CELL-BASED THERAPY

RESEARCH ARTICLE

Plasmid DNA adsorbed onto cationic microparticles mediates target gene expression and antigen presentation by dendritic cells

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Dendritic cells (DC) play a key role in antigen presentation and activation of specific immunity. Much current research focuses on harnessing the potency of DC for vaccines, gene therapy, and cancer immunotherapy applications. However, DC are not readily transfected *in vitro* by traditional nonviral techniques. A novel DNA vaccine formulation was used to determine if DC are transfected *in vitro*. The formulation consists of plasmid DNA adsorbed on to cationic microparticles composed of the biodegradable polymer polylactide-co-glycolide (PLG) and the cationic surfactant, cetyltrimethylammonium bromide (CTAB). Using preparations of fluorescent-labeled plasmid DNA formulated on PLG-CTAB microparticles to study internalization by macrophages and dendritic cells *in vitro* and *in vivo*, we found that most, but not all, of

the fluorescence was concentrated in endosomal compartments. Furthermore, uptake of plasmid DNA encoding HIV p55 gag adsorbed to PLG-CTAB microparticles by murine bone marrow-derived dendritic cells resulted in target gene expression, as detected by RT-PCR. The antigen was subsequently processed and presented, resulting in stimulation of an H-2k^d-restricted, gag-specific T cell hybridoma. Activation of the hybridoma, detected by IL-2 production, was dose-dependent in the range of 0.1–20 µg DNA (10–2000 µg PLG) and was sustained up to 5 days after transfection. Thus, adsorption of plasmid DNA on PLG-CTAB microparticles provides a potentially useful nonviral approach for *in vitro* transfection of dendritic cells. Gene Therapy (2000) 7, 2105–2112.

Keywords: DNA delivery; dendritic cells; cationic microparticles, DNA vaccines

Introduction

Dendritic cells (DC) have been characterized as the most potent antigen presenting cells of the immune system (reviewed in Refs 1 and 2). Specifically, DC efficiently internalize, process and present antigens, and also migrate to lymphoid organs, secrete cytokines, and express co-stimulatory molecules required for lymphocyte signaling. Their central role in initiating potent immune responses by eliciting helper and cytotoxic T cells, antibodies, and IL-12, has made them logical targets for development of both prophylactic and therapeutic applications.² Targeting antigens into dendritic cells *in vivo* or exposing DC to antigen *ex vivo* may enhance the immunogenicity of vaccines or induce T cell-mediated anti-tumor responses for cancer immunotherapy.

The development of methods to generate large numbers of DC has allowed researchers to harness their potent immunostimulating properties.^{3,4} The discovery that granulocyte-monocyte colony-stimulating factor (GM-CSF) was the key cytokine involved in differentiation of hematopoietic progenitors to dendritic cells

made it possible to generate dendritic cells from murine bone marrow (BMDC).^{5,6} Since that time, many preclinical studies using mouse models have elaborated on the important role these cells play in generation of the immune response and demonstrated their potential use in antitumor immunity (reviewed in Ref. 7). Anti-tumor cytotoxic T lymphocyte activity and protection against lethal tumor challenge in mouse models have been demonstrated using cytokine-driven BMDC pulsed with tumor-associated peptides and whole tumor lysates transferred by the subcutaneous route.^{8,9} Several clinical trials have established general safety and feasibility, and more are ongoing to demonstrate clinical efficacy of DC vaccination in several types of cancer including melanoma, prostate, breast, lung, colorectal, and renal cell carcinomas (reviewed in Ref. 7). Yet another strategy currently in development involves the introduction of genes encoding antigens directly into DC, allowing direct priming of both cytotoxic and helper T cell responses.

