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Polymer-Mediated DNA Vaccine Delivery via Bystander Cells Requires a Proper Balance between Transfection Efficiency and Cytotoxicity

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Abstract

Direct targeting of dendritic cells is an ideal goal for DNA vaccine delivery in order to stimulate both arms of the immune system. However, dendritic cells are often difficult to transfect using nonviral polyplexes. Here we show that transfecting bystander cells such as fibroblasts with PEI/DNA complexes leads to efficient cross-presentation of a model antigen by dendritic cells and subsequent activation of antigen-specific CD8⁺ T cells. Maturation of dendritic cells is also stimulated after co-culture with transfected fibroblasts. Such outcomes depend on a proper balance between transfection efficiency and polyplex-induced cytotoxicity in the fibroblasts. In fact, substantial cytotoxicity is desirable and even necessary for cross-presentation and cross-priming of T cells. This study illustrates a new pathway of polymer-based DNA vaccine delivery via bystander cells without direct targeting of antigen-presenting cells and highlights the importance of exploiting polymer-induced cytotoxicity for the benefit of immune activation.

Keywords

DNA vaccine; Polyplex; Gene delivery; Immune response; Cytotoxicity

1. Introduction

Dendritic cells (DCs) are considered the most important antigen-presenting cells and play a central role in modulating immune responses. They are a very attractive target for DNA vaccine delivery because they are able to present antigen on both MHC class I and II molecules for generation of cellular and humoral immune responses. The classical antigen processing pathways lead to MHC class II presentation and a humoral immune response from exogenously obtained antigen, characterized by CD4⁺ T cell stimulation and antibody production. In the case of directly transfected DCs, endogenously expressed proteins are processed through the MHC class I pathway, leading to cellular immune response, characterized by CD8⁺ T cell activation and cytotoxic T lymphocyte (CTL) generation.¹ The CTL response is essential to the defense against viral infections and cancer.² Therefore,

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Supporting Information Available. Additional cell transfection data, flow cytometry results on scattering properties of mixed cell populations, and antigen-presentation.

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targeting DNA vaccine to DCs using properly designed carriers and achieving direct transfection of DCs has been widely accepted to be crucial for the generation of cellular immunity.

Many methods have been explored to efficiently deliver antigen to dendritic cells, including manipulation of carrier size,³ targeting of the carrier,⁴⁻⁶ or targeting of the antigen itself.⁷ Many of these methods have resulted in some increased immune response, confirming that delivery of antigen to DCs is important. Many studies suggested that for DNA vaccine, direct transfection of DCs that leads to antigen expression in these cells is indeed a very effective vaccination pathway. Hattori and colleagues were able to directly demonstrate enhanced in vivo DC transfection using mannose-targeted liposomes,^{4,8,9} leading to some enhancement in T cell stimulation. Intradermal transfection with a DC-restricted expression plasmid also showed enhanced CD8⁺ T cell stimulation when compared to a keratinocyte-restricted plasmid.¹⁰ Unfortunately, DCs remain difficult to target directly because of their relative rarity in vivo.¹ In addition, DCs as well as other APCs, are notoriously difficult to transfect, especially by nonviral vectors. It is possible that they have enhanced protection against nuclear entry by foreign nucleic acids due to their functional role of pathogen uptake and processing.¹¹ Even when DC transfection in vivo is observed, the level is usually very low. Work by Hattori and others, for example, reported transfection detected only through transcriptional amplification by RT-PCR.^{4,8,9} Using cationic polymer carriers, Jilek and colleagues reported a very low frequency of DC transfection of one per 10,000 cells.¹²

An alternative pathway does exist for generating CD8⁺ T cell responses through DNA vaccination without directly transfecting DCs. It has been well documented that DCs can present exogenous antigen on MHC class I molecules in a process known as cross-presentation. The subsequent stimulation of CD8⁺ T cells is known as cross-priming.^{2,13} Cross-presentation is a pathway distinctly different from endogenous MHC class I peptide loading¹⁴ and is regulated, at least in part, by the transporter associated with antigen processing (TAP) protein.^{2,14} Antigen from various sources has been efficiently cross-presented, including soluble protein at high concentrations, protein or peptide associated with particles, as well as protein and peptide from autologous cells.² Also, a particularly effective way of delivering antigen to DCs for cross-presentation is to have it ingested through damaged and dying cells.^{2,15,16} Therefore, if DNA vaccination results in some antigen expression by cells other than DCs, it can still contribute to the overall immune response through cross-presentation and cross-priming.

