Journal of Controlled Release 227 (2016) 82–93

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Cellular biodistribution of polymeric nanoparticles in the immune system

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ARTICLE INFO

Article history:
Received 30 August 2015
Received in revised form 29 January 2016
Accepted 5 February 2016
Available online 10 February 2016

Keywords:
Cellular biodistribution
Immune system
Polymeric nanoparticles

ABSTRACT

The biodistribution of polymeric nanoparticles (NPs) is of crucial importance in the development of nanoparticle-based vaccine delivery or immunotherapy for cancer. The purpose of this study was to investigate the kinetics of cellular biodistribution of polymeric NPs in the immune system. Polystyrene (PS) yellow-green nanoparticles (YG-NPs) 500 nm in diameter were intravenously (*i.v.*) injected into the tail veins of mice, and the kinetics of YG-NP biodistribution was followed by harvesting cells at pre-determined time points from various immune organs, including blood, bone marrow, spleen, and lymph nodes and analyzing them by polychromatic flow cytometry. To observe the location of YG-NPs in the spleen after *i.v.* administration, spleens of mice were isolated at 6 h post-injection (*p.i.*), cryosectioned, immunostained, and examined by confocal microscopy. Our data show that the major phagocytosing cells included granulocytes ($B220^{-}CD11b^{+}Gr\text{-}1^{high}Ly\text{-}6C^{low}$) in the blood and bone marrow and B cells ($CD11b^{-}B220^{+}$) in the spleen. The kinetics of the phenotypic analysis suggest the potential trans-differentiation of the $B220^{-}CD11b^{+}Gr\text{-}1^{low}Ly\text{-}6C^{high}$ subset into $B220^{-}CD11b^{+}Gr\text{-}1^{-}Ly\text{-}6C^{-}$ double-negative (DN) cells expressing the F4/80 macrophage phenotype in the blood and CD115 in the bone marrow after treatment with YG-NPs. Based on the microscopic analysis of spleen cryosections, the majority of YG-NPs were located in the marginal zones (MZ) and red pulp of the spleen at 6 h post-injection (*p.i.*), allowing further interaction with MZ macrophages and granulocytes. The data obtained in this study demonstrate the kinetics of biodistribution of polymeric nanoparticles in the blood, bone marrow, and spleen at the cellular level.

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1. Introduction

The development of nanoparticles (NPs) has been applied in biomedical fields of various scientific disciplines, including disease detection and diagnosis, pharmaceutical drug delivery, and vaccine-based immunotherapy [1,2]. Immunization strategies based on nanoparticulate carriers have also received considerable attention in recent years. In these strategies, polymeric nanoparticles were developed as vaccine carriers to deliver antigens to antigen-presenting cells (APCs) and promote anti-tumor immunity, and the nanoparticles were used to enhance the antigen-specific cellular and humoral immune responses [3–6]. This approach has been widely used to elicit an immune response for effective immuno-modulation [7,8]. One of the most critical issues in nanomedicine for promoting effective immunotherapy or vaccination is the biodistribution of nanoparticles *in vivo*. Despite many studies elaborating on nanoparticle research *in vivo* [9], very little effort has been placed on the investigation of biodistribution of polymeric NPs in the immune system at the cellular level. Studies have shown that proteins encapsulated in poly(lactic-co-glycolic acid) (PLGA) nanoparticles are able to activate cytotoxic $CD8^{+}$ T cells and induce potent anti-tumor activities [10], and the biodistribution of gold nanoparticles has been associated with several cell types in the immune system, including B cells, granulocytes, dendritic cells, and T cells [11]. Immunological imprints and differential uptake of polystyrene nanoparticles by macrophages, B cells, and DCs have also been reported [12]. However, there have been few kinetic studies of cellular biodistribution of polymeric nanoparticles in the immune system.