Although recombinant viral vectors or intracellular bacteria can efficiently deliver genes encoding antigen to DC and induce cell-mediated immunity,^{10–12} issues of safety and manufacturability continue to be addressed.^{13–16} *In vitro* transfection efficiency of DC by nonviral methods has been extremely poor.¹⁷ While progress has been made by the use of electroporation, the efficiency of DC transfection is extremely low and results in substantial loss of

cell viability.¹⁸ To date, no purely chemical method has been shown to be effective. To facilitate transfection of DC and recombinant gene expression, DNA must be delivered to the nucleus, and overcome the initial barriers of uptake and endosomal degradation.¹⁹

DC can capture antigen at peripheral sites via macropinocytosis using membrane ruffling, or may also internalize antigen by receptor-mediated processes involving FcγIII, the mannose receptor, or the C-type lectin DEC-205 (reviewed in Ref. 20). Thus, DC may be targeted by the capture of larger (>250 nm) particulate antigens by phagocytosis. Biodegradable polymer microspheres such as polylactide-co-glycolide (PLG) are readily internalized by phagocytic cells up to a diameter of 5 μm²¹ and have been utilized as carriers for drug delivery systems. The application of synthetic biopolymers for nucleic acid delivery has proven advantageous by protecting DNA against nuclease activity²² and increasing cellular uptake.²³ While polyalkylcyanoacrylate nanoparticles have been used to bind cetyltrimethylammonium bromide (CTAB)-oligonucleotide complexes to deliver antisense oligonucleotides to macrophage cell lines *in vitro*,^{23,24} these vehicles have not been shown to transfect DC with plasmids carrying recombinant genes.

Our approach utilized a novel platform technology for gene delivery involving adsorption of DNA on the surface of PLG microparticles prepared with a cationic surfactant. The strategy of adsorbing plasmid DNA on PLG-CTAB microparticles was originally developed to enhance the immunogenicity of DNA vaccines whose mechanism of action remains poorly understood.²² The goal of the present study was to determine whether cationic microparticles can deliver recombinant genes to antigen presenting cells, specifically dendritic cells, resulting in gene expression, antigen processing, and presentation of epitopes to T cells. Here, using an *in vitro* model system of murine bone marrow-derived dendritic cells, we demonstrate efficient uptake of fluorescent-labeled DNA adsorbed on to PLG-CTAB microparticles, resulting in direct transfection and gene expression. By incorporating an H-2k^d restricted T cell hybridoma into the model system, we show antigen processing and presentation of major histocompatibility complex (MHC) class I epitopes.

Results

Internalization of PLG-CTAB-DNA microparticles by phagocytic cells

The intracellular distribution of rhodamine-labeled plasmid DNA adsorbed onto 1 μm PLG-CTAB microparticles was examined in mouse bone marrow-derived dendritic cells on day 6 of culture from 15 min to 18 h after exposure. Uptake was maximal at 18 h. Confocal micrographs of representative cells are shown in Figure 1. Rhodamine fluorescence was seen predominantly in a sharp punctate distribution (Figure 1a). The late endosomal distribution in the same cells is visualized by immunostaining for lysosomal-associated-membrane protein-1 (LAMP-1) with a Cy2-conjugated secondary antibody (Figure 1b). Superimposition of the two images shows that much of the DNA signal colocalized with LAMP-1 immunoreactivity (Figure 1c). Some diffuse rhodamine fluorescence, which did not colocalize with LAMP-1, was

Figure 1 Internalization of PLG-CTAB-DNA microparticles by BMDC. Murine bone marrow-derived dendritic cells were pulsed with rhodamine-labeled plasmid DNA adsorbed on to PLG-CTAB microparticles. (a) Confocal micrograph showing intracellular distribution of rhodamine-labeled DNA (red); (b) immunofluorescence of anti-lysosomal-associated membrane protein-1 (green); and (c) superimposition of the two confocal micrographs indicating co-localization (yellow-orange).

also present. Similar patterns of internalization were observed with PLG-CTAB microparticles labeled with the fluorescent dye, DiI (3,3'-diiododecylindocarbocyanine), encapsulated fluorescein isothiocyanate-labeled bovine serum albumin, or naked rhodamine-labeled plasmid DNA (data not shown).

Target gene expression in BMDC

To determine whether uptake of plasmid DNA formulated on PLG-CTAB microparticles resulted in transfection of BMDC, we examined plasmid-encoded gene expression by reverse transcriptase (RT)-PCR. Using a plasmid encoding the HIV p55 gag protein under the control of the cytomegalovirus early promoter (pCMVkm2.GagMod.SF2),²⁵ BMDC on day 6 of culture were treated with the naked plasmid, or the same plasmid formulated on PLG-CTAB microparticles. Messenger RNA was isolated 24 h later, and transgene expression was analyzed by RT-PCR.

