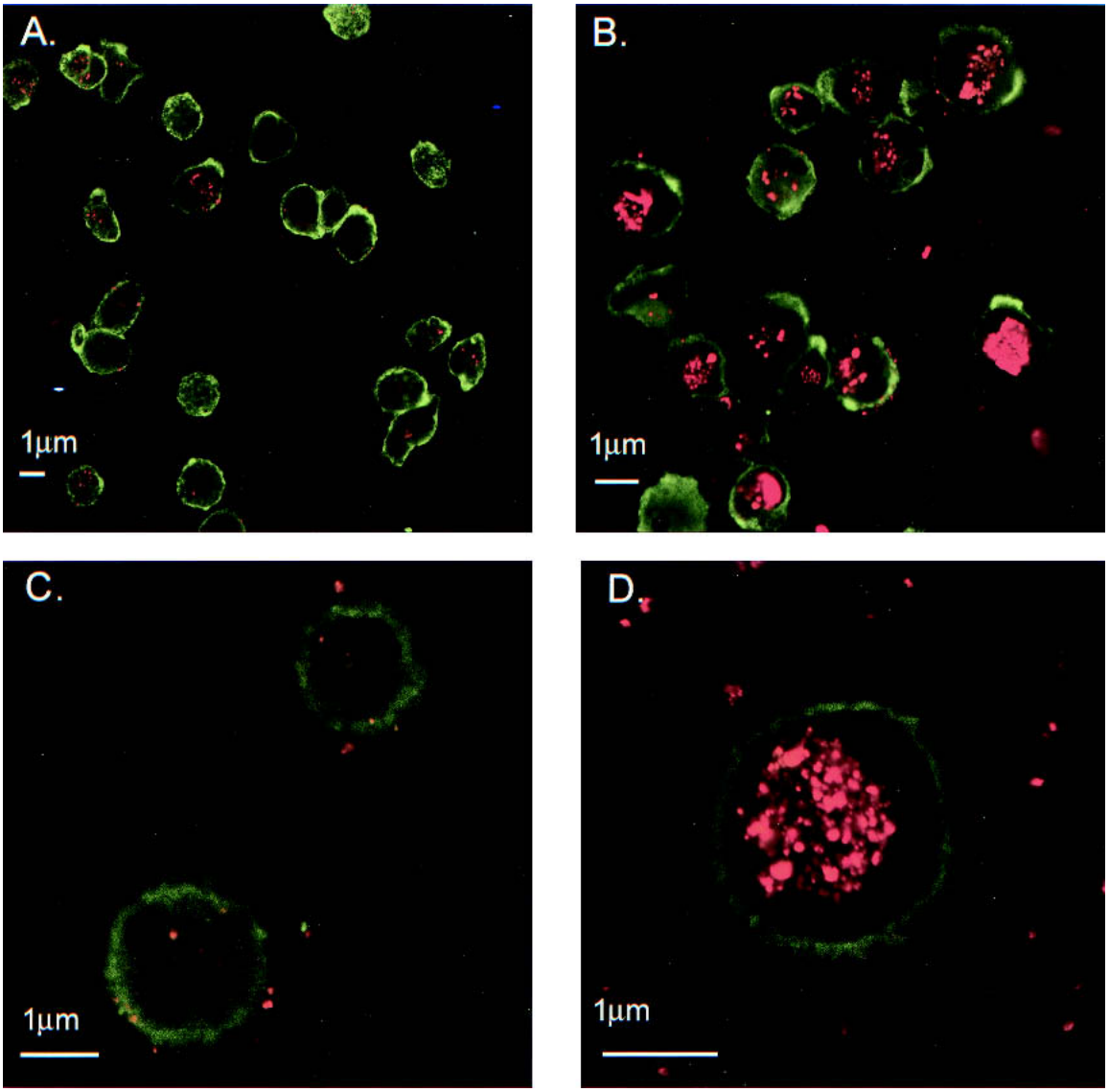

Fig. 3. Uptake of poly(D,L-lactic-co-glycolic acid) nanospheres by human dendritic cells (DCs). On day 4, after 24-h incubation with tetramethylrhodamine-dextran–containing nanospheres, cell membranes of DCs were stained with fluorescein-5-isothiocyanate-labeled concanvalin A and examined using a confocal microscope. Note the veiling on the cell membranes, which is a morphological trait of DCs. Control cells (A and C) were pretreated with cytochalasin B. Test cells (B and D) were untreated.

