

The function and magnetic resonance imaging of immature dendritic cells under ultrasmall superparamagnetic iron oxide (USPIO)-labeling

Wei Zhang · Shuihua Zhang · Wan Xu · Min Zhang · Quan Zhou · Wenli Chen

Received: 21 September 2016 / Accepted: 29 March 2017 / Published online: 10 April 2017
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Abstract

Objective To investigate the effects of ultrasmall superparamagnetic iron oxide (USPIO) labeling on the maturity or immune tolerance of immature dendritic cells (imDCs) as the success of immunotherapy with immature dendritic cells is highly dependent on immune tolerance.

Results The feasibility of tracking implanted USPIO-labeled imDCs in vivo by magnetic resonance imaging (MRI) was explored. The effects of USPIO labeling on the immune tolerance of imDCs was examined. USPIO when higher than 200 µg/ml caused considerable damage to imDCs, induced imDC maturation, and impacted the immune tolerance of imDCs. USPIO labeling caused a dose-dependent increase in autophagosome formation in imDCs, and autophagy inhibitors prevented the maturation of imDCs while stimulating their immune tolerance.

Conclusions We speculate that high concentrations of USPIO can be used to induce imDC maturation, and that this process is likely mediated through an autophagy-related pathway.

Keywords Autophagy · Cell transplantation · Immature dendritic cells · Immune tolerance · MRI · Ultrasmall superparamagnetic iron oxide

Introduction

Dendritic cells (DCs) play a critical role in the initiation of primary immune responses and can be classified as either tolerogenic immature dendritic cells (imDCs), that maintain immune tolerance, or immunogenic mature dendritic cells (mDCs), that are powerful stimulators of the immune response (Griffiths and O'Neill 2008). imDCs are highly efficient in antigen uptake and are capable of inducing T cell anergy or the generation of regulatory T cells (Tregs). Therefore, these cells can be used for specific cell therapies to induce immunological tolerance in transplantation and autoimmunity (Boks et al. 2012). imDCs exert potent immunosuppressive effects in the context of allograft rejection and allergy. However, the application of imDC vaccines in the clinical setting and the precise mechanisms underlying the immunosuppressive effects of these cells remain unclear.

Ultrasmall superparamagnetic iron oxide (USPIO), an iron oxide nanoparticle with diameters of approx. 30–50 nm, yields decreased signals on T2- and T2*-weighted images and has a relatively long half-life, making it an effective contrast agent for use in magnetic resonance imaging (MRI). Indeed, this agent is widely used for disease diagnosis and therapy.

W. Zhang · W. Xu · M. Zhang · W. Chen (✉)
MOE Key Laboratory of Laser Life Science, South China Normal University, Guangzhou, China
e-mail: Chenwl@scnu.edu.cn

S. Zhang · Q. Zhou
Medical Imaging Center, The First Affiliated Hospital of Jinan University, Guangzhou, China

Characterizing the mechanism(s) of DC migration in vivo is crucial for both enhancing our understanding the immune response and treating diseases. While live cells can be labeled and tracked by MRI, direct cell labeling is generally associated with certain drawbacks, including cytotoxicity, suppressed proliferation, and impaired differentiation capacity (Bengel et al. 2005). The efficacy of MRI for visualizing SPIO-labeled monocytes and lymphocytes has been evaluated and has identified concentrations of these cells that are suitable for in vivo tracking; however, the influence of USPIO on cellular functions, as well as the optimal concentration of USPIO-labeled imDCs for in vivo tracking, remain unclear. In this study, we therefore investigated the viability and functionality of USPIO-labeled imDCs.

Autophagy is an intracellular process employed by various types of cell, including DCs, that is involved in packaging long-lived proteins and defective organelles into autophagosomes and delivering them to lysosomes for degradation (Cuervo et al. 2005). While excess autophagy has been suggested to induce the maturation of imDCs, the mechanism underlying this process remains poorly understood. Elevated autophagy is a widespread phenomenon in cells that have been treated with nanomaterials, indicating that USPIO, a type of nanoparticle, might activate this process. Additionally, induction of autophagy can facilitate antigen processing (Jagannath et al. 2009); however, limited data are available on the effects of autophagy on the other functions of imDCs.

In this study, we explored the minimal concentration of USPIO and the lowest number of imDCs required for detection by 1.5T MRI. Furthermore, we investigated whether MRI comprises a feasible method for monitoring transplanted imDCs. Lastly, we investigated whether USPIO promotes imDC autophagy, and whether autophagy might affect imDC maturation and the uptake/processing of antigens by comparing the levels of cytokine secretion and co-stimulatory molecule expression of labeled and unlabeled cells.

Materials and methods

Ethics statement

All experiments were conducted according to the Code of Ethics of the World Medical Association

(Declaration of Helsinki), and all experimental protocols involving animals were approved by the Animal Care and Use Committee of Guangdong Province, China.

Nanomagnetic labeling materials

USPIO particles (diam: 30–50 nm) were provided by the Medical Imaging Center of Southern Medical University (Guangzhou, China). USPIO was incubated for 30 min at 50–2000 µg/ml with poly-L-lysine (PLL; Sigma) at 1.5 µg/ml in serum-free RPMI 1640 medium containing L-glutamine.

Cell preparation, cultivation, and labeling

Dendritic cells (DCs) were derived from peripheral blood mononuclear cells (PBMCs) as previously described (Zhou and Chen 2012). PBMCs were isolated from the peripheral blood of New Zealand white rabbits via the auricular artery and cultured in complete medium (CM) in six-well polystyrene tissue culture plates for 4 h at 37 °C in an atmosphere containing 5% CO₂. After incubation, non-adherent cells were discarded from the wells, and adherent cells (monocytes) were incubated in CM supplemented with granulocyte–macrophage colony-stimulating factor (GM-CSF; PeproTech, Rocky Hill, NJ, USA) at 50 ng/ml and 50 ng interleukin-4/ml (IL-4; PeproTech) for 6 days. Purity of DCs was assessed by expression of CD11c by flow cytometry. This was routinely >80%. For USPIO-labeling, imDCs (5×10^5 cells/well) were co-cultured with 1.5 ml of each USPIO-PLL mixture, respectively, for 12, 24, or 48 h.