As illustrated in Figure 2, gag gene expression was detected only in extracts from cells treated with PLG-CTAB-DNA preparations, and not in cells treated with naked plasmid DNA. Control PCR-only reactions demonstrate that the gag sequence detection depended on RT

Figure 2 Transfection of BMDC by PLG-CTAB-DNA microparticles. Following a 24 h treatment with 1 μ g/ml DNA, alone or adsorbed on to PLG-CTAB microparticles, mRNA was isolated from cell lysates and subjected to RT-PCR for detection of target HIVgag gene expression (294 bp product). Amplified sequences were analyzed by agarose gel electrophoresis. Lane designations are as follows: (1) 500 bp DNA ladder; (2–4) β -actin control RT-PCR reactions from untreated, plasmid DNA, and PLG-CTAB-DNA treated BMDC; (5) 100 bp DNA ladder; (6) untreated mRNA prep with control spike of pCMVkm2.GagMod.SF2; (8–10) p55gag RT-PCR from untreated, plasmid DNA, and PLG-CTAB-DNA treated BMDC; (11–13) PCR control (no RT) from untreated, plasmid DNA, and PLG-CTAB-DNA treated BMDC; (14) pCMVkm2.GagMod.SF2 PCR positive control.

activity and was not the result of plasmid DNA contamination in the mRNA preparation. In an attempt to detect intact reporter gene products, BMDC were treated with a plasmid encoding β -galactosidase adsorbed to PLG-CTAB microparticles. When stained for β -galactosidase activity, we were only able to detect functional enzymatic activity in a control, nonprofessional antigen presenting cell line transfected by lipofectamine–DNA complexes. No β -galactosidase activity was detected in PLG-CTAB-DNA-pulsed BMDC, a professional antigen presenting cell.

Antigen processing and presentation to an H-2K^d restricted T cell hybridoma

Epitope presentation and stimulation of T cells is a sensitive and functionally relevant method for detection of target antigen expression, processing, and presentation. Using an H-2K^d-restricted T cell hybridoma specific for the p7g (199-AMQMLKETI-207) epitope of HIV-1_{SF2} p24gag, we investigated antigen presentation by BMDC. Bone marrow cells differentiated in the presence of GM-CSF for 6 days had the surface phenotype of immature DC as determined by FACS analysis (CD11c⁺, CD11b⁺, H-2K^d+, I-A^{d(low)}, CD80^(low), CD86^(low)). On day 9, the cells had the surface phenotype of mature DC (CD11c⁺, CD11b⁺, H-2K^d+, I-A^{d(bright)}, CD80⁺, CD86⁺).²⁶ Both immature and mature BMDC were cultured for 24 h with pCMVkm2.GagMod.SF2 plasmid DNA alone, or adsorbed to PLG-CTAB microparticles. Treated BMDC were plated with the gag-specific T cell hybridoma and incubated 24 h further to allow for antigen processing and presentation of the p7g epitope to the T cell hybridoma, resulting in the secretion of IL-2 into the culture medium. In a single experiment, we compared production of IL-2 in BMDC of both immature (day 6) and mature (day 9) phenotypes. Treatment of immature and mature BMDC with PLG-CTAB-DNA resulted in relative IL-2 increases of 2.1 ± 0.4 and 2.2 ± 0.1 , respectively. Since there was no significant difference in the capacity for

stimulation by PLG-CTAB-DNA, subsequent experiments were performed only with day 6 BMDC.

As shown in Figure 3, only PLG-CTAB-pCMVkm2.GagMod.SF2 treated BMDC significantly stimulated IL-2 production above background ($P < 0.05$), as determined by ELISA. Relative increases of IL-2 in PLG-CTAB-DNA-treated BMDC ranged from 1.8- to 162-fold over untreated cells, reflecting the considerable variability in this complex system ($n = 15$ experiments; 159 ± 46 pg/ml IL-2). Negative controls included untreated cells and cells treated with PLG-CTAB microparticles alone. Cells were also treated with the pCMV-luciferase plasmid either alone or adsorbed on to PLG-CTAB microparticles to demonstrate antigen specificity. Furthermore, PLG-CTAB-DNA-mediated stimulation of IL-2 production was dependent on the presence of antigen presenting cells, as the hybridoma treated with PLG-CTAB-DNA did not result in detectable levels of IL-2 (data not shown). Naked DNA encoding gag in the presence of free CTAB also did not result in antigen presentation, nor did another traditional means of *in vitro* cell transfection, ie commercial lipid:DNA complexes (Figure 3). Vaccinia-gag infected cells, included as a positive control in a separate experiment, produced 1650 pg/ml IL-2, approximately 10 $\times$ greater than the mean observed with PLG-CTAB-transfected cells.