While most DNA vaccination studies do not differentiate between direct and cross-primed responses, there is evidence that the cross-presentation pathway alone may have been sufficient. For example, it was reported that when antigen expression is limited only to muscle cells, cross-presentation can generate CTL responses.^{17,18} Cho et al. determined that CTL responses were generated more effectively when expression of plasmid DNA delivered by gene gun was under the control of a nonspecific CMV or K14 promoter. When expression was controlled by an APC-specific CD11b or MHC II promoter, both antibody and CTL responses were weakened, suggesting that cross-presentation, rather than direct transfection of DCs, could be the primary mechanism for immune response generated by DNA vaccination.^{19,20} In the case of polymer-based DNA vaccine delivery, it has been speculated that cross-presentation may be important.²¹⁻²³ However, cross-presentation and its importance in generating cellular immune response by polymer-delivered DNA vaccine have not been evaluated. Furthermore, how polymer carrier design might affect cross-presentation and cross-priming is not known.

Here we developed a co-culture system to test the hypothesis that polymer-mediated transfection of non-antigen-presenting bystander cells can be sufficient in generating MHC

class I restricted antigen presentation on DCs and subsequent CD8⁺ T cell stimulation. To mimic the in vivo situation typical of vaccination and infection, our co-culture system consists of bystander cells (fibroblasts), DCs, and naïve CD8⁺ T cells mixed sequentially and cultured for certain periods of time (Fig. 1). We transfected the bystander cells using complexes of polyethylenimine (PEI) and plasmid DNA and quantified the transfection efficiency and toxicity in the bystander cells. Using plasmid encoding a model antigen, ovalbumin (OVA), we further quantified OVA-specific cross-presentation by DCs, their maturation state, and the activation of OVA-specific CD8⁺ T cells. Since cell death is known to have significant impact on cross-presentation, we further investigated the relationship between polymer-mediated transfection efficiency and cytotoxicity in bystander cells, in order to determine how antigen-presentation and DC maturation can be optimized.

2. Experimental Methods

2.1 Cell culture

NIH 3T3 murine fibroblast cell line was cultured in DMEM (1 g/L D-glucose, L-glutamine, 110 mg/L sodium pyruvate, Gibco) media supplemented with 10% fetal bovine serum (FBS, heat inactivated, Gibco) and 100 units/mL penicillin/streptomycin (Gibco). The DC 2.4 murine dendritic cell line (ATCC, provided by Dr. John Ohlfest) was maintained in the same media without additional glucose but also supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4). Mouse CD8⁺ T cells were cultured in RPMI medium (Gibco) supplemented with 10% FBS, 100 U/mL penicillin/streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate). All cells were incubated at 37°C in 5% CO₂.

2.2 Preparation of PEI/DNA polyplexes

Polyplexes were prepared using PEI (branched, 25 kDa, Sigma) and plasmid DNA in 20 mM HEPES buffer (pH 7.4). PEI and DNA solutions were both diluted to 0.1 mg/mL in 20 mM HEPES, mixed in equal volumes to a total of 100 µL, vortexed briefly, and allowed to incubate at room temperature for 30 minutes prior to use. For transfection experiments polyplexes were prepared with a plasmid encoding enhanced green fluorescent protein (GFP) (pEGFP-N1, Elim Biopharmaceuticals). A plasmid encoding the luciferase gene (pCMV-Luc, Elim Biopharmaceuticals) was used for toxicity studies and as a no-antigen control. For antigen presentation and maturation studies, a plasmid encoding the full-length chicken OVA and a CMV promoter was used (Clonetech). The plasmids were either purchased from Elim Biopharmaceuticals with high purity and free of endotoxin, or were amplified in *E. coli* DH5 cells and purified using an Endo-free MAXI plasmid purification kit (Qiagen). Purity and quantity of DNA was verified by UV absorbance at 260 and 280 nm, and endotoxin level was verified to be below 3 EU/mg using a Pyrogen Gel Clot LAL assay (Lonza).

