Published in final edited form as:

Nano Life. 2010 September 1; 1(3-4): 239–250. doi:10.1142/S1793984410000171.

ANALYSIS OF DENDRITIC CELL STIMULATION UTILIZING A MULTI-FACETED NANOPOLYMER DELIVERY SYSTEM AND THE IMMUNE MODULATOR 1-METHYL TRYPTOPHAN

KEVIN P. NIKITCZUK*, EDMUND C. LATTIME†, RENE S. SCHLOSS*, and MARTIN L. YARMUSH*,‡

*Department of Biomedical Engineering, Rutgers University, 599 Taylor Road Piscataway, New Jersey, USA

†The Cancer Institute of New Jersey, Departments of Surgery and Molecular Genetics Microbiology, and Immunology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, New Brunswick, New Jersey, USA

Abstract

Dendritic cells (DCs) play a pivotal role in immune modulation. Therefore, understanding and regulating the mechanism of DC activation is paramount for functional optimization of any immunotherapy strategy. In particular, the paradoxical ability of DCs to secrete the immune suppressive enzyme indoleamine 2, 3-dioxygenase (IDO) and the suppressive cytokine IL-10 during the course of, and in response to, stimulation is of great interest. 1-Methyl-Tryptophan (1 MT) is a known inhibitor of IDO and has thus been administered in numerous *in vitro* and *in vivo* systems to block IDO activity. However, the effect 1 MT has on DCs beyond inhibiting IDO, especially in therapeutic models, has rarely been analyzed. In the current study, we have administered 1 MT via a nanopolymer-based delivery system in conjunction with an antigen (ovalbumin, OVA) and an adjuvant (CpG motif DNA) to determine both the effects of 1 MT on DCs and the resulting efficacy of the polymer-based treatments. 1 MT delivery alone, either via the polymer-based delivery vehicle or dissolved in solution, induced no significant change in DC activation as measured by surface expression of CD80, CD86, and MHCII and several secreted products such as IL-12. These same factors were upregulated however, when 1 MT was delivered in conjunction with OVA and CpG. Although soluble delivery of these components increased the levels of expression and secretion of key proteins, a differential effect of DC stimulation was seen as a result of the polymer delivery system. The T cell suppressive IL-10 secretion was lower with the polymer-based treatments and IL-12 immune-enhancing secretion was increased when 1 MT was supplemented into the polymer system. As a result, including 1 MT in the polymers along with OVA and CpG was seen to have additional effects on DC stimulation and was able to shift DCs to a state more indicative of inducing a Th1-type response.

Keywords

Dendritic cells; DC; PLGA; 1-methyl-tryptophan; 1MT

1. Introduction

Dendritic cells (DCs) play a powerful role in immune system modulation and interact in various ways with many T cell subsets. CpG motif DNA or lipopolysaccharide (LPS) from bacteria stimulate DCs to upregulate a number of critical immunomodulatory molecules, including, for example, increased expression of CD80 and CD86 and increased secretion of IL-12 and TNF- α .^{1,2} DCs interact with specific T cell subsets to initiate or augment antigen-specific immune responses. However, binding of CD80(B7-1)/CD86(B7-2) to different receptors on T cells, specifically CD28 or CTLA-4, result in differential stimulation of T cells. Interaction with CD28 results in an expansion of T cells,^{3,4} while binding of CTLA-4 results in an attenuated T cell response.^{5,6} These divergent responses are essential for the regulation of T cell activity and in coordination with the cytokines secreted by DCs, aid in maintaining T cell homeostasis. Hence, the classic role of DC activation of T cells for the purpose of killing and removing pathogenic cells is now known to be only a partial immunomodulatory role of these potent APCs.

It is known that DCs can directly facilitate and induce immune suppression, for example via the CTLA-4 pathways.^{7,8} DCs can stimulate a subset of T cells now termed T regulatory cells (Tregs), originally identified as a subset of lymphocytes that provided a degree of tolerance.⁹ It is believed that both CD4+ and CD8+ Tregs exist, each performing distinct yet complementary functions.¹⁰ DCs can interact and activate both Treg subsets,^{11,12} typically mitigating the T cell milieu. Studies have begun to investigate methods of blocking this immune suppression, via antibodies against Tregs, which are proving to be effective therapeutic supplements.^{13,14} Biochemical enzymes and pathways are also potential avenues for Treg blockade. The indoleamine 2, 3-dioxygenase (IDO) enzyme pathway has been described as an important tolerogenic target in tumor progression, suppressing T cell immunity and increasing Treg based immune suppression.¹⁵ Stimulated DCs increase their levels of IDO production and can directly inhibit T cell proliferation and activate Tregs, minimizing effector T cell activity.¹⁶⁻¹⁸ With this clear IDO mediated balance within DC activation, there is a need to minimize its activity. Multiple methods have been used to inhibit IDO, the most popular being 1-methyl-tryptophan (1 MT) supplementation, which leads to a decrease in Treg suppression and subsequent increase in effector T cell activity.¹⁹ However, the literature is sparse in details concerning the cellular and biochemical effects 1 MT has on DCs.

