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Macrophage silica nanoparticle response is phenotypically dependent

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Phagocytes are important players in host exposure to nanomaterials. Macrophages in particular are believed to be among the "first responders" and primary cell types that uptake and process nanoparticles, mediating host biological responses by subsequent interactions with inflammatory signaling pathways and immune cells. However, variations in local microenvironmental cues can significantly change the functional and phenotype of these cells, impacting nanoparticle uptake and overall physiological response. Herein we focus on describing the response of specific RAW 264.7 macrophage phenotypes (M1, INF-gamma/LPS induced and M2, IL-4 induced) to Stöber silica nanoparticle exposure *in vitro* and how this response might correlate with macrophage response to nanoparticles *in vivo*. It was observed that variations in macrophage phenotype produce significant differences in macrophage morphology, silica nanoparticle uptake and toxicity. High uptake was observed in M1, versus low uptake in M2 cells. M2 cells also displayed more susceptibility to concentration dependent proliferative effects, suggesting potential M1 involvement in *in vivo* uptake. Nanoparticles accumulated within liver and spleen tissues, with high association with macrophages within these tissues and an overall Th1 response *in vivo*. Both *in vitro* and *in vivo* studies are consistent in demonstrating that silica nanoparticles exhibit high macrophage sequestration, particularly those with Th1/M1 phenotype and in clearance organs. This sequestration and phenotypic response should be a primary consideration when designing new Stöber silica nanoparticle systems, as it might affect the overall efficacy.

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1. Introduction

Macrophages play an important role in nanoparticle processing, and are believed to be primarily responsible for uptake and trafficking *in vivo*. Therapeutic capacity and clearance mechanisms in clinically relevant nanomedicines have been linked with macrophage activity [1,2]. Successful nanoparticle clinical candidates should deliver high payloads to target sites, however in clinical applications, more than 95% of the total injected dose of drug ends up being cleared or residing in non-specific clearance organs [3]. Traditionally this has been attributed to the rapid association of these nanomedicines with elements of the mononuclear phagocytic system (MPS) [4]. To some extent this is a function of the opsonization that the particle undergoes when exposed to the blood and recognition of these opsins via the MPS [5], particularly Kupffer cells and splenic macrophages. If macrophages are indeed responsible for high clearance rates, reduced efficacy due to poor delivery of active drug payloads to specific targets and potential inflammatory mediated events are likely due to macrophage nanoparticle recognition and subsequent processing. If delivery vehicles were designed such that they avoided or harnessed this recognition system, payload delivery and subsequent efficacy could be significantly enhanced. However, in order to design appropriate systems one needs to understand nanoparticle macrophage interactions at a cellular level and how this might impact complete physiological responses.

Rational design of nanomedicines with specific macrophage interactions can be difficult, as local microenvironmental factors and cues *in vivo* can alter the phenotype and differentiation state of

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macrophages. This can drastically influence how they interact with the surrounding environment including nanomaterial processing and subsequent biological responses [6,7]. We currently lack a full understanding of which macrophage phenotypes are important in the discovery and uptake of nanoparticle systems. A more complete understanding of this would be valuable in the design of new drug delivery systems, as this knowledge could guide nanomedicine design to achieve higher specificity towards desired targets.

The Th1/Th2 paradigm introduced decades ago [8,9], illustrates different activation states and different macrophage phenotypes, which could help identify the macrophage phenotype which discovers and uptakes nanoparticles [10,11]. M1 macrophages are generally considered janitorial cells, responsible for clearing foreign materials, pathogens and, potentially, nanomaterials. *In vitro* M1 cells are induced by IFN-gamma and are generally characterized by high iNOS, IL-12 release, and high expression of CD80. In general, M1s represent a Th1 response and, most likely, an inflammatory-mediated response [12]. In contrast, a Th2 state generally has an up-regulation of alternatively activated (or M2) cells. M2 cells are considered wound healing cells, inducing basement membrane breakdown, angiogenesis and general tissue repair. These cells are induced by IL-4 *in vitro* and are characterized by high arginase, IL-10 release and high expression of CD206 [12]. The Th1/Th2 paradigm is a simplified immunological model system. Many macrophage phenotypes *in vivo* lie in between these two states and may even reside outside of them, making it extremely difficult to identify these cells *in vivo* [7]. However, the Th1/Th2 model system does represent the complex *in vivo* biological environment more accurately than traditional unpolarized macrophage models. This model may help explain whether a specific phenotype is responsible for nanomaterial processing and/or biological response and, if so, whether we can select for specific target phenotypes to harness therapeutic responses or reduce toxicity.

Evidence suggests variations in nanomaterial properties can alter macrophage uptake and initiate either Th1 or Th2 responses [13]. Clinically, environmental exposure to nanomaterials correlates directly to induced autoimmune disorders such as scleroderma and rheumatoid arthritis [14,15]. These disease states are generally classified as a Th1 response, suggesting the involvement of M1 phenotypes [16]. In line with these findings, silica and titanium nanoparticles have been shown to induce M1 phenotypes *in vivo*, significantly up regulating inflammatory mechanisms [17,18]. However, a recent study revealed that alternatively activated macrophage M2 phenotypes *in vitro* and *in vivo* took up 300 nm particle replication in nonwetting templates (PRINT™) nanoparticles to a higher extent than M1 phenotypes [19]. In contrast, 200–600 nm poly(lactic acid) particles induced a Th1 response while 2–8 μm particles showed a Th2 response [20]. Poly(lactic-*co*-glycolic) acid (PLGA) nanoparticles showed a Th1 response even after priming *in vivo* for a Th2 response [21]. Interestingly, environmental exposure to crystalline silica has also been linked to silicosis, a disease characterized by fibrosis and a general Th2 response [22]. Injection of silica has also shown increased levels of IgG2a and IgE within serum indicating increased presence of antibodies [23], an M2 mediated adjuvant-response. However, research has shown an inability to induce an M2 activation state after macrophage incubation with superparamagnetic iron oxide particles and, to a limited degree, this effect is observed with silica [24]. In general, evidence suggests nanoparticle characteristics and testing environments can drastically affect the uptake and response within macrophage systems.