In addition to specificity for an H-2K^d-restricted epitope of HIV p55gag, the T cell hybridoma used in this

Figure 3 Specific stimulation of an MHC class I T cell hybridoma by PLG-CTAB-p55gag DNA microparticles. Balb/c mouse bone marrow cells were differentiated into dendritic cells by growth in the presence of GM-CSF. Cells were treated with the indicated formulations of 1 μ g/ml plasmid DNA on day 6 of culture and incubated 24 h further. DNA denotes the pCMVkm2.GagMod.SF2 plasmid encoding HIV-1 gag; plucDNA denotes a non-specific plasmid encoding luciferase. Cells were co-cultured with an H-2K^d-restricted, HIV-1 gag-specific T cell hybridoma at a ratio of 1:10 for 24 h. Specific T cell activation was measured by assaying IL-2 in culture supernatants. Values show mean $\pm$ s.e. of the relative increase in IL-2 secretion over untreated cell background for duplicate samples from separate experiments ($n = 15$).

study was generated using the *lacZ*-inducible BWZ.36 fusion partner harboring the *Escherichia coli lacZ* reporter gene under the control of the nuclear factor of activated T cells (NFAT) enhancer element of the IL-2 gene.²⁷ Thus we were able to confirm the results obtained by IL-2 ELISA by assaying β -galactosidase activity following exposure to treated BMDC. A 4.8-fold increase over background in β -galactosidase-expressing T cells was observed in PLG-CTAB-pCMVKm2.GagMod.SF2-treated BMDC; the average number of β -gal positive cells per 800 μm^2 field was 37 ± 12 versus 180 ± 64 , respectively ($n = 4$; $P \leq 0.01$).

Considering the induction of IL-2 by an epitope-specific T cell hybridoma as an *in vitro* model, we examined the kinetics of antigen presentation after PLG-CTAB-mediated transfection and epitope presentation in the context of MHC class I. BMDC were treated with PLG-CTAB-pCMVKm2.GagMod.SF2 on day 6 of culture and incubated for 1–5 days before addition of the H-2K^d-restricted hybridoma. As indicated in Figure 4, the measured response was greatest at 24 h, but PLG-CTAB-pulsed BMDC stimulated the hybridoma as long as 120 h following internalization. In a parallel experiment, neither naked DNA, nor lipofectamine-complexed DNA stimulated the hybridoma during this time period. The stimulation of IL-2 production by T cell recognition of antigen presented in the context of MHC class I molecules was found to be dose dependent in the range of 0.1–20 μg DNA/ml (10–2000 μg PLG). In Figure 5a, levels of IL-2 production increased with the dose of DNA presented to BMDC, however it is of interest to note that

Figure 4 Kinetic response of PLG-CTAB-DNA-mediated antigen presentation. Balb/c mouse bone marrow cells were differentiated into dendritic cells by growth in the presence of GM-CSF. On day 6 of culture, cells were treated with 1 $\mu\text{g}/\text{ml}$ plasmid DNA in the formulations indicated or left untreated, and incubated for an additional 24 h. DNA denotes the pCMVKm2.GagMod.SF2 plasmid encoding HIV-1 gag. At the indicated time points following treatment, BMDC were plated with a H-2K^d-restricted, HIV-1 gag-specific T cell hybridoma at a ratio of 1:10 and incubated 24 h further. Culture supernatants were assayed for T cell-specific stimulation by IL-2 ELISA. Data points represent the average relative increase IL-2 production over untreated cells from a representative experiment.

at higher doses substantial toxicity was also observed, as shown by trypan blue exclusion (Figure 5b).