Considering the power and versatility of DCs with regard to their ability to modulate T cell activity, understanding how 1 MT affects DC subsets becomes increasingly significant. A major goal in DC therapy is to induce antigen-specific immunity while inhibiting IDO activity, providing a strong effector T cell response, while inhibiting Tregs. In the current study, we aimed to develop an efficient and quantitative method to deliver 1 MT in conjunction with antigen and a known adjuvant, CpG motif DNA, to bone marrow-derived

DCs. The ultimate goal is to provide antigen-specific stimulation, while minimizing and/or inhibiting the suppressive capabilities of DCs. However, in such a process, 1 MT can have collateral effects on DC activation. We sought to elucidate the effects, either negative or positive, of 1 MT treatment on DCs by delivering 1 MT via a polymer delivery vehicle. DCs were treated with 1 MT alone or in conjunction with stimulatory factors and analyzed for secreted products and their cell surface expression of key proteins responsible for costimulation. Our results indicate 1 MT may be playing a larger role in DC stimulation than was previously thought.

2. Materials and Methods

2.1. PLGA polymer characterization

Polymer vesicles were generated using a water/oil/water double emulsion method,²⁰ with the following modification. In brief, 0.1 g of poly(lactic-co-glycolic acid) (Sigma-Aldrich, MW 7–17 kDa, 50:50 ratio) was dissolved in 0.4 mL of chloroform (Sigma-Aldrich) using a sonicator water bath to expedite dissolution. Aqueous phase, containing OVA (ovalbumin, Sigma-Aldrich), CpG DNA (ODN-1668 TCCATGACGTTCTGATGCT, phosphorothiated, IDT) and/or 1 MT (1-Methyl-DL-tryptophan, Sigma-Aldrich), or Dextran-TMR (40 kDa, Invitrogen) was added to the organic PLGA mixture at 0.05 mL per 0.4 mL of chloroform/PLGA. To create the primary emulsion, a microtip sonicator (Branson Ultrasonics) was placed in the first aqueous and organic phase and ran at 60% magnitude for 5-s pulses, followed by 30-s pauses, repeated for 4 cycles. To create the secondary emulsion, the resulting primary emulsion was placed in 2 mL of a 9% PVA solution (Sigma-Aldrich, MW 31–50 kDa, 87%–89% hydrolyzed) and subjected to identical sonication as the primary emulsion. To finalize the secondary emulsion, all of secondary emulsion was added drop-wise to an 8 mL bath of a 9% PVA solution under constant stirring. Solution was kept under constant stirring for at minimum 5 h to evaporate the organic solvent. Final product was collected by centrifuging at $17,500 \times g$ for 2 h, washed twice with dH₂O, resuspended in 2% sucrose and lyophilized.

Particle size was determined by dynamic light scattering using a Zetasizer (Malvern Instruments Ltd). Antigen loading was determined by first lysing the polymer vesicles in 0.1 M NaOH for 24 h then analyzing the supernatant with UV spectroscopy at 280 nm and 260 nm or using a BCA protein assay. Due to interference in the BCA assay, 1 MT loading capacity was estimated based on the OVA encapsulation results. To load 1 MT into PLGA vesicles at specific amounts, the starting concentrations of OVA used to obtain a desired final loading concentration was used as the starting concentration for 1 MT. To determine vesicle weight, 10 mg of polymer vesicle was dissolved in 200 μ L PBS and analyzed via flow cytometry (BD FACSCalibur) at a constant flow rate of 12 μ L/sec for 30 s. Using BD CellQuest software, polymer count was established and the average weight of each polymer capsule calculated.

