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RESEARCH ARTICLE

Physical characterization and *in vivo* evaluation of poloxamer-based DNA vaccine formulations

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Abstract

Background Plasmid DNA (pDNA) vaccines have generated significant interest for the prevention or treatment of infectious diseases. Broader applications may benefit from the identification of safe and potent vaccine adjuvants. This report describes the development of a novel polymer-based formulation to enhance the immunogenicity of pDNA-based vaccines.

Methods Plasmid DNA was formulated with a nonionic block copolymer, poloxamer CRL1005, and the cationic surfactant benzalkonium chloride (BAK) to produce a thermodynamically stable, self-assembling system. The influence of parameters such as polymer concentration and BAK composition on the immune responses was evaluated in mice vaccinated with pDNA encoding influenza nucleoprotein.

Results At concentrations of 7.5 mg/ml CRL1005, 0.3 mM BAK and 5 mg/ml pDNA, CRL1005/BAK/pDNA particles had a mean diameter of 261 ± 0.2 nm and a surface charge of -11.6 ± 0.9 mV. The negative surface charge and atomic force microscopy images suggested that pDNA binds to BAK adsorbed to the surface of poloxamer particles. The CRL1005/BAK/pDNA formulation significantly enhanced antigen-specific cellular and humoral immune responses, and increased transgene levels in muscle and serum. The complexity of the formulation was reduced by replacing the commercial BAK, which is a mixture of four alkyl chains, with a C14 BAK homolog. The substitution yielded an analytically preferable formulation with equivalent physical characteristics and immunogenicity.

Conclusions The results suggest that the CRL1005/BAK/pDNA formulation may enhance immunogenicity by improving the delivery of pDNA-based vaccines. This formulation is currently being evaluated for the prevention of CMV-associated disease in a phase 2 clinical trial. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords plasmid DNA; vaccine; poloxamer CRL1005; block copolymer; gene delivery

Introduction

DNA vaccines are generating significant interest for the prevention or treatment of infectious diseases for human and veterinary applications [1–5]. Although results from preclinical studies of unformulated plasmid DNA (pDNA) have been encouraging, broader human applications may benefit from the identification of safe and potent adjuvants to enhance the immunogenicity of pDNA-based vaccines. One promising

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approach to increase the humoral and/or cellular immune responses is the use of micro- or nanoparticulate delivery systems, which allow delivery of phagocytosable particles to antigen-presenting cells (APCs) [4]. Amongst various particulate delivery systems, adsorption of pDNA onto polymeric micro- or nanoparticles reduces many of the manufacturing hurdles associated with encapsulation. Several clinical trials using such an approach are underway [4,6,7].

A microparticulate HIV pDNA vaccine is being tested in a phase 1 clinical trial for safety and immunogenicity [3]. The vaccine candidate employs a strategy of priming the immune responses using *gag* and *env* DNA plasmids, followed by boosting with a recombinant gp140 envelope protein formulated with an adjuvant. The pDNA prime uses a gene-delivery system in which the pDNA is loaded onto surface-modified poly(lactide-*co*-glycolide) (PLG) microparticles. The PLG microparticles are coated with the cationic surfactant cetyltrimethylammonium bromide (CTAB) during preparation using a standard double emulsion followed by solvent evaporation technique [8–10]. A resulting net positive surface charge of the microparticles allowed the negatively charged plasmids to bind, via electrostatic interactions, with high efficiency and loading capacity [4]. Studies performed to elucidate the mechanism of action of such a formulation have indicated that, after intramuscular administration, the transgene is expressed at the injection site, followed by recruitment of mononuclear phagocytes, activation of APCs, and subsequent presentation of the antigen-derived peptides in the context of MHC class I molecules [4,8,9].

Another prime-boost regimen investigated utilized poloxamer CRL1005 adjuvanted pDNA and an adenovirus type 5 vector boost, both expressing the HIV-1 *gag* gene [11]. Preclinical studies in nonhuman primates have described the immunogenic potential of this approach [6,11–13]. A recent publication [6] described some physical characteristics and immunological properties of a poloxamer formulation consisting of CRL1005 and the cationic surfactant benzalkonium chloride (BAK). The presence of BAK imparts a net positive surface charge to the particles, rendering them capable of binding pDNA through electrostatic interactions. Between 1.5–3% of the total pDNA in the formulation was shown to be associated with the CRL1005/BAK particles, corresponding to approximately 20–40 plasmids per particle.

A CRL1005/BAK-based pDNA vaccine formulation is currently in a phase 2 clinical trial for the prevention of cytomegalovirus (CMV)-associated disease in the allogeneic hematopoietic cell transplant population [14]. This plasmid-based immunotherapeutic vaccine contains two pDNAs encoding the major surface glycoprotein B (gB) and the tegument phosphoprotein 65 (pp65) of human CMV formulated with the nonionic triblock copolymer CRL1005 and BAK in phosphate-buffered saline (PBS).

CRL1005, a triblock POE-POP-POE copolymer with a formula $HO\text{-}(CH_2CH_2O)_8\text{-}[CH_2CHO(CH_3)]_{205}\text{-}(CH_2CH_2O)_8\text{-}H$, contains repeating units comprising a linear chain of hydrophobic polyoxypropylene (POP, Mw = 12 kDa), flanked by two linear chains of hydrophilic polyoxyethylene (POE, 5% of total molecular weight). This poloxamer has inverse solubility characteristics in aqueous media [15–17]. Below the phase transition temperature or cloud point (7–12 °C), this copolymer is water-soluble and forms a clear solution that can be sterile filtered. When a solution of copolymer is warmed and it passes through its cloud point, the increased thermal motion is sufficient to break the hydrogen bonds and, as the copolymer comes out of solution, it self-assembles into micelles or microparticles [18]. The process is reversible. In the presence of the hydrophobic cationic surfactant BAK, CRL1005 self-assembles into nanoparticles with BAK anchored onto the surface of the copolymer. BAK is a mixture of alkylbenzyldimethylammonium chlorides of general formula $[C_6H_5CH_2N(CH_3)_2R]Cl$, where R represents a mixture of alkyls, including $n\text{-}C_{12}H_{25}$ (C_{12} BAK), $n\text{-}C_{14}H_{29}$ (C_{14} BAK), $n\text{-}C_{16}H_{33}$ (C_{16} BAK) and possibly $n\text{-}C_{18}H_{37}$ (C_{18} BAK).