Cell viability and proliferation

The Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assay was used to assess the effects of USPIO-PLL on cell viability. The viability of cells was proportional to A₄₅₀ value as determined using a microplate reader.

Levels of cell apoptosis and necrosis were measured using an Annexin V-FITC/PI Kit (BD Bioscience, Co.; San Jose, CA, USA), according to the manufacturer's recommendation, and a FACSCanto flow cytometer (BD Biosciences).

Determination of intracellular iron content and MRI of USPIO-PLL-labeled imDCs

Iron concentrations within USPIO-labeled cells were quantified using a Ferrozin-based spectrophotometric assay.

For MRI analyses, different numbers of cells labeled with varying concentrations USPIO-PLL for 12 h were transferred into 1.5 ml Eppendorf tubes loaded with 1 ml 1% agarose. Samples were then subjected to MRI using a 1.5 T clinical whole-body MR system (GE Healthcare, Little Chalfont, UK) with a 12.7-cm receive-only knee coil (Zhang et al. 2015). Cells were imaged using the T2 mapping sequence [repetition time (TR) = 1 s; echo time (TE) = 16, 18, 20, 22 ms; band-width = 15.63; phase = 160; number of excitations (NEX) = 2] with a section thickness of 0.5 mm and a field of view (FOV) of 18 mm. Images were obtained with a matrix size of 256×160 . The region of interest (ROI) for the signal intensity (SI) measurement was 19 mm^2 .

For MRI analysis of *in vivo* DC migration to the liver, imDCs were labeled with $200 \mu\text{g}$ USPIO/ml and injected into rabbits via the ear vein (10^6 cells/ml, 2 ml per rabbit); control animals were injected with an equal volume of physiological saline. After anesthesia

and immobilization in the prostrate position, rabbits were scanned by MRI at 1, 3, and 7 days after cell transplantation. Imaging was performed with a knee coil on a 1.5 T MRI with 3/0.5 mm thick slices. MRI tracking was performed using T2-weighted sequences with fat suppression (T2WI-FS) with the following parameters: repetition time (TR) = 5500 ms; echo time (TE) = 106.6 ms; FOV = 14 mm; and matrix size, 256×256 .

Analysis of the expression levels of surface antigens by flow cytometry and laser-scanning microscopy

Cells labeled with different concentrations of USPIO-PLL (0–500 $\mu\text{g}/\text{ml}$) for 12 h were harvested and stained with FITC-conjugated anti-CD86, APC-conjugated anti-CD14, and PE-conjugated anti-HLA-DR (BD Biosciences) for 30 min, and evaluated by flow cytometry and laser-scanning microscopy (LSM510/ConfoCor2; Carl Zeiss, Oberkochen, Germany).

ELISA and mixed leukocyte reaction (MLR)

The concentration of IL-10 in each sample was measured using ELISA. Concentrations were

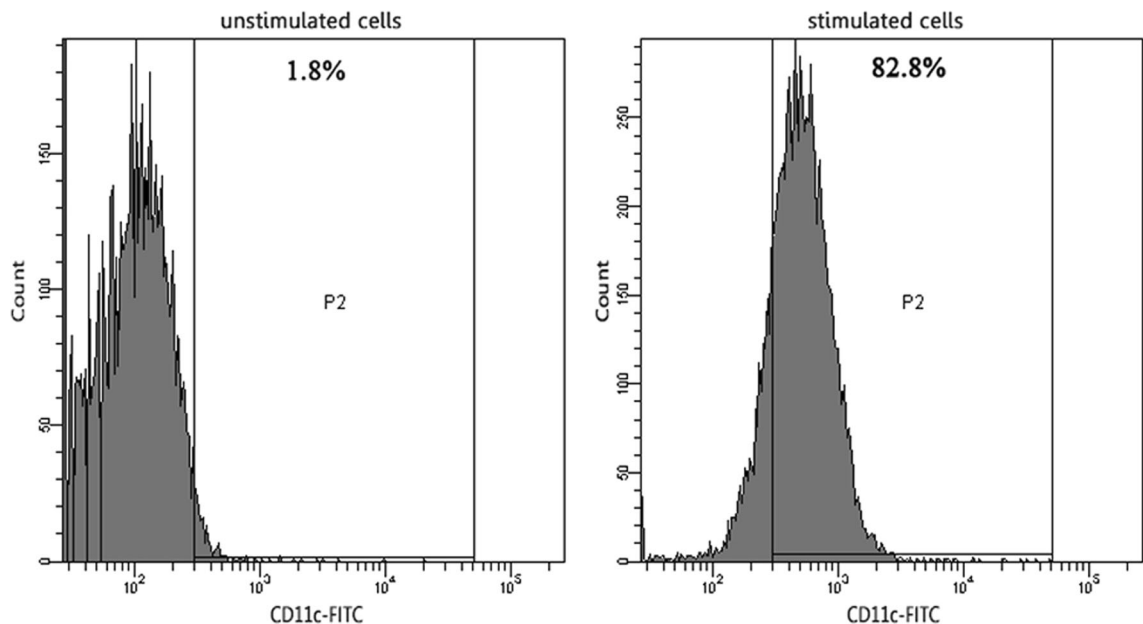


Fig. 1 Detection of surface markers of unstimulated cells and cells before stimulation by flow cytometry. The expression of CD11c on cell membranes before stimulation and cell

membranes before stimulation was detected by flow cytometry analysis using marker-specific antibodies

quantified by comparing the OD₄₅₀ values of the samples to a standard curve.

MLR was assessed using a Cell Counting Kit-8 (Dojindo), according to the manufacturer's instructions. Levels of lymphocyte proliferation were proportional to the OD₄₅₀ value, which was determined using a microplate reader.