2.2. DC generation

Dendritic cells were generated from murine bone marrow as previously described with slight modification.²¹ Briefly, the femurs and tibiae of six- to eight-week-old male C57BL/6 mice

(Jackson Laboratory) were removed and cleaned of excess tissue then washed with 75% EtOH for 60 s. Marrow was flushed out with $1 \times$ PBS using a syringe and 27.5 gauge needle. Bone marrow plugs were dissociated by vigorous inverse pipetting. Red blood cells were lysed using 5 mL of ammonium chloride buffer (0.15 M NH_4Cl , 1.0 mM KHCO_3 , 0.1 mM EDTA) for 5 min. Cells were washed twice with media (Invitrogen Advanced RPMI 1640, 110 mg/L Na Pyruvate, 10% FBS, 50 IU/mL penicillin/streptomycin, and 2 mM L-Glutamine) and filtered through a 70 μm cell strainer. Resulting cell pellet was resuspended to a concentration of 2×10^6 cells/mL in media with 10 ng/mL of GM-CSF and 10 ng/mL of IL-4 (R&D) and plated at 5 mL per well on 6-well plates (BD). Cells were then incubated at 37°C and 5% CO_2 . Every 48 h floating cells were removed and the media was replaced with fresh media and cytokines. On days 7–9, all cells were harvested using a cell scraper and characterized as immature DCs for subsequent analysis and experimentation.

2.3. Polymer treatments

Bone marrow-derived DCs were resuspended at 1×10^6 cells/mL in media at 1 mL per well of a 24-well plate (BD Falcon). Prepared polymer vesicles was weighed, sterilized by UV light for 10 min, and dissolved in media and added to cell suspensions. LPS (055:B5, Sigma-Aldrich) was used a control for stimulation at a final concentration of 2 $\mu\text{g/mL}$. Cells remained in 37°C and 5% CO_2 during the remainder of treatment.

2.4. Immunohistochemistry and flow cytometry

DCs were harvested from culture via vigorous inverse pipetting and washed with PBS. Cells were blocked with FC block (BD Pharmingen) for 10 min then stained in 100 μL with 2 $\mu\text{g/mL}$ of antibodies for the following surface proteins; CD80, CD86, CD11c, MHCII, CD8a, CD11b (BD Pharmingen). Expression levels were analyzed via flow cytometry using a BD FACSCalibur with BD CellQuest or a Coulter Cytomics FC500 and analyzed with the Coulter CXP software. Geometric mean fluorescent intensities are calculated by subtracting the intensities of the respective isotype controls from the Ab treated conditions. Flow cytometry on the Coulter Cytomics FC500 was performed using the CINJ Shared Resource.

2.5. ELISA/Bioplex

Supernatants from cell cultures were harvested and run through an ELISA with paired antibodies or on a Bio-Rad Bioplex Suspension Array System. For the ELISA, a standard sandwich assay was performed. For the Bioplex system, 50 μL of sample was used and the protocol for the assay was followed as per manufacturer's recommendations, analyzing samples in duplicate. Statistical comparison between experimental conditions was done using a two-sample equal variance Student's *t*-test. Differences were considered significant at $p < 0.05$ and thus reported.

3. Results

3.1. Polymer delivery system

We chose to utilize the FDA-approved, resorbable copolymer PLGA due to its versatility and well-characterized properties, that allow one to deliver any specified tumor associated antigen and/or immune stimulant at desired concentrations. Based on the water/oil/water

emulsion technique,²² particles were constructed in an array of sizes starting as low as 200 nm with highest sonication power settings available. Decreasing sonication power to 10% increases the vesicle average diameter to 500 nm. The set time of exposure to sonication strength ensures a homogeneous population at the reported average vesicle size, while shorter exposures results in large polydispersity and heterogeneous sizes. Longer exposures would further decrease the polydispersity, however, this causes excessive heat to develop within the system. Using dynamic light scattering, we verified the reproducibility of particle size generation (Fig. 1(A)) with an average polydispersity below 0.1, demonstrating near homogeneity. Lyophilized polymer was weighed and lysed, and it was determined that up to 10 μ g of OVA could be encapsulated per mg of vesicle when the starting concentration during particle generation is at 100 mg/mL. Starting concentration was incrementally decreased to 25 mg/mL (Fig. 1(B)), yielding vesicles containing as low as 2 μ g/mg of particles. Starting concentrations higher than 100 mg/mL induced aggregation and prevented individual particle formation, while concentrations lower than 25 mg/mL proved difficult with regard to measuring encapsulation efficiency.

To first verify the ability of the polymer vesicles to be internalized by DCs, tetramethyl rhodamine (TMR)-labeled dextran was encapsulated and delivered to DCs over a 30-h period, shown in the flow cytometry plot of (Fig. 1(C)). Throughout the incubation period, polymer vesicles are seen entering the cells, as indicated by an increase in fluorescent levels. As seen in the fluorescent microscopy images of Fig. 2, polymer vesicles are in fact within the cells in high numbers.

