SiRNA for Therapeutic Immuno-modulation: Simultaneous Delivery of Cytokine Targeted SiRNA and DNA Antigens to Dendritic Cells using Polymer Microcarriers

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ABSTRACT

RNA interference mediated gene knockdown is a potential immuno-modulatory tool for tuning dendritic cells (DC) activation and function in-vivo. We have developed a novel multi-tiered delivery system for combinatorial administration of siRNA and plasmid DNA antigens in a single gene carrier system. Our hypothesis is that co-delivery of IL-10 specific siRNA along with DNA antigens to the same dendritic cells would inhibit IL-10 production, enhance DC activation, antigen presentation, and T cell response. Non-toxic, polyethyleneimine (PEI)-functionalized, poly(lactic-co-glycolic acid) (PEI-PLGA) microparticles co-delivering IL-10 siRNA and DNA antigen exhibited significantly higher ($p<0.05$) IL-10 gene knockdown in primary DCs, and DNA transfection, in-vitro. Mice immunized intramuscularly with (IL-10)-SiRNA-HBsAg-PEI-PLGA particles demonstrated successful "switch" towards a stronger Th1 response as compared to naked DNA or HBsAg loaded PEI-PLGA treated animals.

Keywords: siRNA, immuno-modulation, combinatorial delivery, Th1 response, Interleukin-10

1. INTRODUCTION

At the cellular level, immune modulation is controlled through antigen presentation by dendritic cells (DCs) and by the resulting T cell response. For viral infections and cancer, a strong T cell mediated cytotoxic response (helper T (Th1) mediated, Class I restricted) plays an important role in destroying the disease causing cells. A vigorous cytotoxic (CTL) and helper T (Th1) cell response to viral hepatitis B can be readily detectable in patients with acute self limited hepatitis B but is weak or undetectable in patients with chronic infection. Th1 cytokines (interferon-γ (IFN- γ) production following antigen stimulation of T cells in the periphery is weak or absent in chronic HBV infection [1]. Hence there is a need to develop strategies that can enhance as well as preferably direct the immune response towards Th1 type. In recent years short-interfering RNAs (siRNAs) have emerged as potential therapeutic tools [2]. In mammalian cells, RNA interference can be induced by siRNA duplexes (~21-23 nucleotides), which subsequently confers shutting down of gene expression in a sequence specific manner [3]. A potential immuno-modulatory application of siRNAs could be in silencing of specific Th1 suppressor genes (e.g Interleukin 10 (IL-10)) in antigen presenting cells (APCs) and thereby driving the immune response towards the Th1 pathway and enhance cellular immunity. Specifically, IL-10 prevents antigen-specific T cell proliferation by inhibiting the maturation of DCs and switching immature DCs into tolerogenic cells with down regulation of major histocompatibility complex (MHC) class II, CD86, and CD54 expression as well as suppression of IL-1 and tumor necrosis factor (TNF)- alpha[4-7]. We hypothesize that if IL-10 silencing siRNA can be efficiently co-delivered with the plasmid DNA (pDNA) antigens to the same DCs in-vivo, the resulting antigen-specific T cell response can be significantly enhanced and "switched" to a Th1 phenotype with an enhanced interferon gamma (IFN-γ) and decreased IL-4 production by CD4+ T helper.

We have earlier demonstrated that surface functionalized poly(lactide-co-glycolide) (PLGA) microparticle mediated delivery of the idiotype pDNA antigen can generate, even in the absence of specific adjuvants, significant protective anti tumor immunity in a prophylactic mouse model of B cell lymphoma [8]. Here we report in-vitro and in-vivo immuno-modulatory effects of IL-10 siRNA-encapsulated, PEI-PLGA microparticles, surface-loaded with pDNA antigens. Our recent results suggest that such a combinatorial delivery system can further enhance DC activation as well as T cell proliferation *in-vitro* and an improved Th1 response in Hepatitis B animal model.

2. METHODS

2.1 Materials

PLGA RG502H (Mw~11,000 Da) and RG503H (Mw ~29,000 Da) were purchased from Boehringer Ingelheim (Petersburg, VA). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and sulfo N-hydroxysuccinimide (sulfo-NHS) were from Pierce Biotechnology Inc. (Rockford, IL). Branched PEI (bPEI), Mw= ~70,000 Da, was purchased from Polysciences Inc. (Warrington, PA). Custom synthesized mouse IL-10 siRNA targeting the specific sequence 5′-AATAAGCTCCAAGAGAAAGGC, scrambled siRNA and were from Ambion Inc. (Austin, TX). Poly(vinyl alcohol) (Mw ~31,000) was purchased from Fluka.