(Fig. 5A), as evidenced by the fraction of cells with a fluorescent signal, and the average amount of particle uptake per cell (Fig. 5B), as indicated by the mean fluorescence intensity (MFI), was determined by flow cytometry. There were no significant differences between the two types of APC with regard to the number of phagocytic cells or the extent of phagocytosis per cell ($p \geq 0.05$). Control wells for both DC and Mϕ containing cytochalasin B were scarcely above the background fluorescence of unstained cells, confirming that the internalization of particles was because of phagocytosis but not adherence of nanospheres to the outside surface of the cells ($p < 0.0001$).

Effect of Time in Culture on Phagocytosis

To determine the optimal day in culture for assessing phagocytosis of DCs and Mϕ, we initially examined their uptake of PLGA nanospheres over a 24-h period on days 6, 7, and 8. There was little or no difference in the number of phagocytic cells (Fig. 6A) or the average fluorescence per cell (Fig. 6B) between days 6, 7, or 8 in DCs. For Mϕ there was also no significant difference in the number of phagocytic cells on different days in culture (Fig. 6A). However, the number of particles per cell increased with increasing days in culture (Fig. 6B).

Kinetics of Phagocytosis of PLGA Nanospheres

Day 8 cultures of DC and Mϕ were studied for the kinetics of phagocytosis. Cells were incubated with nanosphere formulations, collected at different time points, and analyzed for the number of phagocytic cells and mean fluorescence per cell. No significant differences were seen at any time point between DCs and Mϕ in terms of the number of phagocytic cells (Fig. 7A) or the amount of uptake per cell (Fig. 7B).

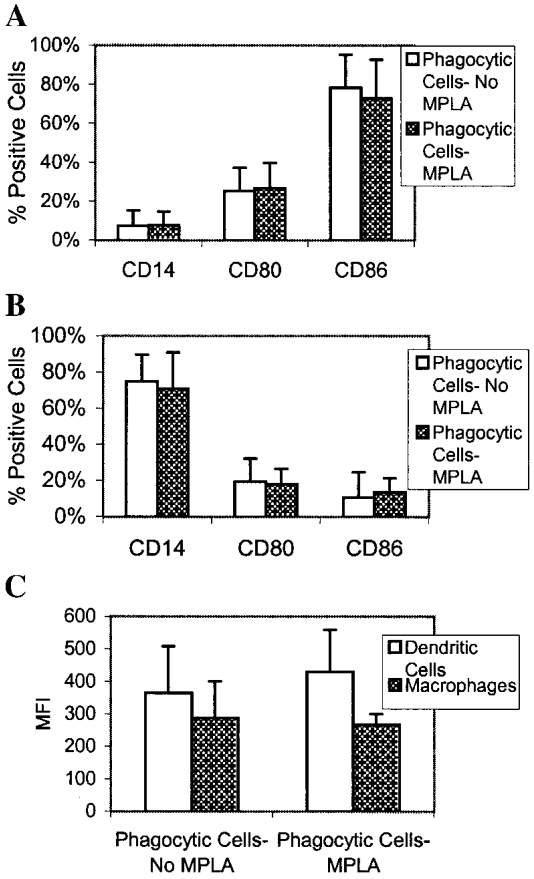

Fig. 4. Day 8 expression of cell-surface markers on phagocytic dendritic cells (DCs) and macrophages (Mϕ). On day 8, after phagocytosis of poly(D,L-lactic-co-glycolic acid) nanospheres containing tetramethylrhodamine-dextran for 24 h, two-color flow cytometry was used to determine the cell-surface maker expression on phagocyte (i.e., tetramethylrhodamine+) cells. A, DC expression of CD14, CD80, and CD86 represented as the percentage of cells positive for fluorescein-5-isothiocyanate (FITC)-labeled antibody. B, Mϕ expression of CD14, CD80, and CD86 represented as the percentage of cells positive for FITC-labeled antibody. C, Level of MHC II on DCs and Mϕ represented as the average amount of fluorescence per cell. MFI = mean fluorescent intensity of the FITC signal. The data are presented as the mean ± SD (error bars) of the values of samples from six different donors.

Incorporation of MPLA appeared to have no effect on the number of phagocytic cells at any time point (Fig. 7A). However the number of particles per cells for the formulation containing MPLA was greater than that without any adjuvant (Fig. 7B). Between 12 and 24 h, the number of phagocytic cells did not increase but the MFI did (data not shown).