2.3 Transfection of bystander cells

NIH 3T3 cells were plated at 5×10^4 cells/well in 6-well plates and allowed to adhere overnight. The cell media was replaced by serum-free media and polyplexes of GFP plasmid were added and allowed to incubate for 4 hours. Four µg of DNA was added per well in 6-well plates. The amount of polymer was varied based on the N/P ratio used (4, 8, 12, 16, 20). For some experiments to reduce cell death, the caspase inhibitor Z-VAD FMK (Enzo Life Sciences) was added to cells at 50 µM 30 minutes prior to adding polymer complexes. After transfection, cells were washed three times with phosphate buffered saline (PBS, pH 7.4) and replaced with media containing 10% serum. Twenty-four hours after initial addition of polyplexes cells were harvested by trypsinization and suspended in FACS buffer (PBS, 1% BSA, 1 mM sodium azide) and analyzed directly by flow cytometry. Samples were run

on either a FACSCalibur or LSR II flow cytometer (Becton Dickson) and analysis was done using FlowJo software. GFP-positive cells were gated against a sample of cells transfected with luciferase-encoding DNA using the same transfection procedure to control for any increased autofluorescence of cells caused by polyplexes nonspecifically. Transfected cells on chamber slides were also mounted on Vectashield to stain for cell nucleus and visualized using an Olympus IX70 inverted microscope equipped with an Olympus DP72 camera and CellSens software.

2.4 Toxicity in bystander cells

NIH 3T3 cells were prepared and treated with polyplex as stated above for transfection experiments. Polyplexes were made with luciferase-encoding plasmid instead of GFP plasmid to avoid GFP signal overlap with cytotoxicity detection. N/P ratios of 4, 8, 12, 16, and 20 were tested. In some cell samples labeled “N/P=8×2”, fibroblasts were transfected with one dose of polyplex containing Luc plasmid plus an equal dose of polyplexes containing OVA plasmid. The dose of polyplex was therefore doubled while the amount of antigen-encoding DNA delivered remained constant. The purpose of this was to increase polyplex-induced toxicity without affecting antigen expression. After the 24 h incubation, cells were harvested, trypsinized, and washed once with 1 mL of Annexin V staining buffer (BioLegend). Cells were then resuspended in 100 µL of Annexin V staining buffer and stained with Alexa Fluor 647-labeled Annexin V (BioLegend) and propidium iodide (PI) (BioLegend), followed by analysis by flow cytometry.

In addition, we have determined cytotoxicity (apoptosis) of the fibroblasts using the TUNEL assay. At the end of the 24-hour transfection, cells undergoing apoptosis and DNA fragmentation were examined using DeadEnd™ Colorimetric TUNEL system (Promega). Briefly, cells were washed twice with PBS and fixed by immersing in 4% paraformaldehyde (Sigma) for 25 min at room temperature. After two 5-min washes with PBS, cells were permeabilized with 0.2% Triton X-100, washed twice with PBS and equilibrated in 100 µL of Equilibrium Buffer for 10 min. Recombinant terminal deoxynucleotidyl transferase (rTdT) of 100 µL was added to each sample, and slides were covered with plastic coverslips to ensure even distribution of the reagent. After a 60-min incubation at 37°C in a humidified chamber, the reaction was stopped by immersing cells in 2-times SSC buffer in a Coplin jar for 15 min at room temperature, followed by three washes with PBS. Cells were then incubated with 0.3% hydrogen peroxide for 5 min, washed three times with PBS, and treated with 100 µL of horseradish peroxidase-labeled streptavidin for 30 min at room temperature. After three more washes in PBS, cells were stained with 100 µL of diaminobenzidine (DAB) for 8 min at room temperature and washed three times with de-ionized water. Slides were mounted using Vectashield and visualized using an Olympus IX70 inverted microscope equipped with an Olympus DP72 camera and CellSens software.