2.2 Mice model, primary APC isolation, and cell lines

Murine APCs were derived and differentiated into myeloid lineage from bone marrow isolated progenitor cells of female Balb/c mice (H-2d, 5-10 weeks old, Jackson laboratories) as described previously by Inaba et al. [9] with modifications and cultured in RPMI-1640 Glutamax® (Invitrogen, Carlsbad, CA) medium, supplemented with mouse GM-CSF and IL-4 (eBioscience, San Diego, CA). Loosely adherent APCs (mostly DCs) were harvested on day 7.

2.3 Synthesis of interleukin 10 siRNA encapsulated cationic microparticles

PLGA microparticles with or without encapsulated siRNA (IL-10 targeted) were synthesized with PLGA (RG502H or RG503H) using a water-in-oil-in-water double emulsion, solvent evaporation technique. A modified EDC chemistry, as reported by us previously [8, 10], was used to conjugate branched polyethylimine (bPEI, 70kDa) to the surface of these siRNA-encapsulated microparticles to obtain cationic PEI-PLGA- siRNA microparticles. The particles were characterized (both before and after PEI conjugation) for encapsulation of siRNA, size, and zeta potential. Plasmid DNA was adsorbed on these PEI modified microparticles in PBS (pH 6.5) [8, 10]. We have earlier shown the efficacy of pDNA loaded PEI modified PLGA microparticles in carrying out efficient in-vitro transfection of macrophage cells [8, 10].

2.4 In vitro siRNA release studies

In vitro release of IL-10 siRNA from PEI modified and un-modified PLGA microparticles (RG502H, RG503H) were conducted at 37 ºC in nuclease free PBS (pH 7.4) and analyzed using Nanodrop spectrophotometer at 260 nm.

2.5 Efficient silencing of IL-10 gene expression in bone marrow-derived primary APCs

Efficacy of siRNA loaded microparticles (RG502H) to carry out long term (15 days) IL-10 gene knockdown in primary APCs was analyzed by isolating RNA and evaluating the IL-10 gene expression levels using real time RT-PCR.

2.6 In vivo immuno-modulation using combinatorial delivery of pDNA and IL-10 siRNA

Th1/Th2 class switching: Balb/c mouse (n=8) immunized with pDNA-PEI-PLGA microparticles with or without co-encapsulated siRNA was used to evaluate whether co-delivery of IL-10 silencing siRNA with DNA antigens can "switch" the immune response towards a more Th1 type phenotype. Specifically CD4+ cells from spleens of immunized or control mice were analyzed to determine their cytokine production profile, i.e. whether siRNA delivery can enhance Th1 cytokine (IFN-γ) production and decrease Th2 cytokine (IL-4) production in these cells.

2.7 Statistical analysis

Student's t-test was used to evaluate significance between pairs of groups and $p<0.05$ was considered to be significant.

3. RESULTS

3.1 Encapsulation efficiency of siRNA in unmodified and PEI modified PLGA microparticles

The effects of different molecular weights of PLGA as well as different amounts of siRNA in the internal aqueous phase on siRNA encapsulation efficiency were evaluated. Measurements were conducted following dichloromethane-based extraction of the encapsulated SiRNA both before and after surface functionalization of the microparticles with PEI in order to determine how much SiRNA is leached away during the PEI conjugation process As shown in **Table 1**, successful encapsulation of siRNA in PLGA microparticles was achieved. RG502H microparticles with 100 µg initial siRNA showed significantly higher encapsulation efficiency compared to RG502H particles

Table 1: Encapsulation Efficiency of siRNA and DNA loading in unmodified and PEI modified PLGA microparticles

Formulation	Theoretical siRNA loading[a]	Encapsulation Efficiency % (Unmodified)[b]	Encapsulation Efficiency % (PEI modified)[b]	DNA Loading % (PEI modified)[b,c]
IL-10 siRNA RG502H®	100 µg	84.64 ± 1.49	62.22 ± 7.80	75.00 ± 0.55
IL-10 siRNA RG502H®	200 µg	67.71 ± 2.01	33.50 ± 7.09	78.00 ± 0.49
IL-10 siRNA RG503H®	100 µg	68.20 ± 0.89	54.60 ± 4.90	82.00 ± 0.76
IL-10 siRNA RG503H®	200 µg	68.91 ± 2.11	56.80 ± 4.11	74.00 ± 2.13

[a] Amount of siRNA (IL-10) per 200 mg of PLGA polymer
[b] Values represents Mean ± S.D of at least three independent samples
[c] Values reported here are for Luciferase pDNA and was consistent for other plasmids used in the study (Beta-Galactosidase and HBsAg)

with 200 µg initial siRNA as well as RG503H particles with either 100 µg or 200 µg initial siRNA. Furthermore, the encapsulation observed following PEI conjugation to particle surface was significantly lower than pre-modification values. However, even after surface modification significant amount of SiRNA was encapsulated within all formulations with highest amount in the RG502H particles with 100 µg initial SiRNA (62.22 ± 7.8 %). PEI was successfully conjugated on the surface of PLGA microparticles using a simple, EDC-NHS chemistry. Surface modification was characterized by zeta potential analysis. All batches of PEI modified microparticles showed highly positively charged surface with zeta potential (~ +31 mV) whereas unmodified particles indicated a negative zeta potential (~ -16 mV) arising from the surface carboxyl groups. Average particle diameter for microparticles centered around 1.6 µm with no significant difference between PEI modified and unmodified microparticles.