Effect of MPLA on Expression of Cell Surface Markers by DCs

We examined the effects of MPLA in the formulation on the expression of cell-surface markers on day 8 in cultures. MPLA had no significant effect on the expression of MHC II (Fig. 4C), CD80, and CD 86 (Fig. 4A).

DISCUSSION

Antigen uptake by DC is a prerequisite for T cell–mediated immune responses. Therefore, effective delivery of antigens to DCs is of paramount significance to therapeutic vaccines designed to activate T cell responses. It has only recently been recognized that DCs are phagocytic, and to date most phagocytosis studies have focused on microorganisms and on nontherapeutically relevant particulate antigens (11,12). PLGA nanospheres are a useful antigen-delivery system capable of formulating a variety of antigens for use in humans (14–18). We set out to demonstrate that DCs are capable of phagocytosing PLGA nanospheres and to begin characterizing this phagocytosis.

Preliminary studies demonstrated qualitatively that DCs could phagocytose PLGA nanospheres (Fig. 3), but we sought a method that could quantify phagocytosis. We selected fluorescent nanospheres and flow cytometry for several reasons. First, compared with other potential techniques, including the use of radiolabeled protein encapsulated in nanospheres, the methodology was simple. This method also eliminated the need for separating free nanospheres from the cells because the flow cytometer does this easily on the basis of size and granularity. The use of a flow cytometer permitted us to gather data on the number of phagocytic cells as well as the amount of particle uptake per cell. Other methods were unable to provide this level of detail. Finally, the use of fluorescent nanospheres allowed us to use dual-color staining to positively identify the phagocytic cell populations in culture on the basis of cell surface marker expression.

We chose to examine parallel cultures of DCs and Mϕ generated from the same individual because this has not previously been performed in studies of phagocytosis by human

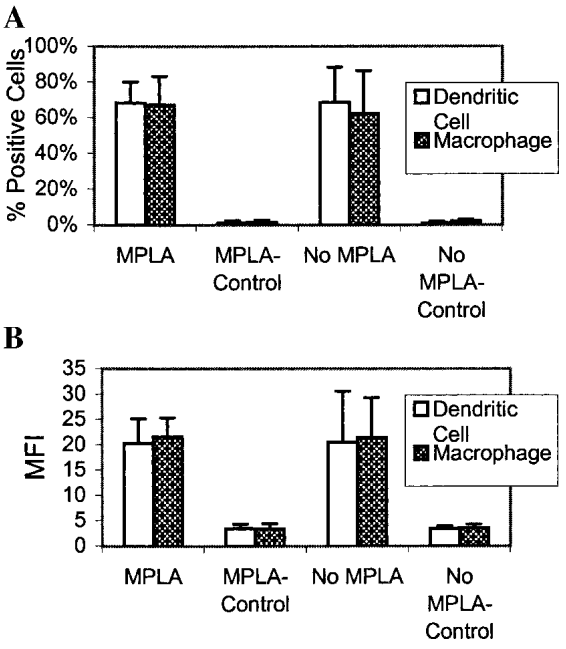

Fig. 5. Dendritic cells and macrophages phagocytose poly(D,L-lactic-co-glycolic acid) nanospheres to the same extent. On day 8, phagocytosis of poly(D,L-lactic-co-glycolic acid) nanospheres containing tetramethylrhodamine-dextran by dendritic cells and macrophages over 24 h was measured. Control wells were pretreated with cytochalasin B. A, The number of phagocytic cells in culture is expressed as the percentage of cells containing tetramethylrhodamine. B, The extent of phagocytosis was measured as the mean fluorescence intensity per cell. Data are presented as the mean ± SD (error bars) of the values of samples from seven different donors.