This paper describes the physicochemical characterization of poloxamer CRL1005-based formulations. The influence of parameters such as polymer concentration and BAK composition on the immune responses in mice vaccinated with pDNA encoding influenza nucleoprotein (NP), as a model immunogen, was evaluated.

Materials and methods

Materials and plasmid constructs

Poloxamer CRL1005 was obtained from CytRx Corporation (Los Angeles, CA, USA). Benzalkonium chloride 50% solution NF (BAK, BTC 50® NF) was obtained from Stepan Company (Northfield, IL, USA). Benzyldimethyldodecylammonium chloride (C_{12} BAK homolog) was obtained from Fluka Chemie (Buchs, Switzerland). Alkyldimethylbenzylammonium chloride, benzylcetyldimethylammonium chloride, and benzyldimethylstearylammonium chloride (C_{14}, C_{16}, and C_{18} BAK homologs, respectively) were purchased from TCI America (Portland, OR, USA). Plasmid VR4700 was constructed by inserting the A/PR/8/34 influenza NP coding sequence into an expression plasmid containing the CMV immediate early 1 promoter/enhancer and intron A, a modified rabbit β-globin transcriptional terminator and an open reading frame encoding the kanamycin resistance gene [19,20]. Plasmids VCL6368 encoding a detoxified CMV antigen pp65, VCL6365 encoding a secreted form of the CMV gB antigen lacking the transmembrane and cytoplasmic domains, VR1255 encoding firefly luciferase, and VR2956 encoding feline erythropoietin (EPO) were constructed as previously described [7,21].

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CRL1005/pDNA formulations

The required concentration of VR4700 pDNA (to produce a final concentration of 0.1 mg/ml) in PBS (0.9% sodium chloride + 10 mM sodium phosphate, pH 7.2) was stirred on ice and the required amount of CRL1005 (to produce a final concentration of 0.15 to 75 mg/ml) was added using a positive displacement pipette. The solution was then thermocycled through the cloud point (7–12 °C) several times to ensure homogeneity, filter sterilized through a Millipore Steriflip disposable vacuum filtration system (Millipore, Billerica, MA, USA) at 4 °C and stored frozen. CRL1005 formulations without pDNA were made as described above by dissolving CRL1005 in PBS.

CRL1005/BAK/pDNA formulations

The required concentration of VR4700 pDNA (to produce a final concentration of 5 mg/ml) in PBS was stirred on ice and the required amount of CRL1005 (to produce a final concentration of 7.5 mg/ml) was added using a positive displacement pipette. The solution was stirred on ice until the poloxamer dissolved and then the required concentration of BAK dissolved in PBS was added (to produce a final concentration of 0.3 mM). The mixture was then thermocycled through the cloud point several times to ensure homogeneity, filter sterilized through a Millipore Steriflip disposable vacuum filtration system at 4 °C and stored frozen. Prior to injection, the vaccine was thawed at ambient temperature (25 °C) and diluted to the required pDNA concentration with PBS above the cloud point of CRL1005. The particle sizes and surface charges of formulations did not change upon dilution (data not shown). CRL1005/BAK formulations without pDNA were made as described above by dissolving the CRL1005 and BAK in PBS.

CRL1005/BAK/pDNA formulations with different particle sizes

The size of CRL1005/BAK/pDNA particles was found to depend on the formulation concentration during storage. The CRL1005/BAK/pDNA formulations with different particle sizes (Z average diameter of 250, 350, 550, 1000, or >1000 nm) tested for immunogenicity were achieved by thermocycling the formulation through the cloud point as described above (at 7.5 mg/ml CRL1005 + 0.3 mM BAK + 5 mg/ml pDNA), diluting the formulation above the cloud point with PBS to various concentrations, and storing them frozen at −80 °C in glass vials. Prior to injections, the formulations were thawed at ambient temperature and diluted with PBS to the final concentration (0.15 mg/ml CRL1005 + 6 μM BAK + 0.1 mg/ml pDNA).

CRL1005/BAK homolog formulations

CRL1005/BAK and CRL1005/BAK/pDNA formulations containing only the C_{14} BAK homolog at a concentration of 0.3 mM were prepared as described in the section before last. CRL1005/BAK formulations containing C_{12}, C_{16} or C_{18} BAK homologs were also prepared as described above (without pDNA) by dissolving the CRL1005 and BAK homologs in PBS.

Particle size and zeta potential measurements

Particle size measurements were made using photon correlation spectroscopy (3000HS Zetasizer, Malvern Instruments, Worcestershire, UK). The instrument was calibrated using a 200 nm latex standard (200 nm latex nanospheres; Duke Scientific, Palo Alto, CA, USA) diluted in PBS at pH 7.2. Prior to analysis, samples were diluted into PBS as per the instrument manufacturer's guidelines. The Z average or the hydrodynamic diameter (nm) and polydispersity of the size range were calculated using cumulants analysis. Zeta potential measurements were made using micro-electrophoresis (Malvern 3000HS Zetasizer). The instrument was calibrated using a −50 mV Malvern zeta potential transfer standard (Malvern Instruments). Prior to analysis, samples were diluted into 20 mM Tris acetate (pH 7.2) as per the instrument manufacturer's guidelines. The zeta potential was derived from the electrophoretic mobility by applying the Smoluchowsky relationship and was quoted in millivolts (mV).

