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The nanomaterial-dependent modulation of dendritic cells and its potential influence on therapeutic immunosuppression in lupus

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Abstract

Targeting dendritic cells with nanoparticles is an attractive modality for instigating immunity or inducing immunosuppression. An important aspect of successful delivery of antigen and immune modulators to these cells is the efficacy of nanoparticle internalization, which can dictate the strength and robustness of immune responses; optimizing particulate uptake is thus key. We compared the internalization of two nanoparticulate platforms: a vesicular “nanogel” platform with a lipid exterior, and the widely-used solid biodegradable poly(lactic-co-glycolic acid) (PLGA) system. We found that nanogels were more effectively internalized by dendritic cells *in vitro*, as demonstrated by fluorescent tracer measurements. Additionally, the magnitude of dendritic cell immunosuppression achieved by nanogels loaded with mycophenolic acid, an immunosuppressant, was greater than similarly drug-loaded PLGA. Although both types of particles could mitigate the production of inflammatory cytokines and the up-regulation of stimulatory surface markers, nanogels yielded greater reductions. These *in vitro* measurements correlated with *in vivo* efficacy, where immunosuppressive therapy with nanogels extended the survival of lupus-prone NZB/W F1 mice whereas PLGA particles did not. Our results highlight the importance of material on nanoparticle uptake by dendritic cells, which impacts the quality of therapeutic immunosuppression.

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1. Introduction

Dendritic cells are crucial mediators of the immune system, where they instigate or suppress immunity by presenting antigen and providing receptor-ligand interactions and cytokines that lead to T and B cell activation [1]. As such, they have been widely studied for their therapeutic potential in vaccination [2], cancer immunotherapy [3, 4], and mediating immunosuppression and tolerance [5–7]. An active area of dendritic-cell based therapies has been to target cells with particles that contain antigen, stimulatory compounds and ligands, or immuno-suppressants [8–15]. The observation that antigen presentation by dendritic cells is more effective when antigen is particulate-bound, opposed to soluble [16], demonstrates the potency of particle uptake. The internalization of particles can be augmented with ligands for CD11c [17], DEC-205 [18], other lectins [19–21], or toll-like receptors [22]. This fundamental need to maximize the interaction of particles with dendritic cells is an important design consideration for nanomaterials.

We previously reported the use of a nanogel-based particle for treatment of lupus [23], an autoimmune disease characterized by systemic inflammation and organ damage. Efficacy was accomplished, in part, by attenuating the inflammatory and stimulatory capacity of dendritic cells [23]. Nanogels consisted of an exterior lipid membrane and a gel-like interior primarily of poly(ethylene glycol), and were loaded with the immunosuppressant mycophenolic acid (MPA). While nanogels were efficacious, we questioned if other classes of nanoparticles could be effective, or if there is a property of nanogels that made them unique. Many types of materials could be compared [24], but we focused specifically on biodegradable, solid nanoparticulate matrices made of poly(lactic-*co*-glycolic acid) (PLGA) because of its popularity in a wide-range of drug and antigen delivery applications [25], including the targeting of dendritic cells [26].

This study provides direct comparisons of PLGA nanoparticles with our previously reported data of nanogels mediating immunosuppression in dendritic cells [23]. We hypothesized that the therapeutic efficacy of nanoparticles is critically linked to their internalization by dendritic cells. We compared the ability of PLGA and nanogel particles to be internalized, and how well MPA-loaded particles could induce immunosuppression among dendritic cells and *in vivo* in lupus.

2. Materials and Methods

2.1. Nanoparticle synthesis and characterization

Nanoparticles were made and characterized according to previously described protocols [23, 27]. Briefly, nanogels were made with liposomes extruded from a lipid mixture of 1:2:0.1 molar ratio of cholesterol: phosphatidylcholine: 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]. Liposomes were lyophilized, and then rehydrated with a mixture of acrylated lactic acid-poly(ethylene glycol)-lactic acid, MPA complexed in non-methylated β -cyclodextrins, and Irgacure 2959. The particles were cured under UV light, rinsed, and centrifuged. Nanogels were functionalized with avidin using sulfo-*N*-hydroxysuccinimide/1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (sNHS/EDC). For PLGA particles, MPA was dissolved with PLGA in ethyl

acetate, and emulsified with poly(vinyl alcohol) and avidin-palmitate using a sonicator probe. PLGA particles were subsequently hardened, washed, and then lyophilized. Biotinylated poly(ethylene glycol) was added to PLGA particles at a ratio of 1.33 μg per mg particle prior to use in experiments.