3.2 In vitro siRNA release studies

siRNA release from PLGA microparticles indicated a biphasic release over a period of 35 days (Figure 1). A rapid initial diffusional release was observed using both RG503H (~29 kDa) and RG502H PLGA resomer with no significant difference between the polymers. A second phase of release was observed after 13 days for RG502H particles and after 20 days for RG503H particles presumably due to their degradation kinetics. These findings suggest that by varying the molecular weight of the polymer the release of encapsulated siRNA can be modulated.

3.3 Efficient silencing of IL-10 gene expression in bone marrow-derived primary APCs

As shown in Figure 2, transfection with IL-10 siRNA-PEI-PLGA microparticles resulted in significant gene silencing (up to 80% compared to untreated cells). The silencing effect was sequence specific i.e. a scrambled siRNA sequence failed to silence IL-10 expression significantly. In addition, for microparticle-treated cells, gene silencing efficacy remained unchanged even after 15 days while a decrease in silencing efficacy was observed with cells transfected using commercial siPORT Amine reagent.

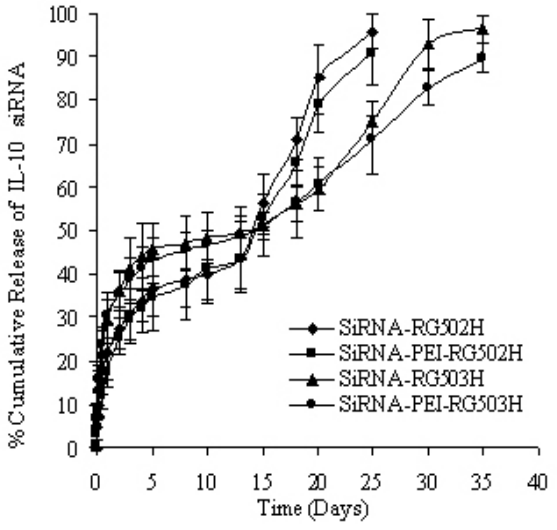

Figure 1: *In-vitro* release of IL-10 *siRNA* from PEI conjugated and unconjugated PLGA microparticles.

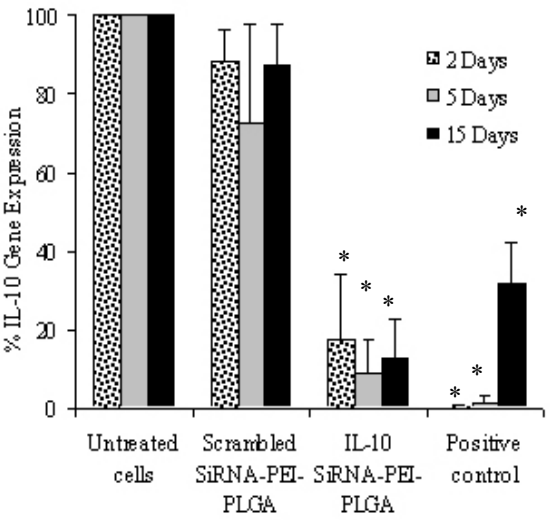

Figure 2: Microparticles mediated Interleukin10 (IL-10) gene knockdown in Antigen presenting cells.* represents $p<0.05$.

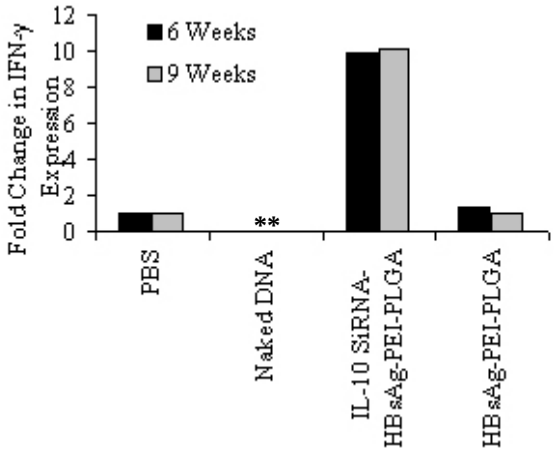

Figure 3**:** Bar graph represents fold change in expression of IFN-γ by pooled splenocytes from immunized mice. * Represents failure to detect any significant amount of T cell activity by a group.