Discussion

Cationic microparticles have been shown to augment both the humoral and cellular immune responses to DNA vaccines in animal models.²² In this study, we examined the uptake, gene expression, and antigen presentation mediated by plasmid DNA adsorbed to cationic microparticles. Using an *in vitro* model system, we have demonstrated direct nonviral transfection of dendritic cells mediated by PLG microparticles prepared with the cationic surfactant, CTAB. The expressed antigen was subsequently processed and presented as peptide epitopes in the context of MHC class I molecules, resulting in stimulation of an epitope-specific T cell hybridoma. These findings demonstrate a chemical method for transfecting dendritic cells *in vitro*, and implicate possible mechanisms for efficient generation of the cytotoxic T cell responses observed *in vivo*.²² Such cell-mediated

Figure 5 Dose response and toxicity of PLG-CTAB-DNA microparticles. BMDC were plated at a density of 5×10^5 per well, and treated with varying doses of pCMVKm2.GagMod.SF2 formulated on PLG-CTAB microparticles. Following a 24 h incubation, live cells were counted by trypan blue exclusion, and plated with the H-2K^d-restricted HIV-1 gag-specific T cell hybridoma. Culture supernatants were assayed for IL-2 production by ELISA (a); data points represent the mean relative increase $\pm$ s.e. of samples assayed in duplicate from a representative experiment ($n = 4$). (b) The percentage of viable cells in the total population following a 24 h incubation with the indicated concentration of PLG-CTAB-DNA.

responses are critical for prophylactic and therapeutic vaccines.

Previous reports have described cytotoxic T lymphocyte activity in mice immunized with plasmid DNA encapsulated in PLG microparticles.²⁸ We have developed novel biodegradable PLG microparticles stabilized by the cationic surfactant, CTAB which have been shown to be a highly effective adjuvant formulation for DNA vaccination. The microparticles were prepared with a mean size of 1 μm , and exhibited a net positive surface charge ($Z = 40 \text{ mV}$) allowing for adsorption of plasmid DNA. Following an initial burst release of about 35%, the DNA was released gradually and was protected from rapid nuclease degradation *in vitro*.²² In the present study, our goal was to determine whether the design of adsorbing plasmid DNA onto a particle would increase functional uptake of DNA by dendritic cells resulting in target gene expression and antigen presentation.

Using rhodamine-labeled plasmid DNA formulated on PLG-CTAB microparticles, we found efficient internalization by phagocytic cells including macrophages and DC. The intracellular distribution of rhodamine signal was primarily localized to endosomal compartments, which is similar to cellular uptake patterns of naked fluorescent-labeled plasmid *in vitro*, and what has been reported for plasmid taken up by Mac-1⁺ cells *in vivo*.²⁹ The presence of the cationic surfactant on the surface of microparticles may contribute to endosomal disruption and increased cytoplasmic or nuclear localization, although the mechanism of endosomal escape remains unclear. Recently, Newman *et al* reported cytoplasmic localization of Texas red labeled dextran encapsulated in PLGA microspheres following phagocytosis by mouse peritoneal macrophages.³⁰

The limit of detection for rhodamine-labeled plasmid DNA used in our studies was estimated at 100 copies per nucleus (P Felgner, personal communication). Although we did not observe fluorescence in the nuclei, we detected plasmid-encoded HIVgag gene expression by reverse transcriptase-PCR, indicating PLG-CTAB-mediated transfection of DC *in vitro*. Further evidence supporting direct transfection of cells by PLG formulations was recently reported by Ciftci and Su³¹ who found PLG microparticles containing a DNA:polycation complex provided controlled release of DNA and enhanced uptake and gene expression in 293 and MCF-7 cells. Our attempts to detect β -galactosidase reporter gene activity in BMDC were unsuccessful, although we have been able to detect intracellular fluorescence with rhodamine-labeled PLG-CTAB-DNA and readily detected HIV gag peptide presentation in PLG-CTAB-transfected DC.