Endocytosis assay

Receptor-mediated endocytosis levels were assayed by evaluating uptake of FITC-dextran (40,000 MW; Sigma). Briefly, cells were labeled with different concentrations of USPIO-PLL (0–500 µg/ml) for 12 h, harvested, washed, and incubated with 100 µg FITC-dextran/ml diluted in PBS for 3 h at 37 °C. Cells

were then washed once with PBS, resuspended in 200 ml PBS, and analyzed immediately by flow cytometry.

Detection of autophagy

Autophagic vacuoles were detected using LysoTracker Red DND-99 stain (Molecular Probes, Inc., Eugene, OR, USA) (Moon et al. 2009). Cells were placed in Prolong Gold antifade with DAPI to stain nuclei. imDCs were incubated with USPIO-PLL (0–500 µg/ml) for 12 h, washed three times with PBS, and then incubated with pre-warmed (37 °C) medium containing LysoTracker Red DND-99 for 1 h, washed once with PBS. Cells were then stained with DAPI. Cells were then examined under a laser-

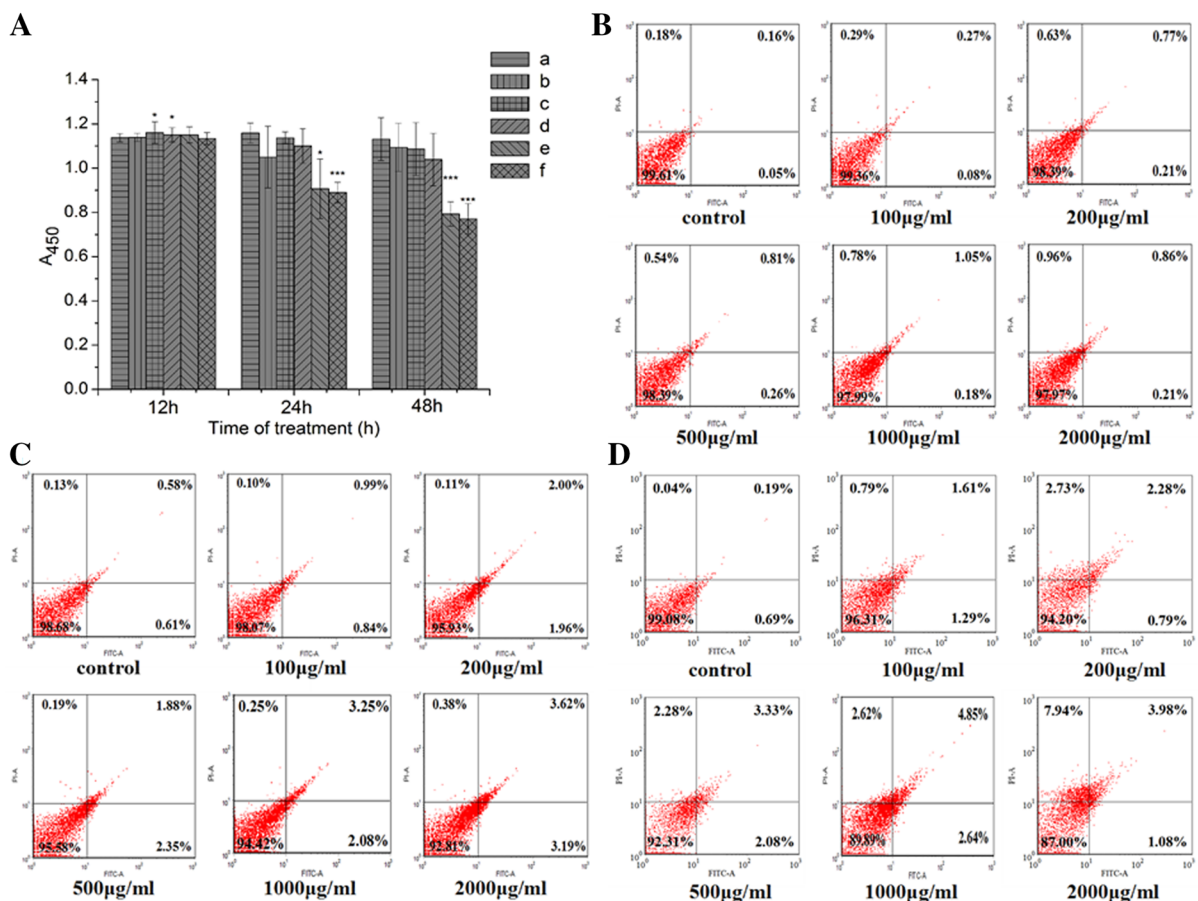


Fig. 2 Effects of ultrasmall superparamagnetic iron oxide (USPIO) labeling on cell proliferation and viability. **a** Immature dendritic cells (imDCs) were incubated with USPIO-PLL (poly-L-lysine) at different concentrations (0–2000 µg/ml) for 12, 24, or 48 h, and CCK-8 assays were used to examine cell

proliferation and viability. (*a–f*) Control, 100, 200, 500, 1000, and 2000 µg USPIO/ml. **b–d** Survival rates of USPIO-labeled imDCs were determined by Annexin V-FITC/PI staining and flow cytometry at **b** 12 h, **c** 24 h, and **d** 48 h after labeling

scanning microscope (LSM510/ConfoCor2); intense red dots within the cytoplasm were representative of autophagic vacuoles.

Statistical analysis

Each experiment was repeated at least three times. Data were presented as means \pm standard deviations (SDs) and were analyzed by two-sample *t*-tests and one-way analysis of variance (ANOVA) with Bonferroni correction using Origin 7 software (OriginLab Corp., Northampton, MA, USA). *p* values < 0.05 were considered statistically significant ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, compared to controls).

Results

Cell differentiation

Compared with unstimulated cells, cells after stimulation exhibited markedly increased expression of CD11c, as determined by flow cytometry (Fig. 1).