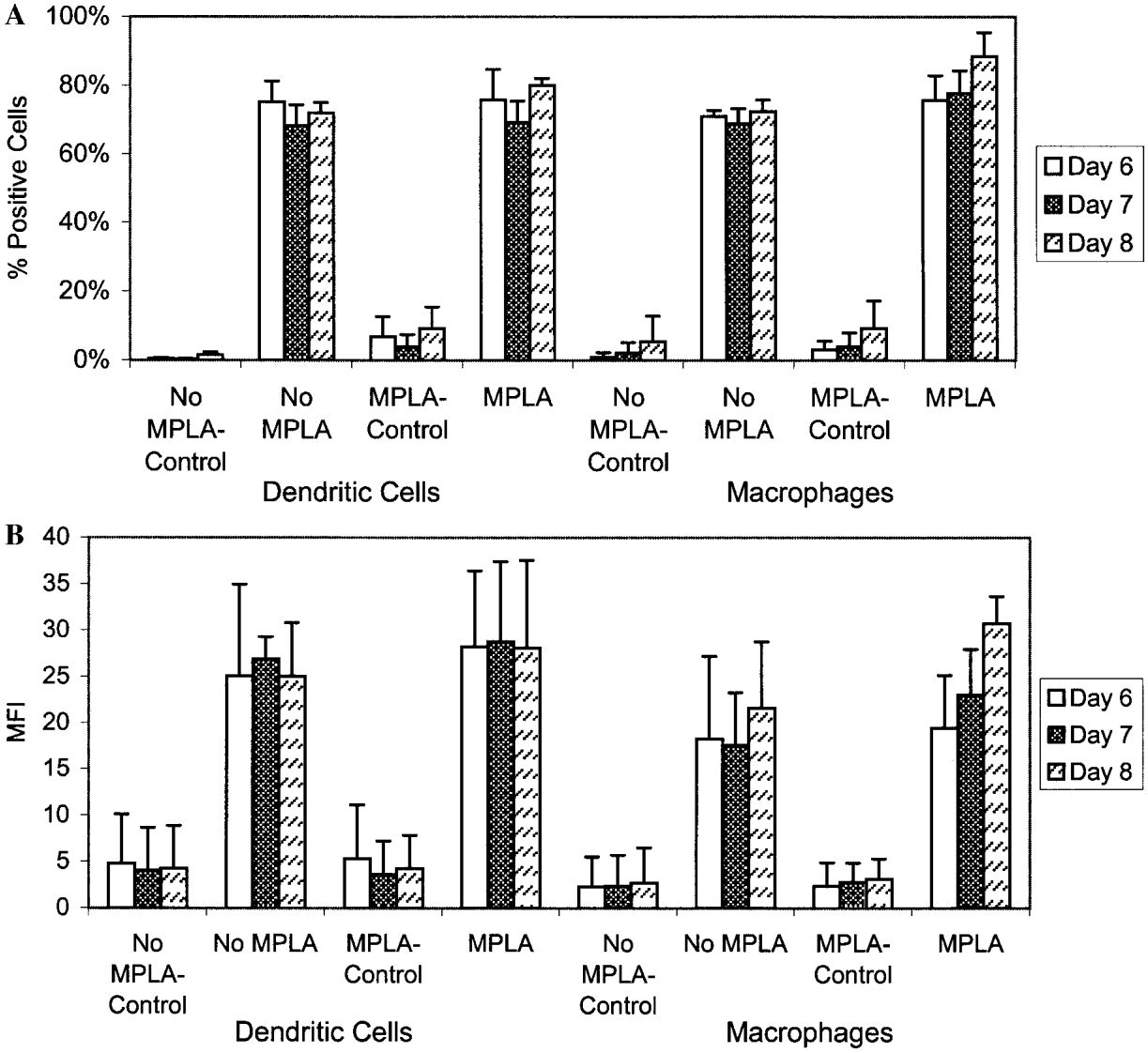

Fig. 6. Effect of time in culture on phagocytosis. On days, 6, 7, and 8, phagocytosis over 24 h was measured in dendritic cells and macrophages. A, The number of phagocytic cells in culture is expressed as the percentage of cells containing tetramethylrhodamine. B, The extent of phagocytosis was measured as the mean fluorescence intensity per cells. The data are presented as the mean ± SD (error bars) of the values of samples from three different donors.

APCs and it allowed us to look for similarities and differences between the scavenger Mϕ and the efficient APC, DC. Our results demonstrated that DCs phagocytose PLGA nanospheres to the same extent as Mϕ. This uptake of fluorescent particles is almost completely blocked by pretreatment with cytochalasin B, an agent that blocks phagocytosis but not pinocytosis or normal metabolism in mammalian cells, including human leukocytes (27). Thus, the uptake was a result of particle phagocytosis and not because of pinocytosis of free TMR or to adherence of nanospheres to the outside of the cells. These results show that PLGA nanospheres are suitable for delivery of antigens to DCs as well as Mϕ.