The MPA amount in particles was measured by fluorescence from the supernatant of dissolved particles. Particle size and molarity (concentration) was measured using Nanosight single particle tracking.

2.2. BMDC culture

BMDCs were cultured from marrow harvested from the femurs and tibias of Balb/c or C57BL/6 mice. BMDCs were cultured with 10 ng/mL GM-CSF and 5 ng/mL IL-4 in complete media (RPMI 1640 media containing 10 mM HEPES, 1 mM L-glutamine, 100 U/mL penicillin, 100 μg /mL streptomycin, 50 μM β -mercaptoethanol, and 10 % heat inactivated FBS) at 37 °C. Media was changed every 2–3 days.

2.3. Nanoparticle internalization studies

Nanogels and PLGA particles were titrated with biotin-fluorescein so that they had similar amounts of fluorescence, as measured by flow cytometry. Seven-day old BMDCs were incubated with particles (not containing MPA) at 37 °C, washed, and then analyzed for the percentage of fluorescein-positive CD11c⁺ cells by flow cytometry. For inhibitors, cells were incubated at 4 °C or treated with 10 μg /mL cytochalasin D or 10 mM of randomly methylated β -cyclodextrin. For confocal microscopy, BMDCs treated with particles were washed, incubated onto charged slides, and then stained with phalloidin-Alexa568 and TO-PRO-3.

2.4. Functional assays for BMDCs

BMDCs from Balb/c mice were treated with MPA-loaded particles beginning on day 1 of culture, with the media changed every 2 days with fresh immunosuppressant. On day 6 of the culture, 50 ng/mL LPS was added for 18 h. The supernatant was subsequently assayed for cytokines by ELISA, and cells surface markers were measured by flow cytometry. For mixed lymphocyte reactions, purified CD4 T cells from C57BL/6 mice were co-cultured with irradiated BMDCs. T cell proliferation was measured by CFSE dilution.

2.5. Lupus animal studies

NZB/W F1 female mice (The Jackson Laboratory, stock #100008) were treated with weekly intraperitoneal injections of 0.625 mg MPA/kg weight animal. For prophylactic studies, treatment was initiated at 18–20 weeks of age. Proteinuria (> 300 mg/dL) was assayed with Uristix, using urine acquired by bladder compression. For studies beginning at later-stage disease, mice were placed on therapy once they had two consecutive days of proteinuria.

2.6. Statistical analysis

All statistical comparisons were performed on GraphPad Prism (version 5.03). Survival was analyzed with log-rank (Mantel-Cox) tests. Two-tailed t-tests and ANOVA with

Bonferonni's post test were performed as indicated. A p-value of 0.05 or less was considered statistically significant.

2.7. Study approval

All animal studies were performed with the approval of the Yale University Institutional Animal Care and Use Committee (New Haven, CT)

3. Results

3.1. Fabrication and characterization of nanoparticles

Nanogels and PLGA nanoparticles encapsulating MPA were prepared using previously reported methods [23, 27]. Nanogels contain an aqueous poly(ethylene glycol)-*co*-lactic acid (PEG-PLA) core where MPA, which is hydrophobic, is solubilized by complexation to non-methylated β -cyclodextrins (Figure 1a). These particles have a greater drug loading than conventional liposomes (Table 1). The nanogels possess a lipid membrane fabricated from a mixture of 2:1 molar ratio phosphatidylcholine and cholesterol, and approximately 3.2 % (mol/mol) amount of PEGylated DSPE lipid. In contrast, PLGA particles are a solid matrix formed from the emulsion of polymer and MPA (Figure 1b).