Effects of USPIO-PLL on cell proliferation and viability

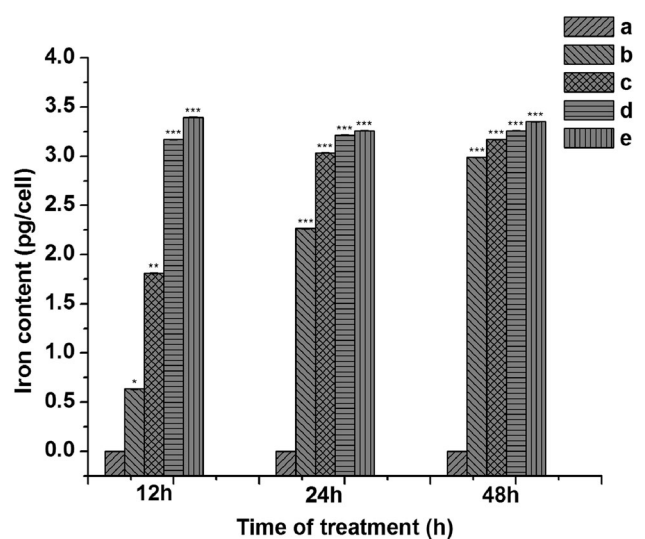
CCK8 assay analyses detected no significant differences between the viability of labeled cells and that of

unlabeled cells after incubation with up to 2000 μg USPIO/ml for 12 h (Fig. 2a). However, statistical analysis of data after 24 and 48 h showed that cell viability decreased significantly as the concentration of USPIO increased to 1000 $\mu\text{g}/\text{ml}$. Consistent with these findings, while we detected no significant differences in the numbers of apoptotic or necrotic cells between labeled and unlabeled cells after 12 h by annexin V-FITC/PI staining (Fig. 2b), incubation with high concentrations of USPIO for 24 or 48 h resulted in increased apoptosis and necrosis (Fig. 2c, d). Together, these results indicate that USPIO affects the viability of imDCs in a concentration- and time-dependent manner.

USPIO-PLL labeling efficiency

In imDCs incubated with USPIO for 12 h, the intracellular iron content increased significantly with increasing concentrations of USPIO from 0 to 200 $\mu\text{g}/\text{ml}$ and remained constant at USPIO concentrations from 200 to 500 $\mu\text{g}/\text{ml}$ (Fig. 3). Notably, no significant differences in labeling efficiency were observed between cells incubated with 200 $\mu\text{g}/\text{ml}$ for 12 h and those incubated with higher concentrations (500 and 1000 $\mu\text{g}/\text{ml}$) for 24 or 48 h, indicating that maximum labeling efficiency with low cytotoxicity could be achieved via incubation with USPIO at 200 $\mu\text{g}/\text{ml}$ for 12 h.

Fig. 3 Cellular iron content in ultrasmall superparamagnetic iron oxide (USPIO)-labeled imDCs. USPIO loading concentrations were as follows (a–e): control, 50, 100, 200, and 500 $\mu\text{g}/\text{ml}$



In vitro and in vivo MR imaging of imDCs

To determine the detection threshold of USPIO-labeled imDCs on a 1.5 T MR scanner, we labeled imDCs with concentrations of USPIO ranging from 0 to 500 $\mu\text{g}/\text{ml}$ for 12 h. As shown in Fig. 4a, the decrease in signal intensity was dependent on both the density of imDCs and the USPIO starting concentration. At 200 μg USPIO/ml, there was a distinguishable decrease in signal intensity (by approx. 31%) for 10^6 imDCs/ml (Fig. 4b), suggesting that injection of 1×10^6 imDCs/ml labeled with 200 μg USPIO/ml

was optimal for distinguishing signal changes on the 1.5 T MRI scanner.

In vivo migration of USPIO-labeled imDCs was measured by MRI. Intravenous bolus injection of USPIO-labeled imDCs resulted in a strong signal loss in the liver tissue (Fig. 4c, d), and the livers of imDC-transplanted rabbits were darker than those of control rabbits. In addition, the minimal reduction in signal within the liver was observed at 1 day after injection, and this reduction increased continuously over the subsequent 7 days; these data indicate that labeled DCs can be used for long-term tracking and that