2.5 Antigen presentation and DC maturation

NIH 3T3 cells were cultured at 2.5×10^4 per well in 12-well plates and allowed to adhere overnight. Cells were incubated with polyplexes of OVA DNA for 4 hours in serum free media as described in the transfection experiments. Two µg of DNA was per well. Afterwards, the fibroblasts were washed three times with 1 mL of PBS, and DC 2.4 cells were added to each well at 1×10^5 /well. All the cells were cultured in media containing 10% serum for another 24 h and then harvested by trypsinization, resuspended in FACS buffer, and stained with PE-labeled anti-mouse OVA-K^b antibody (e-Biosciences) and APC-labeled anti-murine CD40 antibody (BioLegend). Cells were washed once with 1 mL of FACS buffer and analyzed using flow cytometry. The OVA-K^b antibody recognizes MHC class I-restricted SIINFEKL, an OVA-derived peptide. Fibroblasts were gated out using forward and side scatter profiles (see Supporting Information). This gating method was compared to

that based on cells staining positive for CD40 - NIH 3T3 cells were shown to stain negative for this marker - and no significant difference was observed between the two gating methods in terms of antigen presentation.

2.6 CD8⁺ T cell activation

To isolate antigen-specific CD8⁺ T cells, lymph nodes from an OT-1/PL TCR transgenic mouse were harvested, combined, homogenized against a wire mesh, and strained through a 70- μ m cell strainer. The cells were then washed once with PBS containing 2% FBS and resuspended in T Cell media. An aliquot of cells was stained with PE-labeled anti-murine CD8 (BioLegend) and PerCP Cy 5.5-labeled anti-murine CD90 (CD90.1) (BioLegend). The fraction of naïve CD8⁺ T cells present in the whole lymph node cell population was approximately 40% as determined by sizing the population staining positive with both antibodies. To determine T cell activation, NIH 3T3 cells were transfected followed by co-culture with DC 2.4 cells for 24 hours as described above. Lymph node cells from OT-1/PL TCR transgenic mouse were added to the co-culture so that approximately 6×10^5 CD8⁺ T cells were present in each well. After culturing for 3 days, the cell media was analyzed for IFN- γ production by ELISA using a Ready-Set-Go IFN- γ "Femto-HS" kit (e-Bioscience) following standard protocol provided by the manufacturer. In separate experiments, DCs alone were incubated with SIINFEKL peptide (New England Peptide, LLC) at 1 μ M for 1 hour or with lipopolysaccharide (LPS, Sigma) at 2 ng/mL overnight before T cells were added.

3. Results

3.1 Correlation between transfection efficiency and cytotoxicity in bystander cells

NIH 3T3 cells were transfected with PEI/DNA complexes of various N/P ratios for 4 hours in serum-free condition and analyzed by fluorescence microscopy and flow cytometry after 24 hours (Fig. 2A,B). The gate of GFP-positive cells was set based on cells treated with polyplexes of Luc plasmid as the GFP-negative population (Fig. S1), which controls for any increase of cell autofluorescence frequently observed after exposure to cationic polyplexes. As expected, the polyplexes were able to transfect fibroblasts efficiently, especially at lower N/P ratios (4 and 8), but the fraction of GFP⁺ cells decreased at higher N/P ratios (12, 16, 20). Toxicity in polyplex-treated cells was also determined by staining with annexin-V and PI, a common method for quantifying apoptosis and necrosis, and by the TUNEL assay, a common method for determining apoptosis. At relatively low N/P ratios (4 and 8), there was very minor toxicity with over 90% of cells having low levels of annexin-V and PI staining (Fig. 2C,D). With increasing N/P ratio (12, 16, 20), the fractions of apoptotic and necrotic cells increased as shown with high level of either annexin-V or PI staining or both. Comparing transfection efficiency (quantified by the percentage of GFP⁺ cells) with cytotoxicity (quantified by the percentage of annexin-V-high and PI-high cell populations), it is noted that at low N/P ratios (4, 8) when cell death due to apoptosis/necrosis was minimal (<10%), transfection efficiency was high (Fig. 2E). When higher N/P ratios (12, 16, 20) caused more cytotoxicity, transfection efficiency declined accordingly. The same trend was also observed when transfection efficiency was quantified in mean fluorescence intensity (MFI) (Fig. S2). To further elucidate the effect of transfection efficiency and cytotoxicity on immune activation, we focused on N/P ratios of 8 (high transfection/low toxicity) and 16 (low transfection/high toxicity) in the following studies.