3.2. DC stimulation

CpG motif DNA (ODN 1668) and whole OVA protein was first delivered in solution to DCs to determine baseline stimulation capabilities. Delivering OVA alone in solution resulted in no change in levels of any cell surface protein (data not shown). As seen in the histograms depicted in Fig. 3, OVA with CpG induced stimulation of DCs, as indicated by a large increase in CD80, CD86, MHCII and CD11b expression as compared to cells cultured in media alone. CpG delivered alone in solution resulted in the same stimulation as OVA + CpG (data not shown). Although variation was seen in the geometric mean fluorescent intensities in response to the different doses of CpG with OVA delivered, the differences observed were not significant to demonstrate a concentration-dependent effect at those ranges. In addition, concentrations below 0.5 mg of polymer equivalent did not produce significant changes in most surface markers. To ensure comparison between the delivery methods though, overlapping values of 0.5 mg and 1 mg for both soluble form and polymer delivery are reported. Supernatants were also collected and analyzed for secretion profiles, with data normalized to non-stimulated and LPS treated cells. Figure 4 shows that OVA + CpG treated cells increased their secretion of IL-12 (p40 and p70), IFN- γ , TNF- α , MCP-1 and IL-10.

While 1 MT is known to inhibit IDO activity, the effects it has on DC stimulation is still unclear. To gain insight into the effect of 1 MT on altering DC maturation, 1 MT was administered to cells in solution, either alone or with OVA and CpG. 1 MT delivered alone significantly altered CD11b expression, as indicated by higher fluorescent intensities, with

the largest effects seen at highest concentrations (Fig. 3). 1 MT delivered in conjunction with OVA and CpG did not alter the expression of any markers as compared to OVA + CpG. 1 MT alone was also unable to alter the secretion of any measured cytokine and no significant change in secretion was observed when 1 MT was added in conjunction with OVA and CpG.

3.3. Polymer-based stimulation

Although delivering antigen and adjuvants in solution stimulates DCs to a mature phenotype, a polymer-based delivery vehicle provides a controlled environment to deliver desired components at precise amounts to cells. In effect, desired DC stimulation can be achieved and pathways can be altered that are not possible via soluble delivery. Thus, a polymer-based delivery method has proven efficacious in many systems and provides an advantageous therapeutic approach.^{23,24} In verifying this approach for the current system, the first aim was to decouple the effects of delivering OVA, CpG, and/or 1 MT to DCs. DCs were treated with different variations and concentrations of the polymer vesicles. Figure 5 shows representative histograms of CD86 expression on CD11c+ DCs treated with up to 500 μ g of PLGA vesicle per 10^6 cells. In analyzing the immunological effects of PLGA itself, PBS was encapsulated (PLGA-PBS) and delivered to DCs. We noticed no significant change to DC phenotype or secretion profiles in response to empty PLGA particles. Only when doses up to 5 mg of vesicles were administered per 1×10^6 DCs did the empty vesicles elicit a small response from DCs (data not shown). From this we can confirm that any changes in DC properties and/or function resulted from encapsulated components and their route of delivery. OVA and CpG was then encapsulated in PLGA (PLGA-OVA, and PLGA-CpG), but also did not change DC CD86 expression profiles throughout the administered doses. However, DCs treated with PLGA containing both the CpG DNA and OVA (PLGA – CpG + OVA) displayed an increase in CD86 expression. The DC population expressing elevated levels of CD86 was expanded, with more cells expressing higher amounts of CD86 as the dose of polymer vesicle was increased.

To further expand on these findings and determine the effects of delivering 1 MT via the polymer system, DCs were analyzed for CD80, CD86, MHCII, and CD11b expression in response to the polymer treatment. PLGA containing OVA, CpG, and/or 1 MT were administered in increasing amounts to DCs, from 0.2 mg to 1.0 mg per 1×10^6 cells. Figure 6 shows representative histograms of these DCs treated with the respective vesicle conditions. Although the polymer containing OVA and CpG was able to induce stimulation with increasing dose, including 1 MT with the OVA and CpG helped increase the levels of CD80, CD86 and MHCII to even more elevated levels. Secretion profiles of these DCs (shown in Fig. 7) corroborate this finding, as significant increases in IL-10, IL-12, and MCP-1 were observed as a result of adding 1 MT to OVA and CpG in the polymer vesicles. This increase was more evident at the higher concentrations of vesicles. As with delivery in soluble form, 1 MT alone delivered via the polymer vesicles had no significant change in any surface protein throughout the doses (Fig. 6). However, there was arguably some effect in the secretion profiles of DCs treated with 1 MT via the polymer vesicles, as shown in the IFN- γ and GM-CSF profiles (Fig. 7), but variability between experiments was high and thus not statistically significant.