Fluorescence microscopy

Poloxamer particles were visualized with Nile Red (Molecular Probes Inc., Eugene, OR, USA), a dye which becomes fluorescent in nonpolar environments [22]. Nile Red was dissolved in dimethylformamide (Sigma, St. Louis, MO, USA) at 8 mg/ml, and added into CRL1005 or CRL1005/BAK formulations in PBS (final concentration of Nile Red 42 μg/ml). Formulations were placed on poly-L-lysine-coated microscope slides (Electron Microscopy Sciences, Ft. Washington, PA, USA) and examined using a 100× oil immersion objective with a Nikon Optiphot microscope fitted with a Nikon narrow band pass G-1B fluorescence filter (excitation wavelength 546 nm, barrier filter 590 nm), which allowed visualization of the 628 nm emission of Nile Red. Digital phase contrast and fluorescent photomicrographs were taken using a Spot RT color camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA). The size of CRL1005 particles was estimated by measuring the diameter of all poloxamer particles in seven randomly selected phase contrast images (a total of 427 particles were measured) using the Image-Pro Plus software (version 5.1.0.20; BioImaging Solutions Inc., San Diego, CA, USA). The imaging software

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was calibrated using a stage micrometer (Fisher Scientific, Hampton, NH, USA).

Atomic force microscopy (AFM)

Plasmid DNA was diluted to 2.5 μg/ml with 2.4 mM $NiCl_2$. A 10 μl drop was applied to a freshly cleaved mica surface. After incubation for 5 min in a closed environment at ambient temperature, the substrate was washed with nano-pure water (Barnstead Diamond RO water purification system, Garner, NC, USA) and then dried with a stream of nitrogen. The CRL1005/BAK/pDNA formulation was diluted with a solution of 5 mM $NiCl_2$ in PBS, to a final pDNA concentration of 50 μg/ml, and a 40 μl drop was applied to a freshly cleaved mica surface. The preparation was incubated for 120 min and similarly processed for AFM imaging. AFM images were taken in tapping mode using a JEOL JSPM-5200 in air. Topographic and phase images were simultaneously collected. Phase imaging AFM can detect variations in composition, adhesion, friction, viscosity, and is used to supplement information on surface structure.

Vaccination regimen

Groups of 6- to 10-week-old female BALB/c mice (Harlan-Sprague-Dawley, Indianapolis, IN, USA) received bilateral intramuscular injections (on days 0, 20, and 48) into the *rectus femoris* with 5 μg of VR4700 pDNA ± formulation in 50 μl PBS/leg (total dose of 10 μg of pDNA/injection) as previously described [19]. Mice were bled via the ophthalmic venous plexus 61 days after the first injection, and sera were stored at −20 °C until assayed for NP-specific antibodies by enzyme-linked immunosorbent assay (ELISA). Splenocytes were harvested on three consecutive days, 9 weeks after the first injection (on day 62 through 64) and NP-specific $CD8^+$ and $CD4^+$ T-cell responses were measured by IFN-γ ELISPOT assay.

In studies assessing immunogenicity of C_{14} BAK homolog formulations, 8- to 11-week-old female BALB/c mice were vaccinated with the bivalent CMV vaccine containing equal amounts of pp65 pDNA (VCL6368) and gB pDNA (VCL6365). On days 0 and 14, mice received bilateral injections of CRL1005/BAK/pDNA formulations into the *rectus femoris* muscles (20 μg total pDNA in 100 μl PBS/mouse/injection). Splenocytes were harvested on three consecutive days (on days 27 through 29) 4 weeks after the first injections. In each collection, spleens from two mice per test group were pooled, and the pools were assayed for CMV pp65-specific IFN-γ-secreting T-cells using an ELISPOT assay. Mice were bled on day 26, and CMV gB-specific antibodies were analyzed by ELISA. All animal procedures were approved by the Vical Institutional Animal Care and Use Committee (IACUC) and complied with the standards set forth in the Guide for the Care and Use of Laboratory Animals (ILAR, 1996) and the Animal Welfare Act and Animal Care Regulations [23].

Antibody ELISA assays

Serum anti-NP IgG titers were measured in an indirect ELISA using 96-well plates (Corning Incorporated, Corning, NY, USA) coated with 71 ng/well of influenza A/PR/8/34 nucleoprotein (NP) purified from recombinant baculoviral extracts as previously described [19,21]. The anti-gB ELISA was performed as previously described [7]. Endpoint titers were determined as the reciprocal of the last dilution at which the absorbance value of the test serum was at least twice that of the absorbance value of the background established with pre-immune serum.

IFN-γ ELISPOT assays

Mice were sacrificed over three consecutive days. In each collection, splenocytes from three mice out of each test group were isolated, pooled, and the pools were assayed for NP-specific IFN-γ-secreting T-cells. ImmunoSpot plates (Millipore, Billerica, MA, USA) were coated with 5 μg/ml of rat anti-mouse IFN-γ monoclonal antibody (BD Pharmingen, San Diego, CA, USA) and blocked with RPMI-1640 medium containing 10% (v/v) defined fetal bovine serum (FBS, Hyclone, Logan, UT, USA). Splenocyte suspensions were seeded in quadruplicate wells of ImmunoSpot plates at 1×10^6 cells/well in RPMI-1640 medium containing 25 mM HEPES buffer and L-glutamine (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) FBS, 55 μM β-mercaptoethanol (Invitrogen), 100 U/mL of penicillin G sodium salt and 100 μg/mL of streptomycin sulfate (Invitrogen). For detection of NP-specific IFN-γ-secreting $CD8^+$ T-cells, splenocytes were stimulated with the NP class I peptide, TYQRTRALV [24], at 4 μg/ml with 1 unit/ml of recombinant mouse IL-2 (Roche, Indianapolis, IN, USA). For detection of NP-specific IFN-γ-secreting $CD4^+$ T-cells, splenocytes were stimulated with a cocktail of three NP class II peptides, FWRGENGKTRSAYERMCNILKGK, AVKGVGT-MVMELIRMIKRGINDRN, RLIQNSLTIERMVLSAFDERRNK [24–26], at 10 μg/ml each. To assess nonspecific stimulation, splenocytes were plated in the absence of NP peptides, with or without added IL-2. After overnight incubation at +37 °C in 5% CO_2, captured IFN-γ from stimulated cells was detected by the sequential addition of biotin-labeled rat anti-mouse IFN-γ monoclonal antibody (1 : 1000 dilution, BD Pharmingen) and horseradish peroxidase-labeled avidin D (Vector Labs, Burlingame, CA, USA). Spots produced by the conversion of 3-amino-9-ethylcarbazole substrate (AEC, Vector Labs) were quantified using an ImmunoSpot analyzer (Cellular Technology Ltd., Cleveland, OH, USA). For each test group, the average background count derived from unstimulated cells was subtracted from counts obtained from each well of stimulated cells. Data were presented as the number of antigen-specific IFN-γ-producing T-cells, designated as spot forming units (SFU) per million splenocytes (SFU/10^6 cells). ELISPOT assays for the quantitation of

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pp65-specific IFN-γ-secreting T-cells were performed as previously described [7].