Despite their different composition and formulation methods, nanogel and PLGA particles had similar drug loading (w/w basis) with approximately $5.06 \pm 0.62 \mu\text{g MPA/mg nanogel}$ and $6.17 \pm 0.33 \mu\text{g MPA/mg PLGA}$ (mean $\pm$ standard deviation of one representative lot) (Table 1); similar hydrodynamic diameters, $187 \pm 29 \text{ nm}$ for nanogels and $171 \pm 46 \text{ nm}$ for PLGA (Table 1); and similar drug release profiles, with nearly 100 % of drug being released within 24 h (Figure 1c). The endotoxin content of particles was measured by limulus assay, and 0.06 endotoxin units per mg of particle was detected (Supplementary Figure 1).

3.2. Comparison of nanogel and PLGA nanoparticle internalization by dendritic cells

The efficacy of nanotherapies that target dendritic cells is contingent on particle internalization. This consideration has motivated the design of strategies that can increase transport to cells, and the use of targeting ligands or cell penetrating peptides is commonly employed [10, 18, 21, 22, 28, 29]. In contrast to such ligand-based targeting, we explored if there could be intrinsic differences in lipid-based vesicles or solid polymeric particle uptake due to material composition. In order to perform this comparison quantitatively, we used flow cytometry to measure the cellular uptake of fluorescent nanoparticles. Avidin was attached to the surface of particles, which permitted the stable attachment of biotinylated-fluorescein. Particles were then functionalized with biotin-fluorescein so that their mean fluorescent intensity was equivalent (Supplementary Figure 2a, b). We used this method for creating fluorescent particles, as opposed to embedding dye within the particle matrix, because the avidin-biotin linkage is highly stable and would thus better ensure that cell-associated fluorescence was from particles and not from unbound, fluorescent dye released from it (Supplementary Figure 2c). Bone marrow derived dendritic cells (BMDCs) were incubated with varying concentrations of particles for 1 h or 24 h at 37 °C in cell culture medium, and the percentage of fluorescence-positive cells were quantified. We found that nanogels were more readily internalized than PLGA particles, with the half-maximal

effective concentration (EC_{50}) of nanogels to yield 50 % of cells fluorescent positive nearly 100-fold less (Figure 2a). This trend was similar after 1 h and 24 h (Supplementary Figure 3) of incubation. Confocal microscopy confirmed that both nanogels and PLGA particles were internalized and not merely adhered to the cell surface (Figure 2b).

To compare the mechanism of particle uptake, we repeated these experiments with various inhibitors that block different pathways to cellular internalization. Incubation of particles with cells at 4 °C partially reduced the extent of internalization for both nanogels and PLGA (Figure 2c). PLGA but not nanogel internalization was susceptible to 5 μ g/mL cytochalasin D (Figure 2d), which disrupts actin-mediated phagocytosis [30]. Methyl β -cyclodextrin, which sequesters cholesterol and inhibits caveolae-based endocytosis [30], had no effect (Figure 2e).

3.3. Evaluation of MPA delivery by nanogels or PLGA nanoparticles on dendritic cell phenotype

We next investigated the consequences of differential particle uptake by measuring the stimulatory capacity of BMDCs. BMDCs were treated with equivalent doses of drug in particles, 125 μ g/mL MPA, and then challenged with 50 ng/mL of lipopolysaccharide (LPS). MPA caused reductions in cytokine (Figure 3) and surface marker expression (Figure 4), but the extent of suppression was greater with nanogels than PLGA. The levels of TNF- α , IL-12, and IFN- γ (Figure 3a–c) were more reduced with nanogels, though IL-1 β (Figure 3d) reductions were similar. Furthermore, treatment with nanogel-MPA caused greater reductions in the percentage of CD11c⁺ BMDCs that were positive for CD40, CD80, CD86, MHC class I, and MHC class II (Figure 4). This immature phenotype was not associated with increased cell death with either particle, as measured by propidium iodide staining (Supplementary Figure 4).

BMDCs treated with nanogels and PLGA were less potent stimulators of allogeneic CD4 T cell proliferation in a mixed lymphocyte reaction (Supplementary Figure 5), and supported the partial expansion of T regulatory (Treg) cells (Supplementary Figure 6). However, the magnitude of these effects on CD4 T cells was comparable between both types of particles.