3.4 In vivo Th1/Th2 class switching

Intracellular levels of IFN-γ and IL-4 in CD4+ cells (assayed after 48 hours of splenocytes culture with HBsAg are shown in Figure 3. As indicated, IFN-γ production increased significantly when IL-10 siRNA was co-delivered with the surface-loaded pDNA antigen while IL-4 expression was markedly reduced
(later not shown). In fact both naked DNA and DNA loaded microparticles (without siRNA) immunized animals showed

negligible IFN-γ levels while inclusion of siRNA in the formulation showed a 10 fold increase. This data strongly suggests a "switch" in the CD4+ T helper cell response from Th2 to a Th1 phenotype.

4. DISCUSSION

Controlled immune modulation is a fundamental aspect for effective immunotherapy. For effective genetic immunization, pDNA must not only be internalized by host cells but the antigen must also be presented by professional antigen presenting cells, e.g. DCs and macrophages. APC maturation (DCs and macrophages) plays an important role in antigen presentation to naïve T cells. Recent findings report [11] that unmodified pDNA encapsulated PLGA microparticles failed to induce significant DC maturation although these microparticles did manage to present antigen encoded in the pDNA. This limitation of PLGA based genetic vaccines can be circumvented by modifying the surface of PLGA with a cationic polymer and adsorbing pDNA on the surface. pDNA adsorbed on the surface of cationic microparticles can provide a better interaction with toll like receptors and promote maturation of cells. We have developed PEI conjugated PLGA microparticles to serve this purpose. However, there are several other factors that interplay major role in suppression of APC maturation. Production of cytokines like interleukin 10 (IL-10) prevents effective maturation of DCs and possibly macrophages and thereby hampers their antigen presenting abilities. For immunotherapy, instead of manipulating genes *in-vitro* and then using the DCs for *in-vivo* applications, a better delivery system would be one which can perform both the tasks simultaneously *in-vivo.* However to our knowledge there currently exists no successful single delivery system for siRNA and pDNA for immune modulation. Our strategy is to use siRNA, with simultaneous delivery of pDNA antigen in DCs to modulate and control the immune response with possible diversion towards Th1 or Th2 response.

Attempts to explore the potential of siRNA in combination with PLGA have been few [12,13] and we report the first use of PEI conjugated PLGA microparticles as a multi-modal delivery system for DNA vaccines and siRNA. In an attempt to improve the efficacy of specific antigen encoding pDNA loaded PEI conjuagated PLGA MPs we studied the effect of co-deliverying siRNA. In this study we successfully encapsulated siRNA into PLGA microparticles however the efficiency decreased with an increase in initial theoretical loading. A further decrease in entrapped siRNA was observed in PEI modified PLGA microparticles possibly due to initial release of siRNA from microparticles during EDC/NHS activation and PEI conjugation. *In vitro* siRNA release studies indicate slow diffusion of siRNA from polymeric microparticles. High molecular weight polymer showed slightly faster rate of diffusion over a span of thirteen days. Based on a slow release profile and the effects on gene silencing observed it can be inferred that even a small amount of siRNA might be sufficient enough to carry out significant genetic knockdown. In vitro efficacy of this combinatorial delivery system was established with subsequent IL-10 knockdown in antigen presenting cells with IL-10 siRNA along with high luciferase protein expression in model APCs when transfected with pgWizLuciferase DNA (not shown).

Mouse immunized with pDNA-PEI-PLGA microparticles with or without co-encapsulated siRNA was used to evaluate whether co-delivery of IL-10 silencing siRNA with DNA antigens can "switch" the immune response towards a more Th1 type phenotype. Specifically CD4+ cells from spleens of immunized or control mice were analyzed to determine their cytokine production profile, i.e. whether siRNA delivery can enhance Th1 cytokine (IFN-γ) production and decrease Th2 cytokine (IL-4) production in these cells. Intracellular levels of IFN-γ (Figure 3) and IL-4 (not shown here) in CD4+ cells (assayed after 48 hours of splenocyte culture with HBsAg). As indicated, IFN-γ production increased significantly when IL-10 siRNA was co-delivered with the surface-loaded pDNA antigen while IL-4 expression was markedly reduced. In fact both naked DNA and DNA loaded microparticle (without siRNA) immunized animals showed negligible IFN-γ levels while inclusion of siRNA in the formulation showed a 10 fold increase. This data strongly suggests a "switch" in the CD4+ T helper cell response from Th2 to a Th1 phenotype.

5. ACKNOWLEDGEMENTS

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