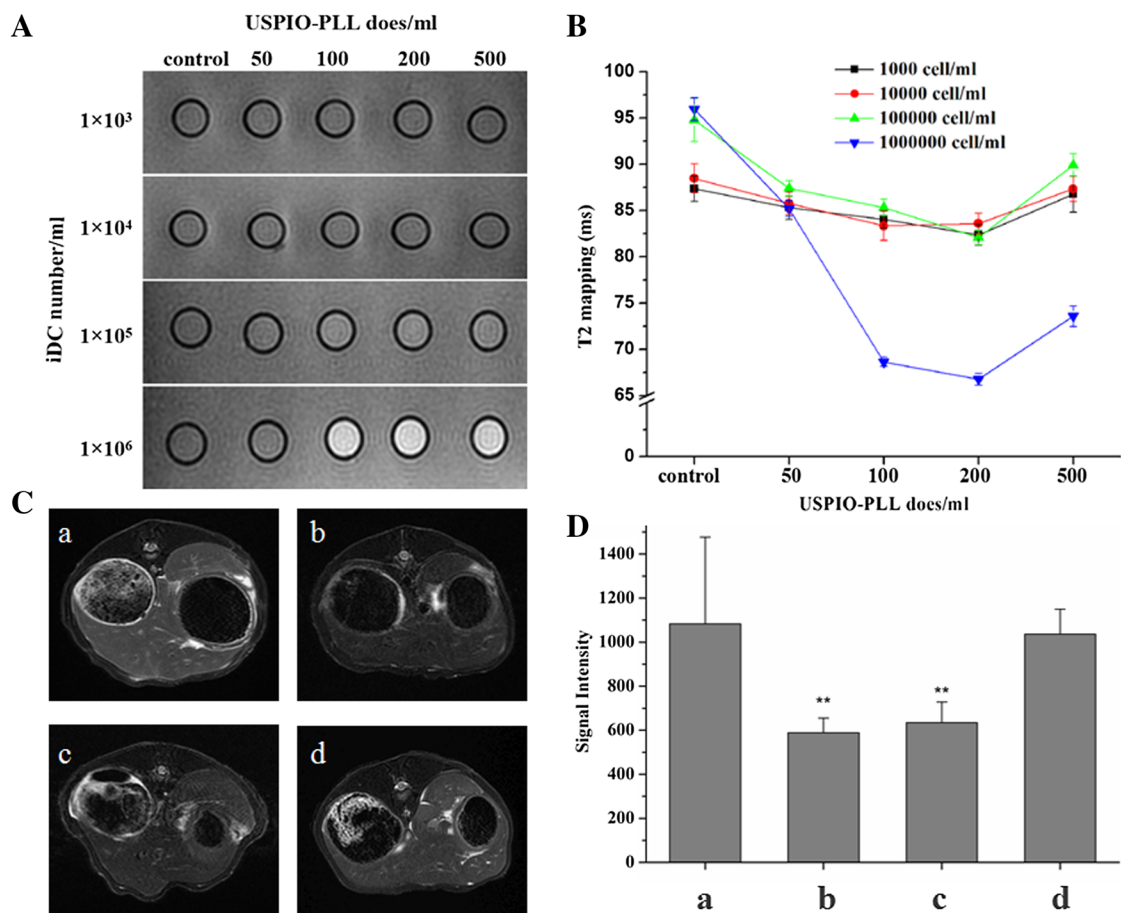


Fig. 4 In vitro and in vivo magnetic resonance imaging (MRI). **a** Different numbers of cells in each condition were embedded in 1 ml of 1% agarose and imaged on a 1.5 T MR system. Cells cultured in the absence of ultrasmall superparamagnetic iron oxide (USPIO) were used as controls. **b** Decrease in signal intensity caused by USPIO labeling of immature dendritic cells (imDCs), imaged with a 1.5 T MR-scanner using T2 mapping. **c** Rabbits were subjected to MRI analysis at different periods

after bolus injection of USPIO-labeled imDCs; images of rabbits prior to bolus injection served as a control. **d** Quantification of the MRI signal intensity of three different slices within a region of interest within the rabbit liver (**a–d** control, 1 day after injection, 3 days after injection, 7 days after injection, respectively). Error bars represent standard deviations of the results obtained from three independent experiments

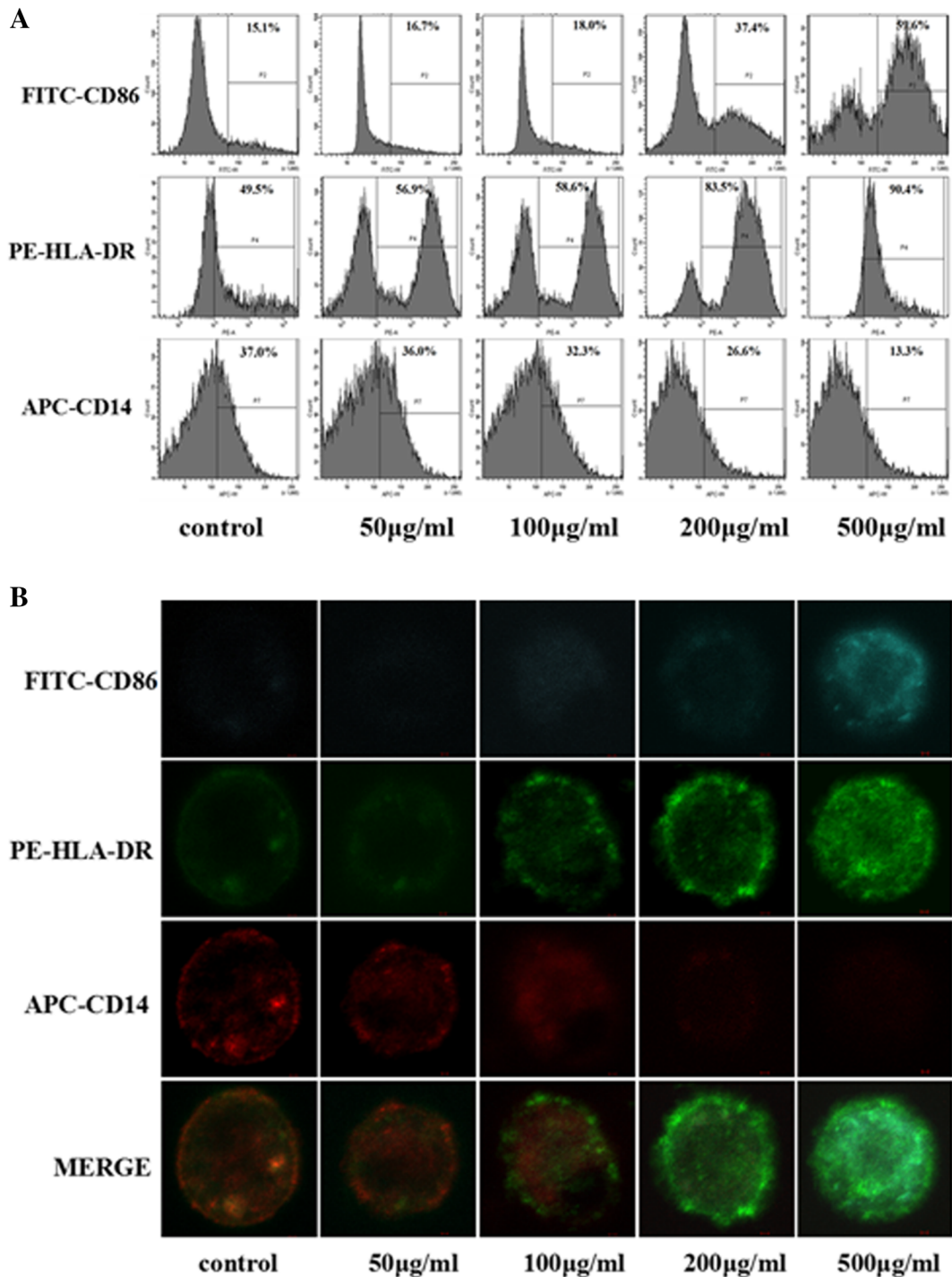


Fig. 5 Detection of surface markers of immature dendritic cells (imDCs) by flow cytometry and laser scanning microscopy. **a** The expression of CD86, HLA-DR, and CD14 on imDC membranes was detected by flow cytometry analysis using marker-specific antibodies. **b** Cells were stained with

fluorescein-labeled antibodies targeting CD86, HLA-DR, and CD14 and were observed under a laser-scanning confocal microscope. *Bars* 1 µm. imDCs were labeled with the indicated concentrations of ultrasmall superparamagnetic iron oxide (USPIO) for 24 h