Reporter gene assays

To quantitate transgene expression levels in the injected tissue, muscles were harvested, pulverized and extracted as previously described [19]. Luciferase activity in muscle extracts was assayed using a Wallac model 1420 Victor^2 (PerkinElmer, Waltham, MA, USA) microplate luminometer as previously described [21]. Erythropoietin (EPO) levels in serum or muscle extracts were assayed using a commercially available kit (R&D Systems, Minneapolis, MN, USA) following the instructions provided in the kit.

Statistical analysis

Statistical analysis was performed using either Kruskal-Wallis one-way analysis of variance (ANOVA), Student's *t*-test, or the nonparametric Mann-Whitney rank sum test (SigmaStat version 2.03; Systat Software, Inc., Point Richmond, CA, USA).

Results

Particle size and zeta potential of CRL1005/BAK/pDNA formulations

To better isolate the physical interactions of the components of the CRL1005/BAK-based pDNA vaccine formulations, the copolymer alone was characterized first. When CRL1005 was dissolved in PBS and cycled through the cloud point, the copolymer self-assembled into particles at room temperature. The size of these spherical particles was dependent on poloxamer concentration, with a trend of increasing particle sizes with increasing CRL1005 concentration (Figure 1A). At 7.5 mg/ml, CRL1005 produced micron-sized particles with a mean hydrodynamic diameter of 1445 ± 32 nm and a negative zeta potential of -4.1 ± 0.5 mV. The size and the charge of the particles could be manipulated with the cationic surfactant BAK (or with other cationic lipids, data not shown) to produce uniform-sized submicron particles with a positive surface charge. Increasing BAK concentration from 0.3 to 0.9 mM had little effect on the size of CRL1005/BAK particles (Figure 1B), but increased the surface charge (Figure 1C). CRL1005/BAK particles, at 7.5 mg/ml CRL1005 and 0.3 mM BAK concentration, had a mean hydrodynamic diameter of 186 ± 0.6 nm and a positive zeta potential of $+8.3 \pm 0.8$ mV (Table 1). Formulating these particles in the presence of 5 mg/ml pDNA produced CRL1005/BAK/pDNA particles with a mean hydrodynamic diameter of 261 ± 0.2 nm and a negative zeta potential of -11.6 ± 0.9 mV. The negative surface charge of CRL1005/BAK/pDNA particles is consistent with pDNA binding to the cationic surfactant anchored onto the surface of the poloxamer particles. Attempts to prepare CRL1005/BAK/pDNA formulations using BAK concentration from 0.52 to 1.2 mM resulted

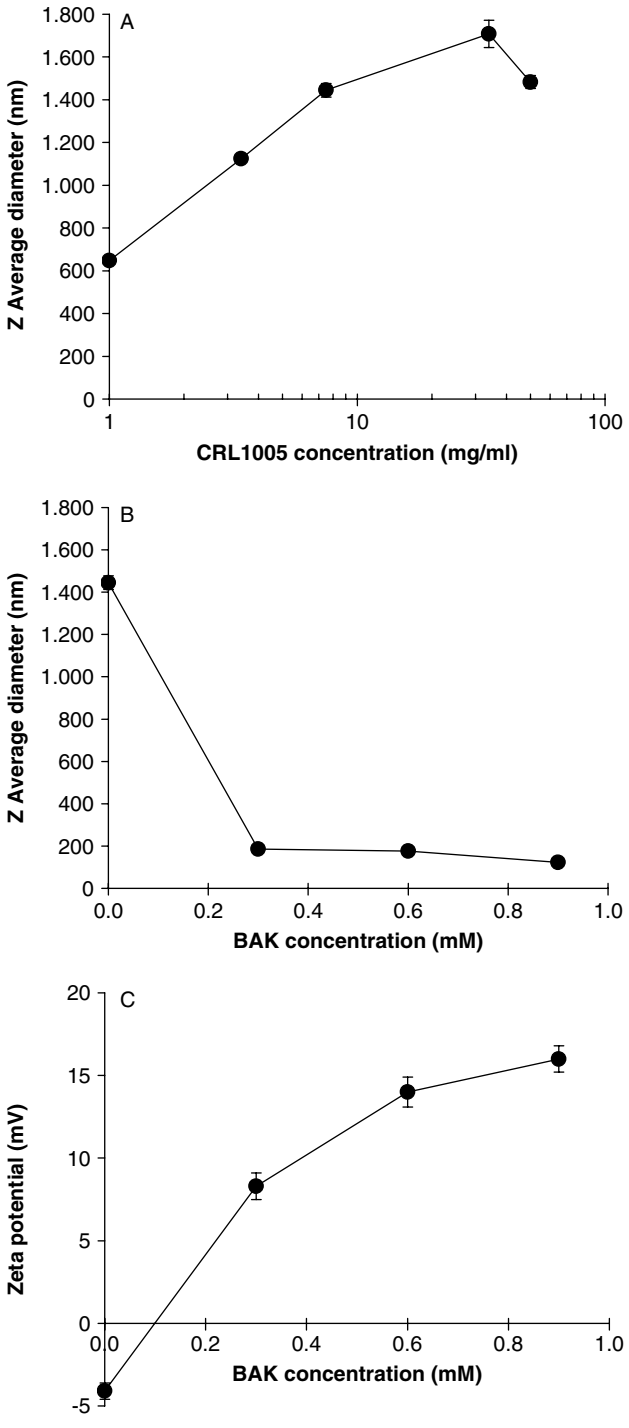

Figure 1. Particle size and zeta potential of CRL1005 and CRL1005/BAK formulations. CRL1005 poloxamer, at concentrations ranging from 1 to 50 mg/ml, was dissolved in PBS and the particle sizes at ambient temperature were determined after cycling through the cloud point (A). The effect of varying concentrations of the cationic surfactant BAK on the size (B; photon correlation spectroscopy) and zeta potential (C; micro-electrophoresis) of CRL1005/BAK particles was determined at a CRL1005 concentration of 7.5 mg/ml