The ability of antigen-presenting cells (APCs) to capture antigens for processing and presentation plays an important role in vaccine targeting of antigens to induce adaptive immunity or immunopotentiation. Classical antigen-presenting cells (APCs) expressing MHC class II molecules, including dendritic cells, B cells, and macrophages, can prime $CD4^{+}$ T cell-dependent immune responses [13]. Polymorphonuclear leukocytes (PMNs), including eosinophils, basophils and neutrophils, on the other hand, are the most abundant cells in the blood and are known to play important roles in inflammation and protection of the host against pathogens [14,15]. Studies have shown that neutrophils not only play roles in innate immunity but also possess the additional function of antigen presentation [16–18]. These cells, along with other cellular effectors for antigen presentation and phagocytosis, were examined in this study to analyze the biodistribution of NPs. We employed fluorescent monodisperse latex beads, which are extensively used in studies of phagocytosis [7], to examine cellular biodistribution of

Abbreviations: APCs, antigen presenting cells; CTL, cytotoxic T lymphocyte; mAb, monoclonal antibody; MMM, marginal metallophilic macrophages; MZ, marginal zone; NPs, nanoparticles; PALS, periarteriolar lymphatic sheath; *p.i.*, post-injection; PMNs, polymorphonuclear leukocytes; PS, polystyrene; YG-NPs, yellow-green nanoparticles.

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http://dx.doi.org/10.1016/j.jconrel.2016.02.011
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polymeric NPs after intravenous (*i.v.*) injection. In view of the potential protection and the immunostimulatory effect induced by nanoparticle-based delivery systems targeting immune cells for vaccination [7,19,20], the kinetics of cellular biodistribution of polymeric nanoparticles was assessed in several immune organs, including blood, bone marrow, spleen, and lymph nodes. The major populations interacting with the YG-NPs, including B cells ($CD11b^-B220^+$), granulocytes ($CD11b^+$ Ly-$6C^{low}$Gr-1^{high}), monocytes ($CD11b^+$ Gr-1^{low}Ly-$6C^{high}$), dendritic cells ($CD11c^+$), and macrophages ($CD11b^+$ F4/80^+), are of greatest interest and were examined.

The spleen, the largest secondary lymphoid organ essential for initiating immune responses to antigens, comprises two major compartments, red pulp and white pulp. The white pulp is composed of the periarteriolar lymphoid sheath (PALS), the follicles, and the marginal zone (MZ), which surrounds the white pulp containing the B cell follicles and the T-cell zone [21]. The metallophilic macrophages of the marginal zone (MZ), an important compartment for antigen processing [21], are highly phagocytic, clean blood-borne antigens of cellular debris, and ingest particulate antigens. CD169 (Siglec-1; Sialoadhesin), the first identified Siglec family member, binds to sialic acids and is involved in cell–cell interactions. CD169 is highly expressed on marginal metallophilic macrophages (MMM) in the MZ of the spleen and subcapsular sinus and on medullary macrophages in lymph nodes [22]. Recent studies have shown that $CD169^+$ metallophilic macrophages are able to activate $CD8^+$ T cells by transferring acquired antigens to $CD8\alpha^+$ dendritic cells for cross-presentation, or directly to the T cells. $CD169^+$ metallophilic macrophages hence play important roles in the generation of T cell responses after vaccination [22,23]. Splenic dendritic cells are potent APCs for initiating T cell responses and inducing the cytotoxic T lymphocyte (CTL) effect, which are crucial in nanoparticle-based vaccination and cancer immunotherapy. To examine the internalization of NPs by the major cell populations in the spleen, splenic sections were prepared after *i.v.* administration of YG-NPs, immunohistochemically stained, and examined by confocal microscopy.

Characterization of the cellular biodistribution of polymeric nanoparticles is not only of critical importance in understanding the cytotoxicity and biodegradability of polymeric nanoparticles, but also crucial in elucidating the mechanism of nanoparticle-based antigen delivery for inducing cross-presentation and the CTL effect, an essential component in the design of nanoparticle-based cancer vaccine therapy. The data presented in this study will be important in understanding the cellular biodistribution kinetics of nanoparticles in the immune system.