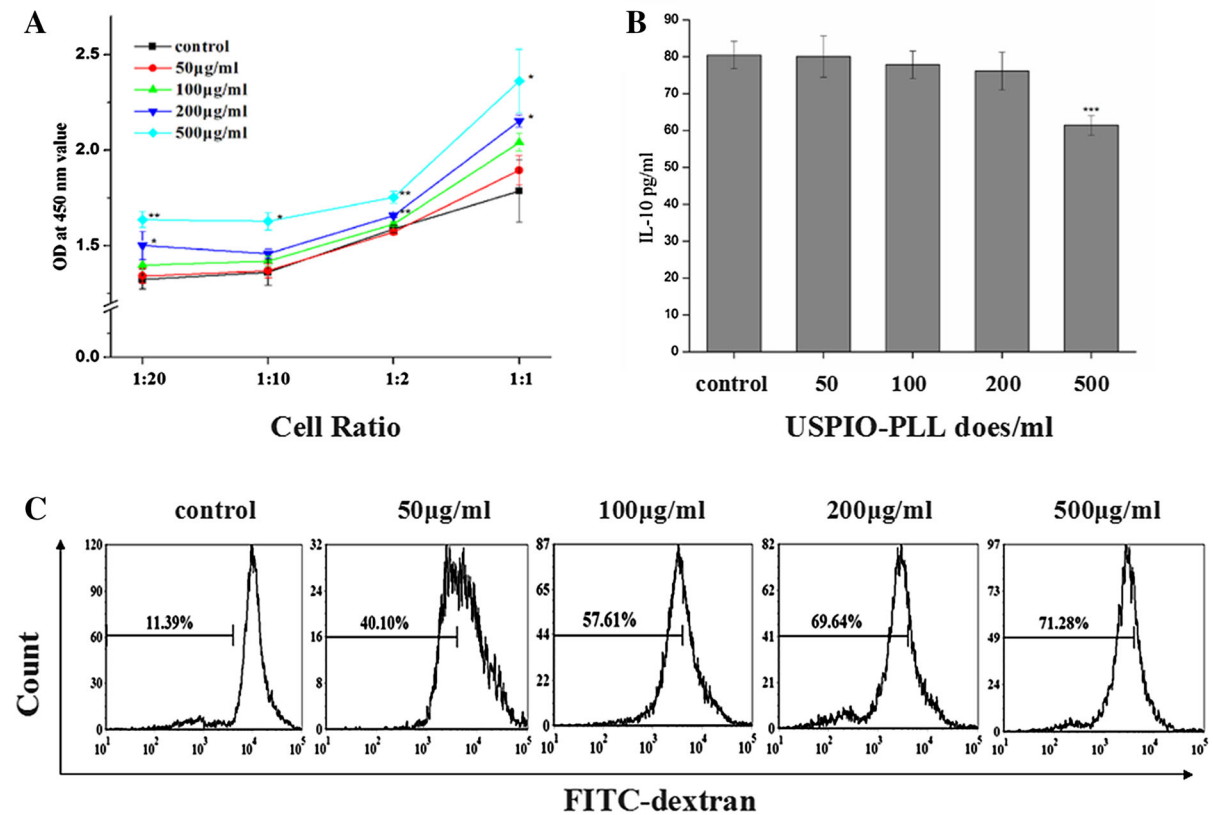


Fig. 6 Detection of immunity of immature dendritic cells (imDCs). **a** Mixed lymphocyte reaction containing imDCs and indicated concentrations ultrasmall superparamagnetic iron oxide (USPIO)-labeled imDCs with peripheral blood mononuclear cells (PBMCs) obtained from an allogeneic donor.

b Analysis of IL-10 levels in 72 h mixed leukocyte reaction (MLR) supernatants (stimulator-responder ratio, 0.1) by enzyme-linked immunosorbent assay (ELISA). **c** Uptake of FITC-dextran by imDCs and indicated concentrations USPIO-labeled imDCs

transplanted DCs labeled with USPIO can be dynamically tracked *in vivo* by MRI.

Phenotypic analysis by flow cytometry and laser-scanning microscopy

Compared with cells cultivated in USPIO-free medium, imDCs treated with 200 μg USPIO/ml exhibited reduced CD14 expression and markedly increased expression of CD86 and HLA-DR, as determined by flow cytometry (Fig. 5a). Moreover, these changes in expression occurred in a USPIO-dose-dependent manner. Similarly, microscopic analyses detected gradual decreases and increases in the fluorescence intensity of CD14, and of HLA-DR and CD86, respectively, in imDCs treated with 200 μg USPIO/ml (Fig. 5b). Together, these results indicate USPIO inhibits the expression of CD14 but promotes the

expression of HLA-DR and CD86 expression, as compared to cells cultured in USPIO-free medium.