3.2 Transfecting bystander cells leads to cross-presentation by DCs

To determine if transfected fibroblasts were able to transfer antigen to DCs to be cross-presented, polyplexes were prepared with DNA encoding the model antigen OVA and transfected 3T3 cells at N/P ratios of 8 and 16. To ensure that only the bystander cells were

transfected and not DCs, fibroblasts were washed repeatedly with buffer to remove any free polyplexes before adding DCs. The subsequent fibroblast/DC co-culture was conducted in serum-containing media because PEI/DNA polyplexes do not transfect well in the presence of serum. After the co-culture, DCs were stained for the OVA-derived peptide SIINFEKL presented specifically on MHC I complexes (Fig. 3). Despite not being directly transfected, DCs did stain positive for antigen presentation (~7%) at N/P 16, which strongly suggests that they were able to obtain the OVA antigen from transfected fibroblasts and process it for MHC I presentation. Fibroblasts transfected with polyplexes of GFP plasmid at N/P 16 did not result in any cross-presentation, confirming that cross-presentation seen with the OVA plasmid was antigen-specific. When transfected fibroblasts alone were stained for antigen presentation, the signal was similar to untreated cells, indicating that DCs are responsible for presenting the OVA antigen. For the flow cytometry analysis of the co-culture, the two types of cell population were separated by their distinct light scattering profiles (Fig. S3) or by different CD40 level (DCs were CD40-high) (Fig. S4). Both methods gave similar results of antigen presentation (~7% for N/P 16). Surprisingly, despite much higher transfection efficiency than N/P 16 (Fig. 1), there was no detectable cross-presentation for polyplexes of N/P 8 (Fig. 3), suggesting that cytotoxicity in bystander cells, rather than transfection efficiency, might be more important in causing cross-presentation.

3.3 Toxicity in bystander cells directly affects cross-presentation by DCs

To further elucidate the correlation between bystander cell toxicity and cross-presentation, we characterized the influence on presentation of OVA antigen in co-culture by either aggravating toxicity in fibroblasts (without altering transfection) or reducing toxicity in fibroblasts through inhibiting apoptosis (Fig. 4). First, we transfected the fibroblasts with polyplexes containing 2 µg of OVA plasmid at N/P 8, which showed very mild toxicity (~10%) (Fig. 4A) and did not result in any antigen presentation (Fig. 4B). We then doubled the dose by adding extra polyplexes containing 2 µg of non-antigen-encoding Luc plasmid at N/P 8, so that cytotoxicity increased to ~45% without altering OVA expression. As a result, OVA antigen presentation went up to ~4%. Moreover, compared to N/P 8, polyplexes of N/P 16 induced much higher cytotoxicity (~60%) as well as much higher OVA presentation (~6.5%). On the other hand, treating the fibroblasts with 50 µM of ZVAD 30 min prior to transfection effectively reduced cytotoxicity compared to those without ZVAD – from ~45% to ~25% (N/P 8×2 double-dose) and >60% to <40% (N/P 16). The amount of OVA presentation after ZVAD treatment went down accordingly, from an average of 4 to <3% (N/P 8×2 double-dose, not statistically significant) and 6.5% to 4% (N/P 16, $p < 0.05$) (Fig. 4B). These observations establish a direct correlation between toxicity in fibroblasts and cross-presentation by DCs.