A correlation should be drawn between variations in nanomaterial properties and how these properties interact with the biological environment to induce either a Th1 or Th2 response and a basic understanding of which macrophage phenotypes could be responsible for these responses. We believe that Stöber silica nanoparticle systems interact with the biological environment and initiate a Th1 phenotypic response, as a function of M1 uptake *in vitro* and *in vivo*. Macrophage phenotype expression is dependent on disease state; understanding the phenotype that specific nanomaterial characteristics target will help to derive a better functional design platform for specific biomedical applications.

2. Materials and methods

2.1. Particle synthesis and characterization

Spherical silica nanoparticles were prepared by previously reported modified Stöber methods [25,26]. All particles were fluorescently labeled with fluorescein isothiocyanate (FITC) to assess cellular uptake. The constructs were sterilized by dry autoclaving. Transmission electron microscopy (TEM) images were taken on a Phillips TECHAI F2 (Hillsboro, OR) at an accelerating voltage of 80 kV. TEM samples were created by evaporating droplets of particles suspended in deionized water off copper grids. After micrograph collection, nanoconstruct size was measured utilizing Adobe Photoshop's pixilation ruler measurement tool (Adobe, San Jose, CA). At minimum the sizes of 300 particles of each type were measured. Particle zeta potential of SNPs dispersed in DI water at a concentration of 1.0 mg/ml was measured using a Malvern Instruments Zetasizer Nano ZS (Westborough, MA). SNPs (50 or 25 mg/ml) were sonicated, vortexed and the final particle dispersions were prepared immediately before use from common stock in culture medium and vortexed before application to the culture cells. All particles were tested for endotoxin levels prior to cellular incubation; levels were below FDA recommended .05 EU/mL.

2.2. In vitro methods

2.2.1. Cell culture

RAW 264.7 murine macrophages were obtained from ATCC (Manassas, Virginia) and maintained in RPMI media supplemented with 10% FBS, at passage numbers 5–25. Cell cultures were incubated at 37 °C in 5% CO_2 and 95% humidified air and kept in logarithmic phase of growth throughout all experiments, never reaching full confluence.

2.2.2. Cell polarization and confirmation

Cells were seeded and allowed to adhere overnight at 37 °C in 5% CO_2. The following day the cells were treated with a M1 cocktail that consisted of LPS (100 ng/mL) and IFN-gamma (300 units/mL), a M2 cocktail of IL-4 (10 units/mL) or an unpolarized cocktail with no additives (Sigma–Aldrich, St. Louis, MO). All cocktails were diluted in fresh media. The cells were incubated for 18 h to obtain sufficient polarization. To confirm polarization, IL-10 and IL-12 were quantified via BD Cytokine Flow Cytometry protocol and colorimetrically for arginase and nitric oxide (described below).

2.2.3. Arginase and nitric oxide evaluation

Following cellular polarization in 96 well plates, cells were treated for 24 h with a range of silica nanoparticle concentrations (5–250 μg/mL). Classen et al.'s colorimetric protocols for the Griess reagent (detection of nitric oxide) and a urea assay (detection of arginase) were followed [27].

2.2.4. Cellular proliferation

Following polarization, cells were exposed to a range of concentrations (5–250 μg/mL) of silica nanoparticles for 72 h in RPMI media supplemented with 10% FBS. Relative cell viability was assessed by utilizing a water-soluble tetrazolium salt, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt], the key component in the Cell Counting Kit-8 from Dojindo Molecular Technologies, Inc. (Rockville, Maryland).

2.2.5. Cell uptake, visualization and quantification

The uptake of silica nanoparticles by cultured cells was visualized by confocal microscopy. Cells were grown on 24 well imaging plates at a density of ~9000 cells/cm^2 polarized and incubated for 24 h with 37.5 μg/ml FITC labeled silica nanoconstructs in RPMI media supplemented with 10% FBS. After incubation, cells were fixed with 4% formalin in PBS. Cell nuclei were stained with 2.5 μM 4′,6-diamidino-2-phenylindole (DAPI) according to the manufacturer's protocol. For CLSM (Olympus FluoView® FV1000, Olympus America Corp., Center Valley, PA), the intensity of the laser beam and the photodetector sensitivity were kept constant in order to compare the relative fluorescence intensities between experiments. Z stacks were collected and used for 3D reconstruction and visualization of intracellular particle localization. All image acquisitions and analyses were performed using FluoView 2.0 software.

Flow cytometry was used to quantify the amount of nanoparticle uptake. Cells were grown on 12 well plates at a density of ~15,000 cells/cm^2 polarized and incubated with 37.5 μg/ml FITC labeled silica nanoconstructs in RPMI media supplemented with 10% FBS for 24 h. Following incubation, cells were scraped to obtain a single cell suspension. Cells were suspended in PBS containing 1% BSA and analysis

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