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in precipitation of insoluble material. Thus the 0.3 mM BAK/CRL1005 formulation was chosen to be tested in the *in vivo* studies.

Microscopy of CRL1005/BAK/pDNA formulations

Phase contrast and fluorescence microscopy confirmed that, in the absence of BAK, CRL1005 particles had a variable diameter (Figures 2A and 2B). The particle size was estimated from phase contrast images and, at 7.5 mg/ml CRL1005 concentration, the average measured diameter was 1274 ± 32 nm ($n = 427$, range 230–3201 nm), in good agreement with the data obtained with photon correlation spectroscopy (Figure 1A). In contrast, formulating CRL1005 in the presence of BAK either without (Figures 2C and 2D) or with pDNA (Figure 2E) resulted in more uniform particle sizes with a typical particle diameter in the submicron range.

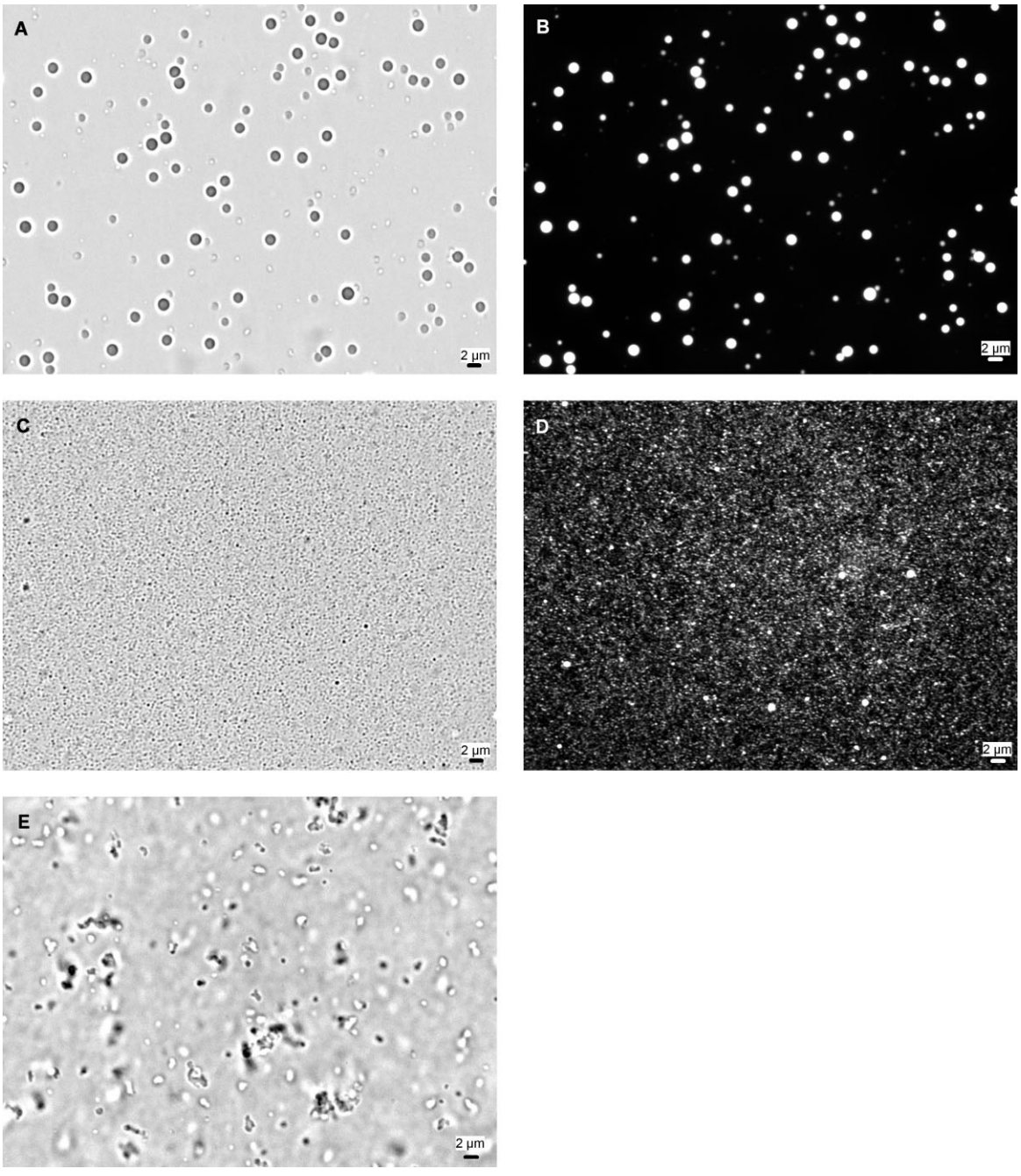

Figure 2. Phase contrast and fluorescence photomicrographs of CRL1005, CRL1005/BAK and CRL1005/BAK/pDNA formulations. CRL1005 at 7.5 mg/ml was dissolved in PBS and cycled through the cloud point in the absence (A, B) or presence (C, D) of 0.3 mM BAK. CRL1005/BAK/pDNA formulation (E) was prepared at final concentrations of 7.5 mg/ml CRL1005, 0.3 mM BAK and 5 mg/ml pDNA. Images were obtained at room temperature, either by phase contrast microscopy (A, C, E) or fluorescence microscopy, after visualizing the poloxamer particles with Nile Red (B, D). Images were taken using a 100 × oil immersion objective. The scale bar corresponds to 2 µm in length