Although the levels of CD86 and MHCII increased as a result of DC treatment both via the polymer vesicles and soluble components, it is interesting to note the ratio of expression of these markers. It is well-known and characterized that T cell binding of DCs occurs in at least two stages, with the initial stage being the binding of MHCII to T cell receptors (TCR), followed by a second stage of CD86 binding to CD28.²⁵⁻²⁷ As shown in Fig. 8, we see an increase in this MHCII to CD86 ratio as a result of the polymer deliver system. This is seen throughout the doses, both with and without inclusion of 1 MT. Even with this increased ratio though, CD86 remains highly elevated in these groups, leading to a DC phenotype able to potentially increase antigen presentation via an elevated MHCII and provide significant costimulation via CD86.

4. Discussion

PLGA has been widely used as a deliver vehicle for drugs, proteins, DNA, and adjuvants for a wide array of applications.²⁸ Utilizing this FDA- approved polymer as a delivery vehicle, detailed and well-documented control over polymer properties can be attained. Via the double emulsion method, polymer vesicles were made ranging from 250–500 nm in diameter (Fig. 1). Vesicle sizes greater than 500 nm are attainable, but endocytosis efficiency and the potential deviation from the observed processing of encapsulated components may vary at larger sizes. Smaller particles, i.e., nanoparticles, offer an advantage of greater cellular uptake as compared to larger microparticles. It has been shown that 100 nm size nanoparticles have 2.5 times greater uptake compared to 1 μ m diameter particles and six fold higher uptake compared to 10 μ m diameter particles.²⁹ Larger particles will also degrade differently, both within cells and in tissue, altering the timing of release and potential stimulation. To minimize this variation, polymer vesicles with average diameter of 250 nm were used throughout our studies. Although a limit for protein encapsulation was found, the limit for DNA encapsulation was not analyzed. Ranges up to a starting concentration of 20 mg/mL was used and proved successful (data not shown), but a starting concentration of 5 mg/mL was sufficient to create polymer vesicles that stimulated DCs. This property is one potential hypothesis for the reason CpG DNA alone in the polymer vesicle was unable to stimulate DCs. Perhaps increasing this DNA starting concentration would allow for vesicles containing CpG alone to stimulate DCs, and will thus be an aim of future studies. From the current results though, we have demonstrated the ability to create a wide range of polymer compositions containing antigen and adjuvant at various ratios. This allows us to fabricate a specifically tailored system to expand various DC populations.

As seen in Fig. 5, particles with either OVA or CpG DNA alone did not induce any significant change in CD86 expression, nor in the cytokine secretion profiles (data not shown). In solution though, this same CpG DNA was able to stimulate DCs to increase CD80, CD86 and MHCII expression (Fig. 3), as well as a vast array of cytokines (Fig. 4). When these concentrations of CpG DNA and OVA were encapsulated simultaneously within the polymer vesicle, the resulting polymer vesicle was able to stimulate DCs. Figures 6 and 7 show that these polymer-based vesicles were able to stimulate DCs to a highly mature state. There are several hypotheses which could explain why CpG requires OVA within the polymer to stimulate the DCs; OVA helps protect CpG from degradation once

internalized within the cell and/or OVA helps increase the loading capacity of CpG DNA within the polymer. Yet another possibility lies in the fact that the internalization and intracellular pathways targeted may differ in DCs as a result of polymer delivery. These results and hypotheses are not unique though, as other researchers have reported similar events. For example, it has been shown that there is enhanced CTL activity against OVA from immunized mice as a result of coencapsulating both OVA and CpG, as compared to decoupled delivery or inoculation in soluble form.³⁰ It has also been shown in studies that delivery of antigen via a polymer vesicle such as PLGA both enhances and prolongs the cross-presentation of exogenous protein on MHC I complexes, a result absent when antigen is delivered in soluble form.³¹ These findings demonstrate the importance and hidden benefits of delivering components to DCs in a combined and controlled manner such as through a polymer delivery vehicle.