3.4 Toxicity in bystander cells directly affects DC maturation

For DCs to effectively activate an immune response against presented antigen, they must be stimulated to mature. To determine if transfecting bystander cells alone with polyplexes could lead to DC maturation, we measured the level of CD40, a receptor that is up-regulated on mature DCs (Fig. 5). We observed higher CD40 expression after co-culture with transfected fibroblasts than untreated DCs and LPS treated DCs (Fig. 5A). CD40 level increased in response to either a higher N/P ratio (16 versus 8) or polyplex dose (single or double-dose at N/P 8), and decreased with the addition of the apoptosis inhibitor ZVAD (Fig. 5B). Polyplexes at N/P 16 containing Luc DNA caused the same degree of DC maturation as OVA DNA. Overall, DC maturation appears to be directly related to fibroblast cytotoxicity but independent of transfection efficiency and antigen expression.

3.5 CD8⁺ T cell activation

To induce an immune response, activated DCs must stimulate antigen specific naïve T cells in the lymph nodes. To determine if DCs could accomplish this after exposure to transfected bystander cells, fibroblasts were first transfected with polyplexes, co-cultured with DCs, followed by co-culture with naïve OVA specific CD8⁺ T cells harvested from an OT-1 transgenic mouse. These T cells express the transgenic T-cell receptor that can recognize the MHC I-restricted SIINFEKL peptide presented on the surface of DCs, thereby becoming activated to secrete IFN- γ . We found that co-cultured DCs were able to induce IFN- γ secretion in the antigen-specific T cells as determined by ELISA (Fig. 6). Compared with untreated cells, T cells stimulated with DCs pulsed with the SIINFEKL peptide and matured by LPS secreted high level of IFN- γ , which serves as the positive control. At N/P 8, the T cells did not produce IFN- γ beyond the level of untreated cells, but cytokine production was significantly increased when fibroblasts were transfected with OVA plasmid at N/P 16 (Fig. 6). This is consistent with our data demonstrating higher DC antigen presentation and maturation at the more cytotoxic N/P 16 (Fig. 4B and Fig. 5B). No IFN- γ was produced after transfection at N/P 16 using the non-antigen-encoding GFP plasmid, suggesting that observed T cell activation with OVA plasmid was antigen-specific and that both antigen expression and DC maturation are necessary. Finally, additional treatment of DCs with LPS during co-culture with fibroblasts transfected by OVA DNA polyplexes at N/P 16 resulted in a further increase in T cell stimulation to the same level of the positive peptide control, suggesting that exogenous adjuvants such as LPS may augment the effect by polyplex-induced cytotoxicity.

4. Discussion

What are the ideal target cells for polymer-based DNA vaccine delivery? Directly transfecting dendritic cells is an attractive means of promoting antigen display on MHC class I molecules for CD8⁺ T cell stimulation and a cellular immune response. However, this strategy requires overcoming intrinsic hurdles associated with DCs, a relatively rare cell type inherently challenging to transfect with foreign genes. Here we have demonstrated an alternative pathway for polymer/DNA vaccine delivery. We have shown that bystander cells such as fibroblasts transfected with PEI/DNA complexes are able to provide antigen to DCs for presentation on MHC class I molecules and trigger subsequent T cell stimulation in vitro. It is envisioned that for in vivo delivery, transfecting bystander cells, such as fibroblasts, at the site of injection may circumvent the difficulties in DC targeting/transfection and potentially generate robust T cell responses.

Another difficulty associated with directly targeting DCs is the cytotoxicity associated with many cationic polymer gene carriers. DCs need to remain functional after acquiring antigen to be able to prime naïve T cells, and toxicity in DCs resulted from direct transfection will compromise the immunostimulatory capacity and limit the life-time of these cells. When bystander cells are the intended target, however, the limitations of cytotoxicity of the polymer carrier can be lifted. The role of non-antigen-presenting bystander cells such as fibroblasts in immunomodulation comprises promoting immune tolerance to self-antigen under normal conditions and inducing immune activation during abnormal conditions such as inflammation, tissue injury, infections, and autoimmune diseases, both via communications with APCs.^{2,14,15} The context of antigen production by bystander cells including cell surface proteins and secreted factors is key to determining whether or not the APCs are activated. Along this line, we have shown here that some bystander cell death is necessary for efficient antigen presentation and DC activation. Dying cells are known to be a particularly effective source of antigen for cross-presentation.² Kurt et al. demonstrated that DCs obtaining antigen from apoptotic cells were as efficient at stimulating CD8⁺ T cells as directly transfected cells.²⁴ Perhaps the reason is that peptides may form complexes with