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Atomic force microscopy images revealed pDNA molecules mainly in a supercoiled state (Figure 3A). The measured height ranged from 0.4 to 0.8 nm, and the approximate length ranged from one to several micrometers. The width of pDNA molecules measured using height profiles in AFM images were in the 20–30 nm range. An AFM topographic image of the CRL1005/BAK/pDNA formulation (Figure 3B) showed that the entire mica surface was covered with entangled pDNA molecules. Rounded particles associated with entangled pDNAs were also present. Their morphology was clearly distinct from that of pDNA, and their diameter was in the 200–250 nm range. These particles were homogenously distributed on the mica surface with a density of about 0.4/μm^2. Increasing the concentration of pDNA 20-fold, to 1 mg/ml, resulted in higher numbers of immobilized molecules on the mica substrate. The AFM phase image of the CRL1005/BAK/pDNA formulation (Figure 3C), taken simultaneously with the topographic image, showed a similar surface morphology, composed of entangled pDNA and round particles. However, the particles were more contrasted in the phase image than in the topographic image. CRL1005 and CRL1005/BAK samples which did not contain pDNA could not be immobilized on mica using Ni^{2+} ions. After incubation and washing, AFM imaging confirmed that nothing remained on the mica surface.

Immunogenicity of CRL1005/pDNA formulations

The immunogenicity of CRL1005/pDNA formulations, in the absence of BAK, were evaluated first since similar poloxamer/pDNA formulations have previously been reported to increase immune responses in non-human primates [12,13]. The particle size and zeta potential of CRL1005/pDNA formulations at 0.1 mg/ml pDNA concentration varied from 462 nm and −3.7 mV (obtained with 0.15 mg/ml CRL1005) to 2030 nm and +0.2 mV (obtained with 75 mg/ml CRL1005), respectively. Compared to unformulated pDNA injected in PBS, CRL1005/pDNA formulations enhanced antigen-specific $CD8^+$ T-cell (Figure 4A) and antibody (Figure 4C) responses by up to 2- and 5-fold, respectively, in a poloxamer concentration-dependent manner. CRL1005/pDNA formulations did not have a significant effect on the number of antigen-specific IFN-γ-secreting $CD4^+$ T-cells compared to vaccination with pDNA alone (Figure 4B).

Table 1. Physical characteristics of CRL1005/BAK formulations prepared using BAK homologs

Formulation[a]	Z average diameter (nm)	Poly-dispersity	Average surface charge (mV)
BTC 50® NF	186	0.02	+8.3
C_{12} BAK	924	0.27	+1.2
C_{14} BAK	207	0.05	+0.8
C_{16} BAK	471	0.27	+3.4
C_{18} BAK	760	0.58	+15.2

[a]The CRL1005 concentration was 7.5 mg/ml, and the BAK (BTC 50® NF) or BAK homolog concentration for all formulations was 0.3 mM.

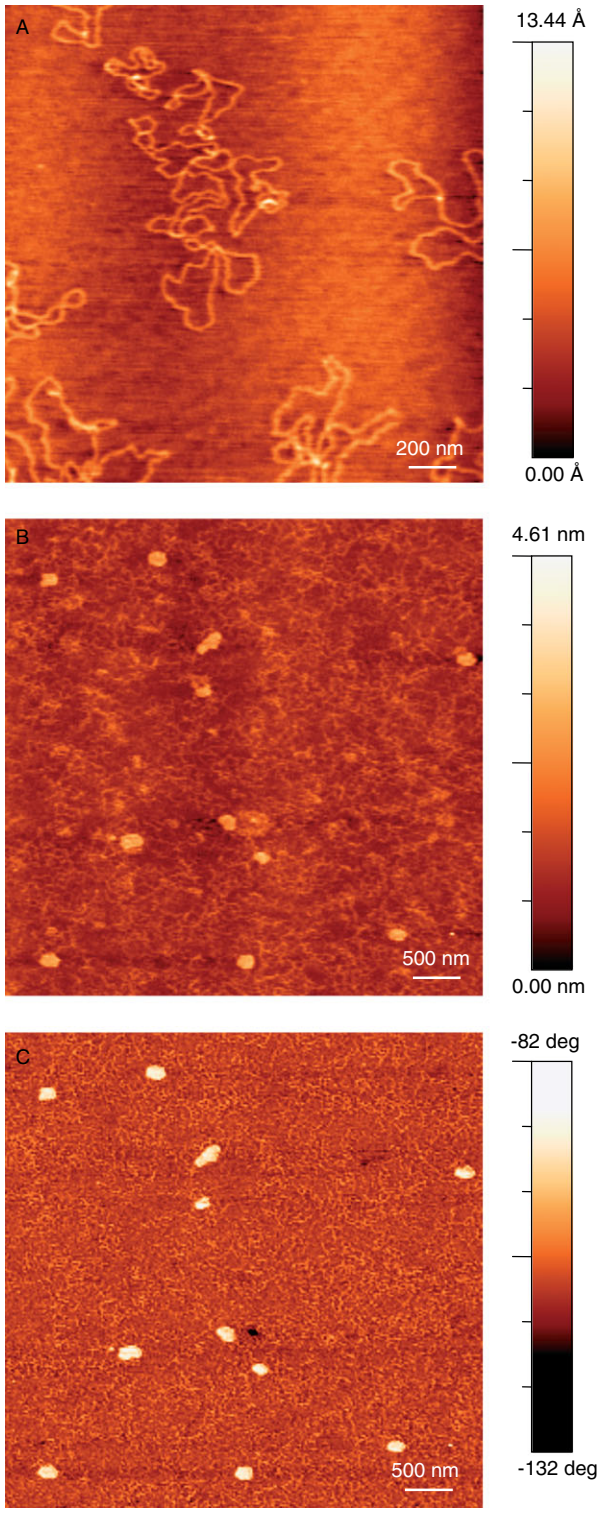

Figure 3. Atomic force microscopy of pDNA and CRL1005/BAK/pDNA formulations. AFM topographic image of pDNA (2.5 μg/ml) molecules immobilized by Ni^{2+} on mica substrate (A). AFM topographic (B) and phase (C) image of CRL1005/BAK/pDNA formulation immobilized on mica surface at pDNA concentration of 50 μg/ml. The scale bar corresponds to 200 nm (A) or 500 nm (B, C) in length