Stimulation of lymphocyte proliferation and ELISA for detection of cytokine expression in imDCs

The capacity of rabbit imDCs to induce allogeneic T-cell proliferation from PBMCs was compared in an MLR (Fig. 6A). imDCs labeled with low concentrations of USPIO (0–200 $\mu\text{g/ml}$) did not exhibit obvious stimulation of proliferation when co-cultured with allogeneic PBMCs. Conversely, vigorous proliferation was observed in co-cultures of allogeneic PBMCs and imDCs labeled with high concentrations of USPIO (≥ 500 $\mu\text{g/ml}$). In addition, mixtures containing higher ratios of imDCs and T cells showed even greater proliferation rates upon PBMC stimulation. Moreover,

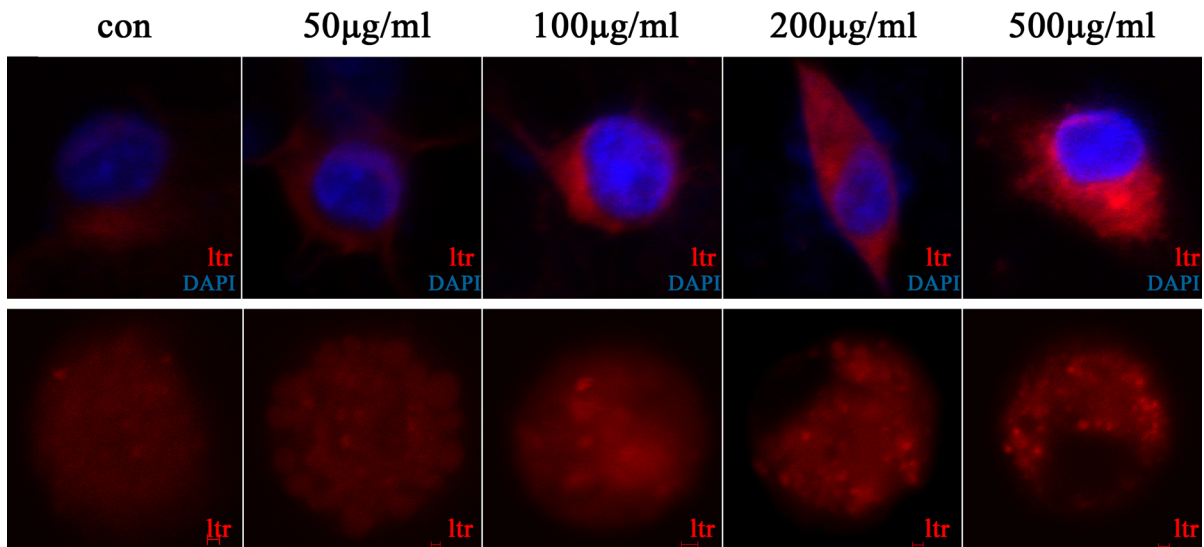


Fig. 7 Indication of autophagy in immature dendritic cells (imDCs) after labeling with different concentrations of ultra-small superparamagnetic iron oxide (USPIO) for 12 h. *Red dots*

represent autophagic lysosomes in imDCs. *Bars* 1 μm . *Blue dots* represent nucleus in imDCs. *Scale bars* 1 μm

as shown in Fig. 6b, incubation with low concentrations (≤ 200 $\mu\text{g/ml}$) of USPIO had almost no effect on IL-10 production in imDCs, while higher concentrations (500 $\mu\text{g/ml}$) of this compound dramatically decreased IL-10 concentrations.

Receptor-mediated endocytosis activity

Receptor-mediated endocytosis activity was assayed by measuring the uptake of FITC-dextran by flow cytometry (Fig. 6c). Exposure to USPIO substantially inhibited the phagocytosis of the antigen. Furthermore, increasing concentrations of USPIO were associated with greater inhibition of endocytosis. Thus, USPIO-treated DCs exhibit significantly lower endocytic capacity for FITC-dextran than untreated imDCs.

USPIO labeling induced autophagy in imDCs

To measure the ability of USPIO to induce autophagy in imDCs, cells were treated with LysoTracker Red, which specifically labels acidic compartments, such as autophagic vacuoles. In addition, to detect the condition of imDCs, cells were treated with DAPI, that stains the nucleus of cells. As shown in Fig. 7, exposure of imDCs to USPIO for 12 h stimulated the production of acidic autophagic lysosomes in a concentration-dependent manner.

Autophagy influenced surface marker expression, T-cell stimulatory capacity, and endocytic capacity of imDCs in vitro

The expression levels of the cell surface co-stimulatory molecules CD86 and HLA-DR were obviously downregulated, while the expression level of CD14 was substantially upregulated, following addition of 3-methyladenine (3-MA, autophagy inhibitor) (Fig. 8a, b). In addition, 3-MA treatment resulted in increased production of IL-10 and decreased T-cell stimulatory capacity by imDCs (Fig. 8c, d). Furthermore, the endocytic capacity of imDCs treated with 3-MA was markedly higher than that of untreated cells (Fig. 8e). Finally, the number of autophagic vacuoles of imDCs treated with 3-MA was markedly less than untreated cells (Fig. 8f). Collectively, these results suggested that autophagy influences the surface marker expression, T-cell stimulatory capacity, IL-10 production, and endocytic capacity of imDCs in vitro. We therefore conclude that autophagy has influences the immunologic function of imDCs.

Discussion

imDCs can act as potent immunosuppressors after allotransplantation by inducing immune tolerance.