heat shock proteins (HSPs) produced by cells under stress, which have been shown to increase cross-presentation when used to deliver antigen to DCs.^{25,26} It has been shown that HSPs are better able to stimulate CD8⁺ T cell effector function rather than that of CD4⁺ T cells when complexed to peptide antigen.²⁷ In fact, the addition of hsp70 has been observed to enhance immune response when delivered along with cytotoxicity-inducing DNA.^{28–31} Dying cells could also be a potent source of antigen for cross-presentation simply because the protein antigen is rendered into particulate form by association with cellular debris.² Apoptotic bodies themselves have been shown to enhance cross-presentation.¹⁵ We also observed that transfection of bystander cells at N/P ratios high enough to cause significant cytotoxicity enhanced DC maturation. This suggests that bystander cell toxicity could be used not only to enhance cross-presentation but also as an adjuvant. Apoptotic and necrotic cells are known to release many factors (“endogenous adjuvants”) capable of both recruiting and maturing DCs, including HMGB-1, HSPs and uric acid, among others,³² which can activate immature DCs. While cytotoxicity of the polymer carrier is undesirable in many gene therapy applications, it could in fact be an advantage within the context of DNA vaccine delivery and immune activation. Thus, as depicted in Fig. 7, we propose a two-fold mechanism for how polyplex-induced bystander cell death can lead to CD8⁺ T cell response – by enhancing DC cross-presentation of acquired antigen and by stimulating phenotypic maturation of DCs.

The idea of causing toxicity in healthy cells deliberately in exchange for enhanced immune response and therapeutic benefit is not unheard of.³³ It has been shown that delivering naked DNA encoding a cytotoxic gene to healthy cells can cause regression of tumors of the same cell type.^{28–31} It was also determined that unlike a bacterial adjuvant such as Complete Freund's Adjuvant, cytoplasmic content damaged cells enhances both CD4⁺ and CD8⁺ responses specifically against particulate antigen.^{34,35} By analogy, polymer carriers of DNA vaccine could be designed and used in such a way to transfect bystander cells and to promote the death of these same cells to achieve immune activation against antigens. In light of this, future designs of DNA vaccine carriers for cancer therapy may incorporate the following considerations: 1) targeting not only healthy somatic cells, but also stromal cells within the tumor microenvironment or even cancer cells themselves; 2) maintaining a proper balance between cellular stress and transfection efficiency; 3) modulating the pathway of cell death to optimize antigen acquisition and DC maturation; and 4) delivering additional synergistic signals (such as TLR ligands) to boost the cross-priming of CD8 T cells. As we have demonstrated here the feasibility of using polymer carrier to target DNA vaccines to bystander cells as an effective means to immunotherapy, the ultimate test will be whether this strategy can be implemented effectively *in vivo* using appropriate disease models. We envision that certain modifications to the design of the polymer carrier will be necessary for *in vivo* application. For example, targeting moieties specific for fibroblasts or tumor stromal cells³⁶ may be conjugated to the polymer carrier to achieve selective uptake of the DNA vaccine by bystander cells. The extent of bystander cell death *in vivo* may also need to be fine-tuned through polymer-mediated co-delivery of cytotoxic agents³⁷ or cell death inhibitors³⁸ along with the DNA vaccine. Finally, the optimal route of administration^{39,40} of the polymer-DNA vaccine needs to be identified to maximize the potential benefit of bystander cell targeting.