3.4. Impact of nanoparticle platform on MPA-based treatment of lupus

Dendritic cells contribute to lupus pathology by augmenting autoreactive T and B cell responses [31] and contributing to the inflammatory milieu that leads to tissue damage [32–34]. We next examined if the graded immunosuppression of BMDCs observed between nanogels and PLGA would correlate to their efficacy *in vivo*. To test this, New Zealand Black $\times$ White (NZB/W) F1 female mice, a lupus-prone model, were administered weekly intraperitoneal doses of 0.625 mg MPA per kg weight of animal (mpk) delivered in nanogels, PLGA, or saline buffer. We previously demonstrated that nanogels were effective in extending survival by 3 months as a prophylactic, and by 2 months when administered after the onset of severe proteinuria (> 300 mg/dL) [23]. In contrast, we found that PLGA did not extend the mean survival as a prophylactic (Figure 5a, b) or after the onset of proteinuria (Figure 5c, d). Urinalysis was consistent with this trend, as MPA-loaded

nanogels could suppress the development of proteinuria whereas MPA-loaded PLGA did not (Supplementary Figure 7).

4. Discussion

Targeting dendritic cells is a key objective of many drug delivery vehicles that modulate immunity. In this manuscript, we demonstrated that material composition affects the extent to which nanoparticles are internalized, the degree of immunosuppression induced in dendritic cells, and the efficacy of autoimmune nanotherapies, as exemplified with lupus, a prototypical inflammatory disease whose pathology involves antigen-presenting cells. A nanogel with a lipid membrane exterior was internalized more efficiently than nanoparticles made of a solid matrix of PLGA. We reason that the lipid surface of the nanogels, compared to that of PLGA, is more amenable to fusion with cell membranes and thus permits greater uptake. This is consistent with our observation that treatment of BMDCs with cytochalasin D, which interferes with actin-mediated endocytosis but not lipid membrane fusion [35], inhibited the internalization of PLGA particles but not nanogels. This biophysical interaction is likely similar to how liposomes and other fatty acid-based vesicles can readily fuse with cells [35–37].

Some nanomaterials are proinflammatory by virtue of their composition, and their use for immunosuppressive contexts requires particular scrutiny as they could hypothetically worsen disease. For example, carbon nanotubes and silica particles have been shown to elicit production of IL-1 β , IL-6, or TNF- α [38–41], and some reports describe materials as having an inherent “adjuvant effect” that can modulate their usefulness in vaccine or anti-inflammatory applications [42, 43]. However, we reason that any immunosuppressive or immunostimulatory potency of the nanogel and PLGA vehicle composition had minimal contributions in our experiments. Although BMDCs treated with empty nanogels produced greater IFN- γ and IL-12 than empty PLGA particles, the encapsulation of MPA in nanogels still yielded better reductions of these cytokines and TNF- α following LPS challenge. Moreover, empty nanogel and PLGA particles did not affect surface marker expression of CD40, CD80, CD86, MHC class I, and MHC class II.

Despite the differences between PLGA and nanogels, we emphasize that our findings do not exclude the successful use of PLGA for immunosuppression. We have demonstrated in allograft transplantation that PLGA nanoparticles were able to extend skin graft survival and suppress the stimulatory capacity of dendritic cells, albeit with much greater doses (approximately 100-fold more MPA per kg animal per week) and increased dosing frequency over a shorter study time (approximately five days of treatment spread across two weeks) than what was performed in our lupus studies [27]. We believe that the higher dosage and shorter time course in transplant studies likely made it possible for PLGA to be successful in delaying transplant rejection. Thus, a caveat is that it may be possible to compensate for the poor uptake of a nanoparticle by increasing the dose. Further studies regarding the dose-response effect among different platforms and their efficacy in immune suppression could address this point more fully.