This benefit of utilizing PLGA was further exemplified when 1 MT was introduced into the polymer system. 1 MT is commonly utilized to inhibit or minimize the activity of IDO either within DCs or Tregs. It has thus been proposed as an agent for use in antitumor therapies to abrogate the immunosuppressive effects of Tregs. In relevance to this study, reports show that stimulating DCs with CpG can induce immune suppression of T cells via the IDO pathway, but can be reversed with 1 MT treatment.³² Although delivering CpG can stimulate DCs to increase costimulatory molecules and induce an inflammatory cytokine milieu, CpG DNA can also paradoxically increase IDO secretion, thus potentially creating a suppressive environment. This finding, coupled with the understanding that secreted IDO can encourage Treg activity, leads to a need to block IDO activity during DC activation, and antigen presentation and costimulation. However, very little work has been performed to analyze the effects 1 MT has on DCs in addition to directly blocking IDO activity. To emphasize the importance of this, studies have claimed that 1 MT delivered with LPS or TNF- α decrease the stimulation levels of DCs as measured by, among other things, CD80 and CD86 expression and secretion of key cytokines.^{33,34} Hence, 1 MT may be inhibiting beneficial factors required for a successful therapy. We therefore sought to analyze the delivery of 1 MT in a therapeutically relevant system; PLGA containing antigen (OVA) and adjuvant (CpG DNA).

We observed that IL-10 was increased significantly as a result of the polymer-based treatment, even more so when 1 MT was introduced into the polymer vesicles. It should be noted though that these levels were lower than those levels observed with the soluble delivery method. Although it is not abnormal for levels of IL-10 to increase after stimulation, IL-10 is an accepted immune suppressant and has been known to skew lymphocytes from a Th1 to a Th2 response. In addition, our group has reported an IL-10-dependent inhibition of DC function in a model of tumor-induced immune suppression.²¹ Effects of increased IL-10 would consequently be detrimental in most systems. For example, IL-10 has been demonstrated to not only show autocrine effect, but also downregulate CD86 and MHCII expression and decrease the amount of IL-12 secreted from DCs.³⁵⁻³⁷ These effects would normally further encourage Th2 responses. However, as a result of the polymer-based delivery system, a deviation from this pattern was observed, unlike that seen in soluble delivery. Introducing 1 MT into the polymer vehicle with OVA and CpG was also

able to greatly increase IL-12 secretion (Fig. 7) and not only maintain but also increase the expression of CD86 and MHCII (Fig. 6), seemingly reversing the effects of having elevated IL-10 levels. Whether this is a result of 1 MT inhibiting IDO or influencing another alternative biochemical pathway will require further studies.

It is also unknown what effects increasing the MHCII to CD86 ratio will have on T cell interactions (Fig. 8) without further studies. It has been shown that when this ratio is elevated, a decrease in immune competence can be observed.³⁸ Although an increase in this ratio was observed as a result of the polymer-based treatments, CD86 was still significantly high. Perhaps this elevated level of both cell surface proteins, in addition to the increased ratio and elevated IL-12 secretion, will provide an overall stronger TCR binding and subsequent costimulation, allowing superior antigen priming and/or activation of T cells. Summarized in Table 1, these DCs generated as a result of the polymer-based treatment provide an advantageous environment to skew lymphocytes to a Th1 phenotype. The levels of cell surface proteins CD80, CD86, and MHCII are elevated along with secretion of IL-12. Although IL-10 level is also elevated in each OVA and CpG treatment, the fold increase is significantly less than that of LPS-stimulated cells. The resulting DC population generated by the polymer-based delivery system will potentially greatly improve the efficacy of antigen-specific therapy. This hypothesis would require further testing, where cytotoxic T cell assays and mixed lymphocyte reactions would help verify the claim.

In light of these results, the effects of 1 MT delivery via PLGA on DCs extend beyond direct IDO inhibition. When designing a therapeutic system that includes 1 MT, attention should now also be focused on the effects 1 MT has on DC stimulation properties. Our results indicate that including this compound with OVA and CpG in PLGA enhanced several aspects of the delivery system. PLGA encapsulating OVA, CpG, and 1 MT was able to generate a DC phenotype indicative of a Th1-inducing environment. The functional effect of these treated DCs on T cell stimulation is currently unknown. Experiments are currently underway to verify their functional capabilities. This study verifies the efficacy of delivering 1 MT and the advantages of doing so in a PLGA-based polymer delivery vehicle and warrants further exploration of stimulating DCs in a controlled and multifaceted manner.

Acknowledgments

Studies were supported by NIH grants R01CA42908, P30CA07270 and fellowships from the NSF IGERT DGE 0333196 and the New Jersey Commission on Cancer Research.