Our *in vitro* model system used an H-2K^d-restricted T cell hybridoma to detect presentation of the HIV-1 gag-p7g epitope by antigen presenting cells. DC pulsed with PLG-CTAB microparticles formulated with the pCMVKm2.GagMod.SF2 plasmid encoding the HIV gag protein could specifically stimulate the antigen-specific T cell hybridoma to produce IL-2, while equivalent doses of the naked plasmid could not. Furthermore, the presentation of epitope was dose-dependent and sustained up to 5 days following transfection. These data led to two conclusions: first, direct transfection of DC may be the mechanism of action of increased immunogenicity observed following immunization with PLG-CTAB-

DNA,²² and second, *ex vivo* transfection of DC with plasmid adsorbed to PLG-CTAB microparticles may be a feasible approach for passive transfer immunotherapy.

We have shown that the use of cationic detergent-stabilized microparticles, which bind and deliver plasmid DNA, result in greater transfection than unmodified plasmid DNA or standard lipofection methods using the highly sensitive T cell hybridoma-based readout. The assay can detect as few as 10 BMDC pulsed with synthetic p7g peptide (unpublished results). By comparing levels of IL-2 production in peptide-pulsed- to PLG-CTAB-DNA-treated BMDC, we estimate that less than 10% of the cell population express and present antigen mediated by PLG-CTAB-DNA. Although PLG-CTAB-DNA treatment resulted in direct transfection of DC *in vitro*, IL-2 production (as a measure of transfection efficiency) was approximately 2% of that observed with a recombinant vaccinia virus expressing the HIV-1SF2p55gag gene (39 and 1648 pg/ml IL-2, respectively). Whereas pulsing surface MHC class I molecules with synthetic peptide epitope was shown to be highly efficient at stimulating the T cell response, even in untreated BMDC, this is not a feasible approach to genetic immunotherapy due to the polymorphism of MHC class I epitopes in an outbred population. Alternatively, treatment of dendritic cells with PLG-CTAB-DNA directly delivers the gene encoding antigen to the antigen presenting cell resulting in potential presentation of multiple class I and class II MHC epitopes.

Production of cytotoxic T cell activity is a desirable goal of immunization, particularly for protection against intracellular and viral pathogens, as well as cancer immunotherapy. Antigen presenting cells (APC) process and present peptide epitopes in the context of both class I and class II MHC molecules resulting in the induction of cytotoxic T lymphocytes facilitated by CD4⁺ T cell help. Direct priming of the cellular immune response by endogenous expression of genes encoding target antigens in APC may potentially result in the generation of 'naturally processed' epitopes presented on MHC class I molecules. Dendritic cells are logical targets for immunization since they are the most potent APC in the immune system, and are unique in their ability to activate naive T cells.¹ Akbari *et al*³² have suggested that transfection and activation of DC are key events for immunity following DNA vaccination by scarification of the ear skin in mouse models. The use of APC-transfecting nucleic acid-based vaccines therefore eliminates the limitations of peptide-based vaccines imposed by genetic polymorphism in MHC epitope selection, and reduces the need to direct protein-subunit vaccines into the endogenous antigen processing pathway for eliciting cytotoxic T cell responses. Therefore, targeting antigens to dendritic cells, either *in vitro* or *in vivo*, is important in facilitating the induction of potent immune responses.

In vitro generation of DC has been optimized sufficiently so that genetic immunotherapy based on passive transfer of DC has been extensively documented in clinical studies.^{3,4} Recombinant viruses can be used to deliver the gene of interest to cultured DC for such applications. While safety issues are considered a concern for such applications,³³ development of safer viral vectors is in progress.¹³⁻¹⁶ Physical and chemical methods of transfection (electroporation, lipofection, CaPO₄) which may offer advantages in safety as well as manufacturability

are useful for a variety of cell types, but ineffective for dendritic cells.¹³ Therefore, development of a purely chemical means of efficiently delivering genes to these potent APC for generation of immune responses either by passive transfer or direct immunization is desirable. We have demonstrated that DNA delivery by cationic microparticles is superior to that achieved with the standard lipofectamine procedure. Further optimization of transfection efficiency may ultimately provide a PLG-CTAB formulation suitable for transfection of APC for therapeutic applications.