Preliminary experiments demonstrated that maximal levels of phagocytosis are reached within 24 h in DCs and Mϕ, so it was of interest to identify any differences between the cell types at earlier time points. Mϕ have been shown to be more efficient at particulate uptake (11), so it was important to determine whether this was true for our formulation of PLGA nanospheres. Our results demonstrate that there are no significant differences between the cells with regard to the rate of phagocytosis or the time required to reach the plateau of maximal phagocytosis. It was interesting to note that the number of phagocytic cells did not increase after 12 h, but the MFI (representing the number of particles per cell) continued to increase until 24 h. This indicates that all cells capable of phagocytosis of particles have taken up particles by 12 h but that they continue to phagocytose additional particles for up to 12 more hours.

The maturation stage of DCs affects their ability to phagocytose particles (11,13). To determine the optimal timing for further experiments, we examined the effect of time-in-culture on phagocytosis. Phagocytosis of PLGA nanospheres by DCs during a 24-h period was examined on days 6, 7, and 8 in culture. No difference was seen between days 6, 7, and 8 with respect to either the number of positive cells or the number of particles taken up per cell. Previous studies in our

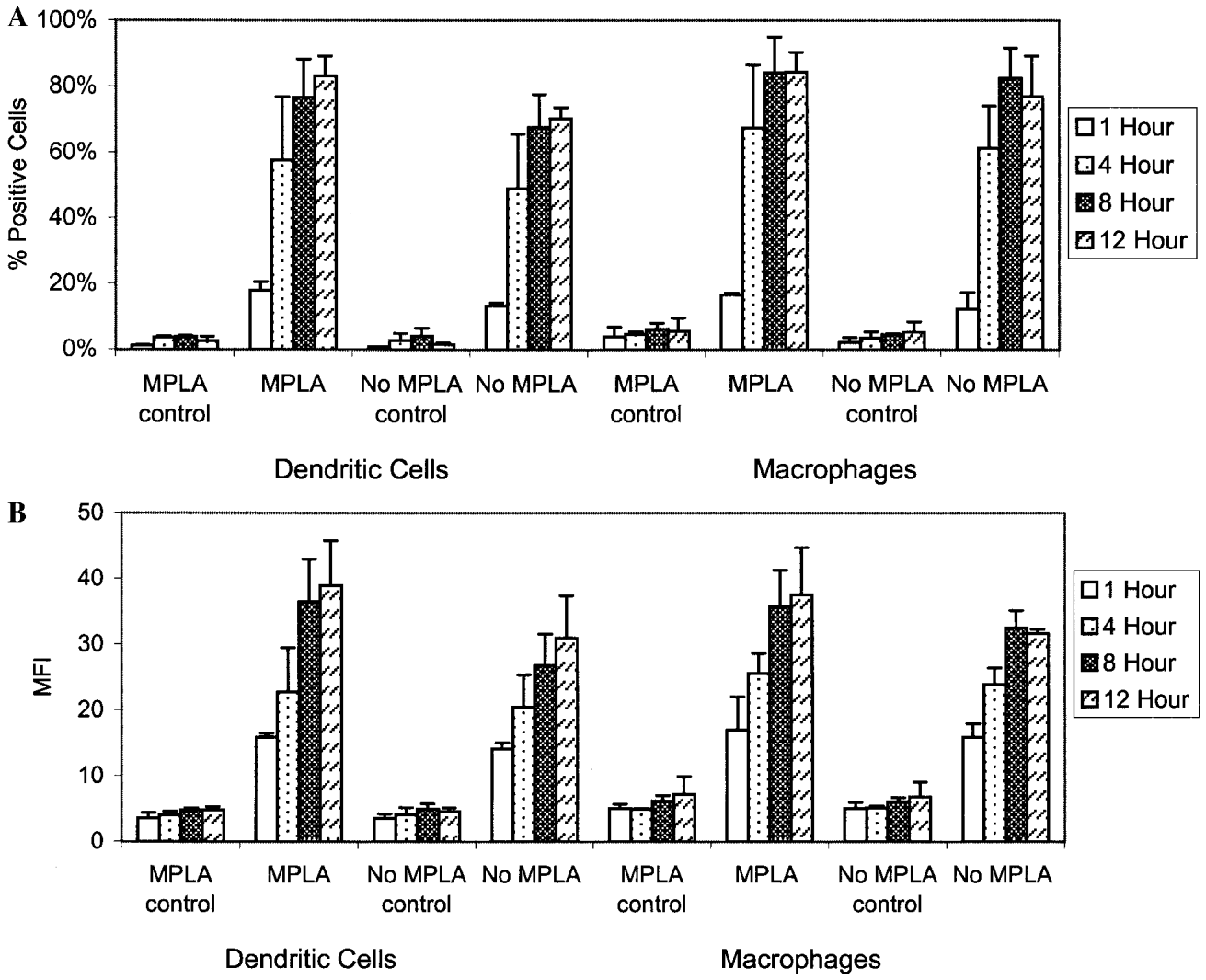

Fig. 7. Kinetics of phagocytosis of poly(D,L-lactic-co-glycolic acid) nanospheres. On day 8, phagocytosis over 1, 4, 8, and 12 h was measured in dendritic cells and macrophages. A, The number of phagocytic cells in culture is expressed as the percentage of cells containing tetramethylrhodamine. B, The extent of phagocytosis was measured as the mean fluorescence intensity per cells. The data are presented as the mean ± range (error bars) of the values of samples from two different donors.

laboratory examining DC phenotype indicate that maturation occurs from day 9 in culture onwards (unpublished results), so phagocytosis may decrease after this point. In contrast, there was no difference in the number of phagocytic cells on different days in Mϕ cultures, but the number of particles taken up per cell seemed to increase as the time in culture increased.

It can be argued that phagocytosis in DC cultures is a result of contaminating Mϕ. To eliminate this possibility, we used two-color staining with TMR-dextran–containing nanospheres and FITC-labeled antibodies to cell surface markers to identify the phagocytic populations in the DC and Mϕ cultures. We compared the expression of cell surface markers on all live cells with expression on the phagocytic population only (TMR^+ cells) to classify the phagocytic subset of cells in the cultures. For all surface markers studied, there were no differences in expression between all live cells and the phagocytic cells within the culture. The cells of the DC cultures were MHC II^{hi}, $CD86^{hi}$, $CD80^+$, and $CD14^{-/low}$, identifying them as DC rather than Mϕ. The cells of the Mϕ cultures were $CD14^{hi}$, MHC II^+, $CD86^{low}$, and $CD80^{-/low}$, confirming their identification as Mϕ.