References

1. Jakob T, et al.
2. Schulz O, et al. *Immunity*. 2000; 13:453. [PubMed: 11070164]
3. Collins M, Ling V, Carreno BM. *Genome Biol*. 2005; 6:223. [PubMed: 15960813]
4. Carreno BM, Collins M. *Annu. Rev. Immunol.* 2002; 20:29. [PubMed: 11861596]
5. Greenwald RJ, Freeman GJ, Sharpe AH. *Annu. Rev. Immunol.* 2005; 23:515. [PubMed: 15771580]
6. Greenwald RJ, Latchman YE, Sharpe AH. *Curr. Opin. Immunol.* 2002; 14:391. [PubMed: 11973140]
7. Bhatia S, et al. *Immunol. Lett.* 2006; 104:70. [PubMed: 16413062]
8. Gabriel EM, Lattime EC. *Clin. Cancer Res.* 2007; 13:785. [PubMed: 17289867]

9. Gershon RK, Kondo K. Immunology. 1970; 18:723. [PubMed: 4911896]
10. Smith TR, Kumar V. Trends Immunol. 2008; 29:337. [PubMed: 18514574]
11. Dhodapkar MV, et al. J. Exp. Med. 2001; 193:233. [PubMed: 11208863]
12. Jonuleit H, et al. J. Exp. Med. 2000; 192:1213. [PubMed: 11067871]
13. Kohm AP, et al. J. Immunol. 2006; 176:3301. [PubMed: 16517695]
14. Goforth R, et al. Cancer Immunol. Immunother. 2009; 58:517. [PubMed: 18719913]
15. Prendergast GC. Oncogene. 2008; 27:3889. [PubMed: 18317452]
16. Hwu P, et al. J. Immunol. 2000; 164:3596. [PubMed: 10725715]
17. Chen W, et al. J. Immunol. 2008; 181:5396. [PubMed: 18832696]
18. Fallarino F, et al. Int. Immunol. 2002; 14:65. [PubMed: 11751753]
19. Hou DY, et al. Cancer Res. 2007; 67:792. [PubMed: 17234791]
20. Newman KD, et al. J. Biomed. Mater. Res. 2002; 60:480. [PubMed: 11920673]
21. Yang AS, Lattime EC. Cancer Res. 2003; 63:2150. [PubMed: 12727833]
22. Elamanchili P, et al. J. Immunother. 2007; 30:378. [PubMed: 17457213]
23. Ali OA, et al. Nat. Mater. 2009; 8:151. [PubMed: 19136947]
24. Diwan M, et al. Curr. Drug Deliv. 2004; 1:405. [PubMed: 16305402]
25. Lenschow DJ, Walunas TL, Bluestone JA. Annu. Rev. Immunol. 1996; 14:233. [PubMed: 8717514]
26. Greenfield EA, Nguyen KA, Kuchroo VK. Crit. Rev. Immunol. 1998; 18:389. [PubMed: 9784967]
27. Bluestone JA. Clin. Transplant. 1996; 10:104. [PubMed: 8680045]
28. Panyam J, Labhasetwar V. Adv. Drug Deliv. Rev. 2003; 55:329. [PubMed: 12628320]
29. Desai MP, et al. Pharm. Res. 1997; 14:1568. [PubMed: 9434276]
30. Schlosser E, et al. Vaccine. 2008; 26:1626. [PubMed: 18295941]
31. Shen H, et al. Immunology. 2006; 117:78. [PubMed: 16423043]
32. Wingender G, et al. Eur. J. Immunol. 2006; 36:12. [PubMed: 16323249]
33. Jung ID, et al. J. Immunol. 2009; 182:3146. [PubMed: 19234212]
34. Hwang SL, et al. Cell Res. 2005; 15:167. [PubMed: 15780178]
35. De Smedt T, et al. Eur. J. Immunol. 1997; 27:1229. [PubMed: 9174615]
36. Corinti S, et al. J. Immunol. 2001; 166:4312. [PubMed: 11254683]
37. Buelens C, et al. Eur. J. Immunol. 1995; 25:2668. [PubMed: 7589143]
38. Girndt M, et al. Kidney Int. 2001; 59:1382. [PubMed: 11260399]

Fig. 1.

(A) PLGA polymer vesicle sizes based on sonication power settings. (B) By modulating the starting concentration of OVA, the final encapsulated concentration can be controlled and measured. (C) Polymer encapsulating TMR-rhodamine was delivered to DCs over 30 h and analyzed via flow cytometry to determine uptake of the polymer vesicles over time.

Fig. 2.

Representative images of polymer uptake by DCs. Rhodamine-labeled dextran was encapsulated within the PLGA particles then delivered to DCs. After 24 h DCs were stained with FITC-conjugated anti-CD11c and visualized via fluorescent microscopy.

Fig. 3.

Representative flow cytometry histograms of cell surface expression of specified markers and corresponding geometric mean fluorescent intensities. Cells were treated for 48 h with OVA, CpG, and/or 1 MT in solution form at concentrations equivalent to 0.5, 1.0, and 2.0 mg of PLGA polymer. Cells were dual-stained for CD11c and indicated surface molecules with the geometric mean intensities of Abs reported on CD11c+ cells. Cells treated with OVA alone reported identical histograms as the negative control (media) and cells treated with CpG alone showed identical histograms as the OVA + CpG condition (data not shown).