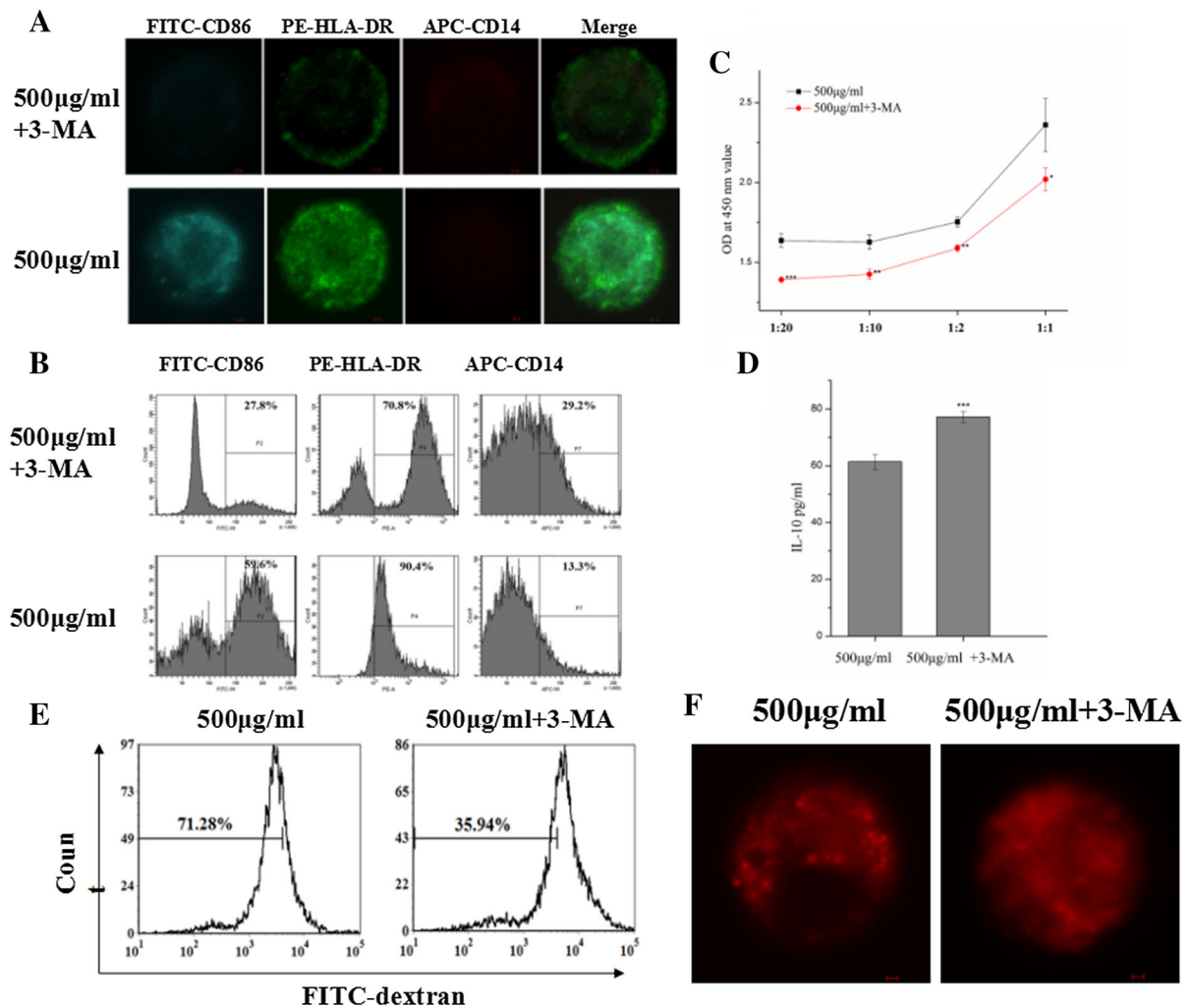


Fig. 8 Autophagy affects the immunity of immature dendritic cells (imDCs). imDCs treated with the autophagy inhibitor 3-methyladenine (3-MA) exhibited deficiencies in immune activity. **a, b** Effects of 3-MA on surface marker expression in imDCs, as determined by **a** laser-scanning microscopy and

b flow cytometry. **c–e** The effects of 3-MA on **c** the stimulation of T-cell proliferation, **d** the production of IL-10, and **e** the uptake of FITC-dextran, as determined by mixed leukocyte reaction (MLR), enzyme-linked immunosorbent assay (ELISA), and flow cytometry analyses, respectively. Scale bars 1 µm

However, we currently lack an effective method for monitoring transplanted imDCs in vivo. MRI has been utilized for tracking cells in vitro and in vivo. In particular, the use of MRI for detecting cells labeled with USPIO particles, which provide strong changes in signals on T2 mapping and are composed of biodegradable, biocompatible iron (Corot et al. 2006), may represent a novel noninvasive method for tracking transplanted cells and tissues in the clinical setting.

In this study, imDCs incubated with 200 µg USPIO/ml for 12 h were readily detectable by MRI after injection in rabbits. This effect on MRI signal

intensity in T2WI images was dependent on both the concentration of iron oxide per cell and the cell density. From these analyses, we concluded that incubation of 10^6 cells/ml with 200 µg USPIO/ml for 12 h yielded optimal detection of imDCs by whole-body MRI scanning. Notably, this signal was sustained for at least 7 days; however, whether the USPIO-loaded imDCs remained immature during this time period remains unclear.

imDCs are efficient in antigen phagocytosis and rarely express the required accessory signals for T-cell activation. Antigen presentation in the absence of co-

stimulatory molecules can lead to clonal T-cell anergy (Lutz and Schuler 2002). Due to the Ag-dependent immunosuppressive ability of imDCs, these cells represent a promising candidate for clinical applications in cell transplantation therapy. Therefore, we next investigated whether USPIO influenced the immunosuppressive capacity of imDCs. We found that cells labeled with high concentrations of USPIO showed upregulation of HLA-DR (MHC class II) and CD86, as well as downregulation of CD14 and IL-10. Moreover, these cells exhibited reduced capacity for receptor-mediated endocytosis and were potent stimulators of allogeneic T-cell proliferation in mixed lymphocyte reactions. Therefore, we concluded that USPIO should be used at concentrations of 200 µg/ml or less for labeling of imDCs.

Nanomaterials, such as, USPIO cause different degrees of autophagy (Man et al. 2010). In this study, autophagy occurred following treatment of cells with USPIO, particularly at high concentrations, for 12 h. Moreover, autophagy influenced the surface marker expression, T-cell stimulatory capacity, IL-10 production, and endocytic capacity of imDCs in vitro. Therefore, we speculated that high concentrations of USPIO could induce imDC maturation, possibly through the autophagy pathway.

In conclusion, strong evidence supporting the use of USPIO-labeled imDCs for MRI-based monitoring of cell tracking in vivo is provided. It remains unclear whether USPIO-loaded imDCs are stimulated to a mature state in vivo after transplantation. If so, future studies will be needed to develop methods to ensure that these cells remain in an immature state after transplantation. Likewise, further studies are still required to confirm whether this potential new treatment modality may have applications in the clinical setting.

Acknowledgements This study was supported by the National Natural Science Foundation of China (81471659 and

81630046). USPIO was kindly provided by Professor Xu Yikai (Department of Medical Imaging Center, Nan Fang Hospital, Southern Medical University, No. 1838 Guangzhou Avenue North, Guangzhou, Guangdong, 510515, China).

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