PLG-CTAB-DNA formulations have been shown to elicit robust immune responses in small animal models.²² The formulation of plasmid DNA on cationic microparticles may allow gene delivery and provide additional adjuvant activity necessary for augmenting the immune response to DNA vaccines (reviewed in Ref. 34). In this study, we have demonstrated rapid internalization, target gene expression, and antigen presentation in DC pulsed with PLG-CTAB-DNA *in vitro*. Studies are underway to delineate the mechanism of action of PLG-CTAB-DNA *in vivo*. Future studies will investigate the potential use of PLG-CTAB-DNA formulations in passive transfer experiments with transfected DC for both prophylactic and therapeutic applications.

Materials and methods

Plasmids and DNA formulations

The pCMVkm2.GagMod.SF2 plasmid encoding the HIV-1 p55 gag protein under the control of the cytomegalovirus early promoter²⁵ was purified by ion exchange chromatography using a Qiagen Endo Free Giga Kit (Qiagen, Valencia, CA, USA) and determined to be endotoxin free (<2.5 EU/ml). For uptake and reporter gene expression experiments, plasmid DNA labeled with a rhodamine-peptide nucleic acid (PNA)-clamp encoding β -galactosidase was purchased from Gene Therapy Systems (San Diego, CA, USA). PNA-clamp does not alter the function or conformation of plasmids.³⁵ Plasmid DNA was adsorbed on to PLG-CTAB microparticles as previously described.²² Briefly, CTAB-stabilized microparticles were formulated by evaporation of methylene chloride from a water/oil/water emulsion containing 0.5% w/v cetyltrimethylammonium bromide. Microparticles were suspended in a 1 mg/ml plasmid DNA solution, washed, lyophilized, and stored dry at 4°C. DNA load was measured by an HPLC approach, which determined total DNA on the particles after dissolution of the polymer;²² PLG-CTAB-DNA formulations used in this study had an actual DNA load ranging from 0.64–0.81% (wt/wt). The size distribution of the microparticles was determined using a particle size analyzer (Malvern Instruments, UK); formulations used in this study had a mean size of $1.54 \pm 0.20 \mu\text{m}$. Lyophilized particles were suspended in saline, vortexed and sonicated immediately before use.

Cell culture

All cells used in this study were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 0.05 mM 2-mercaptoethanol at 37°C in a humidified 7% CO₂ incubator. The murine

T cell hybridoma 12.2 was generated by fusion of splenocytes from HIVgag-immunized CB6F1 mice with the BWZ.36 fusion partner (gift of N Shastri, University of California Berkeley), followed by cloning and selection based on IL-2 production in response to antigen presenting cells loaded with gag-derived peptides. The 12.2 T cell hybridoma is an H-2K^d-restricted line, which recognizes the p7g epitope (AMQMLKETI) of the HIV-1_{SF2}p24gag protein.³⁶

Bone marrow isolation

Female Balb/c mice, 6–8 weeks old, were obtained from Charles River Laboratories (Holister, CA, USA). All experimental procedures were approved by the Chiron Animal Care and Use Committee. Bone marrow was flushed from the femurs and tibias, washed and frozen (–80°C) in heat-inactivated FBS supplemented with 10% cell-culture grade dimethyl sulfoxide (DMSO) at a density of 2×10^7 cells/ml.

Generation of bone marrow-derived dendritic cells (BMDC)

Frozen cell aliquots were rapidly thawed and washed to remove DMSO. Cells were plated in 150 mm suspension culture dishes containing 20 ml supplemented RPMI with the addition of 200 U/ml murine GM-CSF (Peprotech, Rocky Hill, NJ, USA). On day 3 of culture, cells were again supplemented with murine GM-CSF, and on day 5, one-half of the culture volume was centrifuged to replace fresh medium containing GM-CSF. BMDC were harvested by gentle pipetting. Unless otherwise indicated, bone marrow derived DC were pulsed with the gene encoding antigen on day 6 and incubated for an additional 24 h. BMDC were analyzed for cell surface markers by FACS and were characterized as immature by staining positive for CD11c, CD11b, H-2K^d, I-A^{d(med)}, CD80^(low), and CD86^(low) (PharMingen, San Diego, CA, USA).