5. Conclusions

Our results demonstrate how the material composition of a nanoparticle can affect its capacity to be internalized by dendritic cells and treat autoimmunity. These studies comprehensively compare two different platforms and elucidate mechanisms for how they may function in the emerging field of nanotherapy for autoimmune disease. Although the ability of dendritic cells to internalize particles is well-known, we have shown that there can be profound, formulation-dependent differences that influence the success of immunomodulatory therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Description of nanoparticles. (A) Nanogels contain a hydrophilic interior core composed of photo-linked poly(lactic acid-co-ethylene glycol-co-lactic acid). A lipid membrane surrounds the nanogel. MPA was primarily complexed within β -cyclodextrins located in the core, but may also partition within the lipid membrane. (B) PLGA particles are a solid matrix, with MPA embedded throughout the matrix. Avidin-palmitate was incorporated onto the surface of PLGA particles to permit the attachment of biotinylated poly(ethylene glycol). (C) The release of MPA from particles in 10 % FBS at 37 °C. Results are representative of three experiments, where data showing MPA release from nanogels previously reported in reference [23]. Error bars represent the standard deviation. Drug release profiles were not significantly different by two-tailed t-test.

Figure 2.

Internalization of nanoparticles by BMDCs. (A) BMDCs were treated with fluorescein-labeled nanogels or PLGA particles for 1 h at 37 °C. The extent of particle uptake was measured by flow cytometry. (B) Confocal imaging of BMDCs treated with particles for 1 h at 37 °C. Green is fluorescein-labeled particles, blue is TO-PRO-3 dye (specific for DNA), and red is actin-specific phalloidin-Alexa568. Scale bar represents 50 μm. (C–E) Internalization studies were conducted at (C) 4 °C, or with (D) cytochalasin D, or (E) methyl β-cyclodextrin. Experiments were repeated 2–3 times. * indicates $p < 0.05$ or less by two-tailed t-test (for (A)) or by ANOVA with Bonferroni post-test (for (C–E)).

Figure 3.

MPA-loaded nanogels suppressed dendritic cell inflammatory cytokine production better than PLGA nanoparticles. BMDCs were treated with 125 ng/mL MPA, stimulated with lipopolysaccharide (LPS) for 18 h, and then assayed for production of (A) IFN- γ , (B) IL-12, (C) TNF- α , and (D) IL-1 β . * $p < 0.05$ or less by ANOVA with Bonferroni post-test. Error bars are the standard error measurement, with results representing the average of three independent experiments. The data for groups with nanogel and control treatments was previously reported in reference [23].

Figure 4.

MPA-loaded nanogels attenuated the up-regulation of dendritic cell surface markers more effectively than PLGA particles. Dendritic cells were treated with 125 ng/mL MPA and then stimulated with LPS. Nanogel treatment resulted in greater reductions of (A) CD40, (B) CD80, (C) CD86, (D) MHC class I, and (E) MHC class II expression compared to PLGA treatment. * $p < 0.05$ or less by ANOVA with Bonferonni post-test. Error bars are the standard error measurement, with results representing the average of three independent experiments. The data for groups with nanogel and control treatments was previously reported in reference [23].

Figure 5.

Treatment of lupus with MPA loaded in nanogels was more effective than in PLGA particles. NZB/W F1 mice, a lupus-prone model, were treated with weekly doses of 0.625 mg MPA per kg animal beginning at 18–20 weeks of age, with (A) Kaplan-Meier survival curves and (B) the mean survival age shown. The sample size is 9–18 animals per group. In addition, therapy was administered after the onset of proteinuria, with corresponding (C) survival curves and (D) mean survival time shown, with a sample size of 4–11 animals per group. In (B) and (D), the log-rank statistical comparisons are indicated, with * representing $p < 0.05$ or less. The data for groups with nanogel and control treatments was previously reported in reference [23].

Table 1

Characterization of nanoparticles. Values are from one representative lot of particles, which are similar to all batches of particles used in this manuscript (n = 26 for nanogels; n = 18 for PLGA particles). Error bars represent the standard deviation.

Type of nanoparticle	nanogel	liposome	PLGA
MPA loading ($\mu\text{g}/\text{mg}$)	5.06 ± 0.62	2.03 ± 0.14	6.17 ± 0.33
Encapsulation efficiency (%)	3.79 ± 0.46	1.37 ± 0.09	6.17 ± 0.33
Hydrodynamic diameter (nm)	187 ± 29	180 ± 38	171 ± 46