MPLA is an adjuvant that aids in directing the immune response towards a T cell response. Because of its potential use in therapeutic vaccine formulations, it is important to understand what effects this adjuvant may have on the acquisition of antigen by DCs and the presentation of antigen by DCs to T lymphocytes. The first step in studying these effects is to delineate any consequences of MPLA content on the phagocytosis of PLGA nanospheres. The results showed that the incorporation of MPLA into the formulation led to increased phagocytosis by DCs at early time points but that it may have negative consequences on phagocytosis after 24 h in culture. Previous studies with the Mϕ cell line J774A in our laboratory showed a negative impact of MPLA on phagocytosis at 24 h and later but an enhancement of phagocytosis at earlier time points (18). This is a significant area for further investigation.

We also examined the influence of MPLA on the expression of cell surface molecules. The incorporation of an adjuvant into a vaccine formulation may enhance the immune response by altering the presentation of antigen to T cells or the stimulation of T cells by DCs. Any change in the presen-

tation of antigen to T cells is likely to be caused by a change in the expression of MHC II on the surface of the APC. Altered expression of the costimulatory molecules, CD80 and CD86, will affect the ability of the APC to stimulate T lymphocytes. We found that the incorporation of MPLA did not affect the expression of MHC II molecules on day 8 in culture. At this point in culture, the expression of MHC II is quite high and it is possible that any difference is difficult to discern. Preliminary data on the effect of MPLA on the expression of cell surface markers at earlier times in culture has been collected. These data suggest that MPLA may influence the maturation state of the cells as well as their ability to present antigen and stimulate T cells. Further investigations will clarify how to formulate antigen for achieving peak immune activation.

Our investigations conclusively demonstrate that DCs phagocytose PLGA nanospheres. The phagocytic population was unambiguously identified as DCs on the basis of expression of relevant cell surface markers. Because DCs are the key professional APCs capable of stimulating naive T cells, our data support the view that PLGA nanospheres can serve as an efficient delivery system for vaccines designed to activate T cell–mediated immune responses. This finding has significant implications for the development of therapeutic vaccines for cancer and chronic viral infections.

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