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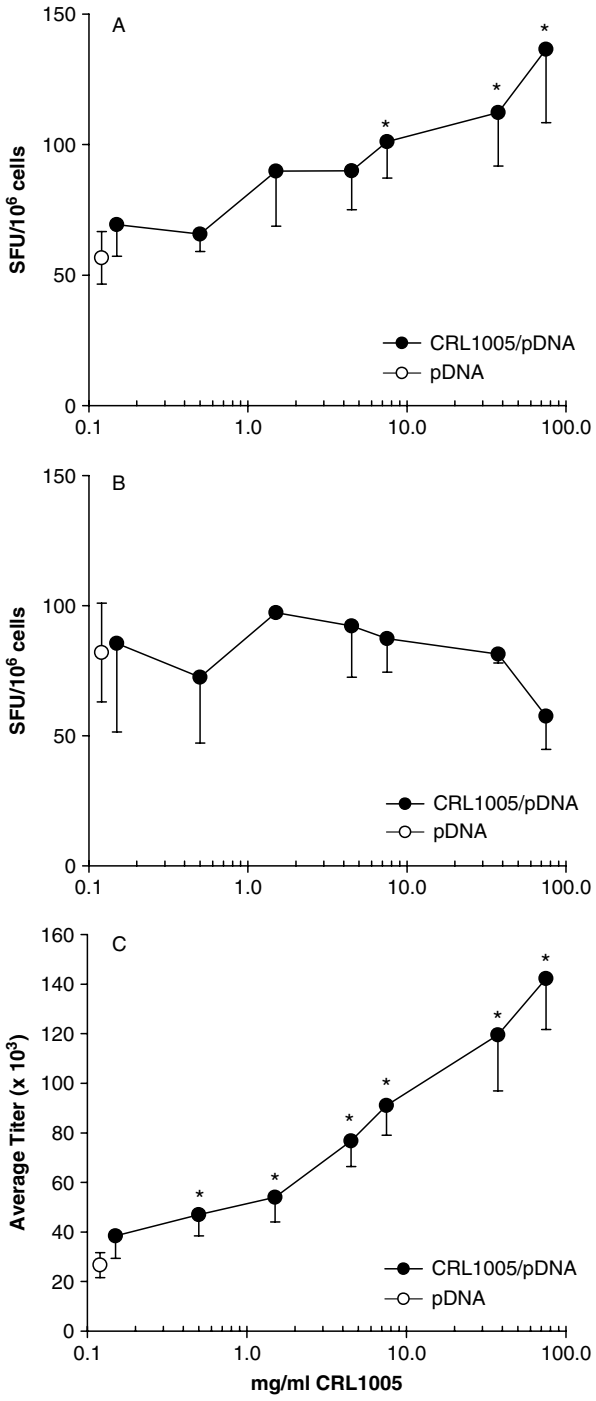

Figure 4. Poloxamer CRL1005 dose response. CRL1005/pDNA formulations were prepared as described, stored frozen at −80 °C and thawed at ambient temperature prior to injections. The concentration of VR4700 pDNA was 0.1 mg/ml in each formulation. In two separate experiments, each with an unformulated pDNA control group, mice (n = 9 per group) received intramuscular injections of 10 μg of pDNA in 100 μl PBS/mouse ± CRL1005 on days 0, 20, and 48. On days 62 through 64, spleens from three mice per test group were harvested, spleens were pooled, and the pools were assayed for NP-specific IFN-γ-secreting $CD8^+$ (A) or $CD4^+$ (B) T-cells using an ELISPOT assay. Each data point represents the mean ± s.e.m. of 2–6 pools. NP-specific antibodies were analyzed by ELISA (C). Each data point in the ELISA represents the mean ± s.e.m. of 9–18 serum samples. *Significantly different from the pDNA control group, $p < 0.05$

Immunogenicity of CRL1005/BAK/pDNA formulations

To assess the ability of BAK-containing formulations to enhance immune responses, the CRL1005/BAK/pDNA formulation was prepared at final concentrations of 7.5 mg/ml, 0.3 mM and 5 mg/ml, respectively. CRL1005 at 7.5 mg/ml was chosen since that concentration resulted in a significant increase in $CD8^+$ T-cell (Figure 4A) and antibody (Figure 4C) responses. BAK at 0.3 mM concentration was chosen to ensure homogeneity of the formulations without visible precipitate (see first section of "Results" above). The results from a dose response study suggested that the CRL1005/BAK/pDNA formulation increased immune responses compared to unformulated pDNA (Figure 5). Due to animal-to-animal variability in immune responses, however, the differences between unformulated pDNA and the CRL1005/BAK/pDNA formulation did not always reach statistical significance in a single study. When the CRL1005/BAK/pDNA formulation was tested in nine separate experiments using 10 μg pDNA dose, the formulation significantly enhanced antigen-specific $CD8^+$ and $CD4^+$ T-cell responses by 1.9- and 1.7-fold, respectively (Figure 6A, $p < 0.01$). No significant enhancement in $CD4^+$ T-cell responses was previously observed with CRL1005/pDNA formulations (Figure 4B), suggesting that BAK increased the immunogenicity of the vaccine formulation. Antibody responses in mice vaccinated with the CRL1005/BAK/pDNA formulation were 1.6-fold higher compared to mice vaccinated with unformulated pDNA (Figure 6B, $p < 0.001$).