5. Conclusions

Fibroblasts transfected with PEI/DNA complexes are effectively able to transfer antigen to DCs for presentation on MHC I molecules and to achieve subsequent T cell stimulation *in vitro*. Antigen presentation and T cell stimulation are both more effective when fibroblasts are transfected at high polyplex N/P ratios, despite lower protein expression in these conditions. This suggests the balance between transfection and polyplex-induced toxicity in

the fibroblasts is important for stimulating immune responses. Fibroblasts transfected at higher polyplex N/P ratios also induce higher DC maturation. These experiments demonstrate an alternative pathway to directly targeting DCs and raise the possibility of harnessing cytotoxicity of the polymer carriers for the benefit of DNA vaccine therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Schematic illustration of a co-culture system for the study of polymer-mediated DNA vaccine delivery to bystander cells (fibroblasts), dendritic cells (DCs), and antigen-specific CD8⁺ T cells, mixed sequentially and cultured for certain periods of time. It allows for the quantification of key cellular events such as transfection, cell death, antigen presentation, DC maturation, and T cell activation.

Fig. 2. PEI-mediated transfection efficiency in bystander cells is inversely correlated to toxicity. (A) A representative set of images of 24-h transfected and untreated NIH 3T3 cells. GFP positive cells are green and the nuclei of all cells are blue. Scale bar: 100 μ m. (B) Dot plots of GFP-transfected cells by flow cytometry. (C) Cytotoxicity of transfected cells determined by the TUNEL assay. Shown is a representative set of images of transfected cells with brown staining indicative apoptotic cells. Scale bar: 100 μ m. (D) Dot plots of apoptotic and necrotic cells by flow cytometry after staining with annexin V and PI. (E) Quantification of transfection efficiency and cytotoxicity (apoptosis/necrosis). Experiments were performed in triplicate and statistical significance between samples (*) was determined as $p < 0.0001$.

Fig. 3. Cross-presentation of OVA antigen by DCs co-cultured with transfected bystander cells. MHC-I-restricted SIINFEKL peptide was detected by immuno-fluorescence staining and analyzed by flow cytometry. Shown are a representative set of dot plots (A) and quantification of three experiments (B). *Statistical significance of the “N/P 16” sample compared to all other samples was determined as $p < 0.0002$.

Fig. 4.

Cross-presentation of SIINFEKL peptide by DCs is dependent on bystander cell toxicity. Fibroblasts were incubated with either an extra dose of polyplex at N/P=8 (Luc DNA) or treated with ZVAD during transfection. Fibroblasts were analyzed for cytotoxicity (A) and quantified for cross-presentation after co-culture with DCs (B). Experiments were performed in triplicate and statistical significance (*) was determined by (A) $p < 0.01$, (B) $p < 0.05$.

Fig. 5. Effect on DC maturation state by bystander cell transfection. DCs were incubated with fibroblasts after transfection, stained for CD40 expression, and analyzed by flow cytometry. Shown are a representative set of histograms of CD40 level (A) and quantification of the mean fluorescence intensity (MFI) of treated cells (B). Experiments were performed in triplicate and statistical significance (*) was determined by $p < 0.05$.

Fig. 6. Transfecting bystander cells leads to activation of antigen-specific naïve CD8⁺ T cells. SIINFEKL-specific OT-1 T cells were co-cultured with DCs and transfected fibroblasts and the amount of IFN-γ secretion was determined by ELISA. Experiments were performed in triplicate and statistical significance (*) was determined by p<0.001.

Fig. 7.

A proposed mechanism of polymer-mediated DNA vaccine delivery through targeting bystander cells to achieve T cell activation. Solid circles depict cellular events that have been quantified in this study. Effective T cell activation requires antigen-presentation by mature DCs. Polymeric carriers can achieve high transfection efficiency and antigen expression in bystander cells. Expressed antigen is then acquired by immature DCs and cross-presented (blue arrows). On the other hand, intrinsic polymer-induced cytotoxicity in bystander cells may enhance antigen acquisition and maturation by DCs (black arrows). Finally, maintaining a proper balance between transfection efficiency and cytotoxicity in bystander cells is crucial, because too much cell death reduces antigen expression (red blunt arrow).