Microparticle uptake and confocal microscopy

BMDC were plated at a density of 10^6 cells in 2 ml medium in six-well culture dishes. Rhodamine-labeled DNA in the form of naked plasmid or formulated on PLG-CTAB microparticles was added to the wells at 1 μg DNA/ml. Following overnight incubation, cells were washed, fixed, and permeabilized using the Cytotfix/Cytoperm kit according to manufacturer's instructions (PharMingen) and stained with rat anti-mouse IgG clone 1D4B for the lysosomal-associated membrane protein 1 (LAMP-1, PharMingen), followed by incubation with goat anti-rat conjugated Cy2 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Cells were washed and applied to Superfrost microscope slides (Fisher Scientific, Tustin, CA, USA), mounted in Vectashield (Vector, Burlingame, CA, USA) and visualized using a Zeiss LSM 410 confocal microscope equipped with a krypton-argon laser and optimized photomultiplier tubes (Carl Zeiss, Thornwood, NY, USA). Images were documented as digital image files and printed on a digital printer (Fujix Pictography 3000, Fuji Film, Tokyo, Japan) from Adobe Photoshop (Adobe Systems, Mountain View, CA, USA).

RNA isolation and RT-PCR

BMDC were plated at a density of 0.5×10^6 cells/ml in RPMI + GM-CSF on day 6 of culture. Cells were

untreated as negative control or incubated in the presence of 1 µg/ml pCMVkm2.GagMod.SF2 DNA alone or formulated on PLG-CTAB microparticles. Following a 24 h incubation, 2×10^5 cells were removed, washed twice in cold PBS (Life Technologies, Rockville, MD, USA), then lysed per manufacturer's instructions for the mRNA Capture kit (Roche, Indianapolis, IN, USA) and frozen at -80°C . Samples were thawed on ice with the addition of RNase-free DNase and RNase inhibitor (Roche). The mRNA Capture kit isolation protocol was then followed to isolate oligo dT-biotin-hybridized mRNA in streptavidin-coated PCR tubes. The Promega Reverse Transcription System (Madison, WI, USA) was utilized for cDNA synthesis according to manufacturer's instructions. The reaction was run at 45°C for 45 min, followed by heat inactivation at 99°C for 5 min. As a control for contaminating plasmid DNA, duplicate samples of mRNA captured on coated PCR tubes were treated as stated above, but without the addition of AMV-reverse transcriptase. For PCR, primers specific for a 294 bp region of the HIV *gag* gene, or β -actin (BioSource International, Camarillo, CA, USA) as a positive control, were added to the RT-PCR reactions and amplified using standard PCR conditions.

IL-2 production by T cell hybridoma

The T cell hybridoma 12.2 was plated at 10^5 cells per well in 96-well, U-bottom microtiter plates. Varying numbers of BMDC were treated 24 h previously with the indicated naked or formulated plasmid DNA or a recombinant vaccinia virus encoding HIV-1_{SF2}gag (2–10 multiplicity of infection).³⁷ BMDC were washed and plated with the hybridoma in a total culture volume of 200 µl. Each experimental condition was performed in duplicate. After a 24-h co-culture period, the plates were centrifuged and the supernatants were removed and stored at -80°C until further assay for IL-2 production. The hybridoma used in this assay was generated using the *lacZ*-inducible BWZ.36 fusion partner that contains the *Escherichia coli lacZ* reporter gene under the control of the nuclear factor of activated T cells (NFAT) enhancer element of the IL-2 gene.²⁷ Following removal of culture supernatants, remaining cells were stained for β -galactosidase activity using the Invitrogen β -gal staining kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Relative numbers of stimulated hybridoma cells were determined by averaging the numbers of β -galactosidase positive cells counted under an 800 µm² field of an inverted light microscope. To assay levels of IL-2 secreted into the medium, culture supernatants were thawed at room temperature and plated on pretreated mouse IL-2 ELISA microtiter plates and analyzed per manufacturer's instructions (Endogen, Woburn, MA, USA). Following development of the colorimetric substrate, microtiter plates were read by a Molecular Devices vmax kinetic plate reader and analyzed with SoftMax software (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

Data are reported as mean $\pm$ standard error of the mean (s.e.). Significant differences between mean values of β -galactosidase positive cells were estimated using the Student's paired *t* test for unequal variances. Statistical sig-

nificance of differences among groups was estimated by analysis of variance (ANOVA).

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