2. Materials and methods

2.1. Materials

Fluoresbrite® Yellow Green microspheres/nanoparticles (YG-MPs or YG-NPs) having a mean diameter of 0.50 μm (= 500 nm) in a 2.5% aqueous suspension were purchased from Polysciences Inc. (Warrington, PA, USA). The wavelengths of the fluorescence excitation and emission spectra maxima were 441 nm and 486 nm, respectively. All cell culture media were obtained from Gibco/Life Technologies. A modified Wright–Giemsa staining reagent was obtained from Sigma-Aldrich (St. Louis, MO, USA). Most fluorochrome-conjugated anti-mouse antibodies for flow cytometry were purchased from BioLegend (San Diego, CA, USA) or eBioscience (San Diego, CA, USA), including: APC-Cy7- and PerCP-Cy5.5-anti-mouse CD11b (M1/70), biotin-conjugated and PerCP-Cy5.5 anti-mouse CD11c (N418), biotin-conjugated and APC-anti-mouse CD45R/B220 (RA3-6B2), PE-anti-mouse PE-CD115/CSF-1R (AFS98), purified anti-mouse CD169 (3D6.112), biotin-conjugated and APC-anti-mouse Gr-1 (RB6-8C5), PE-Cy7 anti-mouse Ly6C (HK1.4), and PE anti-mouse F4/80 (BM8) antibodies. Goat anti-rat IgG (H + L)-Cy3 and ChromePure Rat IgG whole molecule were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA), and streptavidin-Dylight 649 was obtained from Biolegend (San Diego, CA, USA).

2.2. Scanning electron microscopy (SEM)

To examine the morphology of Fluoresbrite® Yellow Green nanoparticles (YG-NPs) by SEM, the samples were fixed onto stubs by double-adhesive tape and coated with gold under vacuum with a Hitachi E-1010 ion sputtering device. The morphology of the YG-NPs was examined using a Hitachi S-4800 scanning electron microscope operated at 15 kV.

2.3. Kinetics of cellular biodistribution of polymeric nanoparticles in the immune system

Six- to ten-week-old C57BL/6J mice were intravenously injected with 100 μl 2.5% Fluoresbrite® Yellow Green nanoparticles (YG-NPs) (with an average diameter of 500 nm) into the tail veins. At the predetermined intervals (3, 6, 12, 24, 48, and 96 h *p.i.*), 100 μl of blood was collected by mandibular sampling and mixed with 100 μl of solution from a BD Vacutainer® blood collection tube. Animals were sacrificed at the desired time points, and the bone marrow cells were collected by flushing the femur and tibia with RPMI 1640 medium. Blood cells were then removed using ACK (ammonium-chloride–potassium) lysing buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2EDTA) [24]. Cells from the spleen and lymph nodes were harvested by mashing the tissue between two frosted microscope slides and subsequently passing them through nylon sieves into test tubes. Cells were washed with PBS and centrifuged. The viable cells were counted by trypan blue exclusion, stained with fluorochrome-conjugated mAbs, and analyzed with a BD FACSVerse two-laser (488 nm and 640 nm) flow cytometer (BD Biosciences). Cytometric analysis was performed with the FlowJo 7.6 software and kinetics of cellular uptake of nanoparticles was determined. Biodistribution of nanoparticles was calculated by dividing cell number of the $FITC^+$ subset at each time point by the total number of cells in the blood, bone marrow, or spleen. The percentage of the phagocytic subset, on the other hand, was calculated by dividing the number of $FITC^+$ subset by the total number of each given subpopulation in that organ.

2.4. Sorting and cell staining with Wright–Giemsa stain

C57BL/6J mice were intravenously injected with 100 μl 2.5% Fluoresbrite® Yellow Green (YG) nanoparticles into the tail veins. The

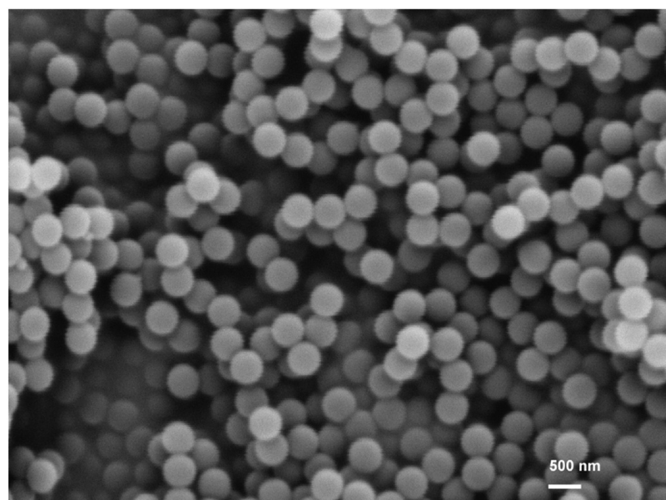

Fig. 1. Scanning electron micrographic image of YG-NPs, showing the mean diameter of approximately 500 nm. Scale bar = 500 nm.

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