Fig. 4.

Secretion profiles of DCs. Cells were cultured at $1 \times 10^6/\text{mL}$ in 1 mL volume and treated for 48 h with OVA and CpG in solution (■), OVA, CpG and 1 MT in solution (■), and 1 MT alone in solution (■) at the indicated polymer equivalent concentrations. Supernatants were harvested and run on ELISAs or the Bio-plex assay. Concentration values were normalized to untreated and LPS-treated cells. Error bars indicate significant error on $n = 2 - 3$ experiments.

Fig. 5.

Representative flow cytometry histograms of CD86 expression on CD11c+ cells. DCs were treated for 48 h with PLGA polymer encapsulating PBS (PLGA-PBS), OVA (PLGA-OVA), CpG (PLGA-CpG), or OVA+CpG (PLGA-OVA+CpG). As controls, cells were either not treated (media), or cultured with CpG or LPS in solution at 2 μg/mL. Cells were dual stained for CD11c (one representative example of CD11c expression shown) and CD86 and gated on the CD11c+ population.

Fig. 6.

Representative flow cytometry histogram of cell surface expression of specified markers and corresponding geometric mean fluorescent intensities. DCs were treated for 48 h with 0.2 to 1.0 mg of polymer vesicles containing OVA + CpG (P-OC), OVA + CpG + 1 MT (P-OCM), and 1 MT (P-MT). Cells were dual stained for CD11c and the following Abs; CD80, CD86, MHCII, and CD11b. Cells were gated for the CD11c+ population to obtain the geometric mean fluorescent intensities of the reported Abs.

Fig. 7.

Secretion profiles of DCs. Cells were cultured at $1 \times 10^6/\text{mL}$ in 1 mL volume and treated for 48 h with OVA and CpG in PLGA (■), OVA, CpG and 1 MT in PLGA (■), and 1 MT alone in PLGA (■). Supernatants were harvested and run on ELISAs or Bio-plex assays. Concentration values were normalized to untreated and LPS-treated cells. Error bars indicate significant error on $n = 2 - 3$ experiments with p values determined by an unpaired one tail equal variance Student's t -Test.

Fig. 8.

Representative expression ratios of MHCII to CD86 on DCs stimulated with OVA, CpG, and/or 1 MT either in solution or in polymer form. The ratios of mean fluorescent intensities of binding Abs for MHCII and CD86 (MHCII/CD86) were compared on 1×10^6 CD11c+ DCs treated (A) in solution form with OVA + CpG (OC, ●), OVA + CpG + 1 MT (OCM, ■) and 1 MT (MT, ▲) at the indicated polymer equivalent amounts, or (B) in polymer form containing OVA + CpG (POC, ●), OVA + CpG + 1MT (POCM, ■), and 1MT (PMT, ▲) at the indicated polymer amounts.

Table 1

Summary of the fold increase of DC surface protein expression (CD80, CD86, MHCII, CD11b) and cytokine secretion (IL-10, IL-12) in response to respective treatment condition.

Condition	CD80	CD86	MHCII	CD11b	IL-10	IL-12
LPS	3.8 ± 0.8	4.7 ± 1.1	1.7 ± 0.4	0.99 ± 0.2	61.6 ± 28	129 ± 16
1 MT	1.1 ± 0.2	1.1 ± 0.1	0.9 ± 0.1	2.7 ± 1.3	1.0 ± 0.6	0.8 ± 0.1
OVA + CpG	3.9 ± 0.9	3.9 ± 0.7	2.0 ± 0.3	1.7 ± 0.7	17.6 ± 4	159 ± 21
OVA + CpG + 1 MT	3.9 ± 1.2	3.7 ± 0.4	1.9 ± 0.2	1.6 ± 0.6	24 ± 3	134 ± 33
P-1MT ^a	1.1 ± 0.1	1.4 ± 0.1	1.3 ± 0.3	0.9 ± 0.02	1.1 ± 0.6	0.8 ± 0.1
P-OVA + CpG ^a	1.6 ± 0.5	1.8 ± 0.4	1.4 ± 0.3	0.9 ± 0.01	9.1 ± 7	62 ± 19
P-OVA + CpG + 1 MT ^a	2.1 ± 0.4	2.6 ± 0.6	1.9 ± 0.6	0.98 ± 0.06	32 ± 13	213 ± 94

Values and corresponding standard errors represent the fold increase of protein expression versus non-treated cells.

^aWithin PLGA vesicle.