To determine whether the particle size of CRL1005/BAK/pDNA formulations affects cellular or humoral immune responses, formulations with a range of particle sizes (Z average diameter of 250, 350, 550, 1000, or >1000 nm) were prepared as described in the Materials and methods section. When compared to the same dose of unformulated pDNA, CRL1005/BAK/pDNA formulations increased antigen-specific cellular and humoral immune responses in mice by up to 1.8- and 2.0-fold, respectively. However, there were no statistically significant differences among the groups vaccinated with CRL1005/BAK/pDNA formulations with different particle sizes (data not shown).

Immunogenicity of a simplified BAK homolog formulation

The current CRL1005/BAK-based CMV pDNA vaccine formulation used in a phase 2 clinical trial is a mixture of poloxamer CRL1005 (7.5 mg/ml), cationic surfactant BAK (0.3 mM) and two plasmids encoding CMV pp65 (VCL6368) and gB (VCL6365) antigens at a total pDNA concentration of 5 mg/ml in PBS [14]. The BAK component of this formulation is a mixture of four alkyl chain homologs: C_{12} (50%), C_{14} (30%), C_{16} (17%), and C_{18} (3%). This BAK mixture is commercially available as BTC 50® NF. A simplified formulation

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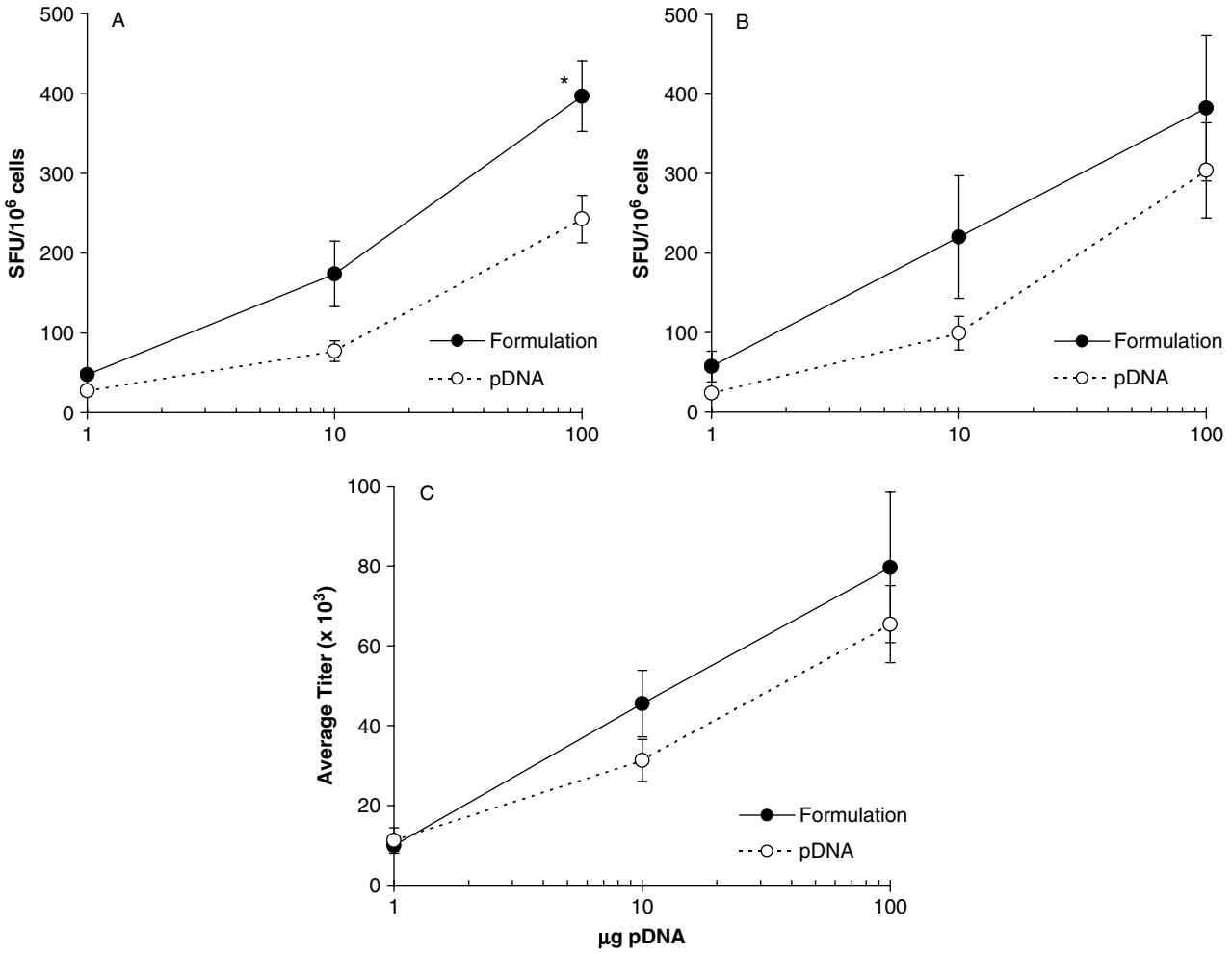

Figure 5. Dose responses for unformulated pDNA and CRL1005/BAK/pDNA formulation. CRL1005/BAK/pDNA formulation was prepared to produce final concentrations of 7.5 mg/ml CRL1005, 0.3 mM BAK and 5 mg/ml pDNA. The formulation was stored frozen at −80 °C, thawed at ambient temperature, and diluted to the required pDNA concentration with PBS above the cloud point of CRL1005 prior to injections. On days 0, 20 and 49, mice (n = 9 per group) received intramuscular injections with the indicated doses of either pDNA (open circles) or CRL1005/BAK/pDNA formulation (closed circles) in 100 µl/mouse. On days 63 through 65, spleens from three mice per test group were harvested, spleens were pooled, and the pools were assayed for NP-specific IFN-γ-secreting $CD8^+$ (A) or $CD4^+$ (B) T-cells using an ELISPOT assay. Each data point represents the mean ± s.e.m. of three pools. NP-specific antibodies were analyzed by ELISA (C). For the ELISA, each data point represents the mean ± s.e.m. of nine serum samples. *Significantly different from the unformulated pDNA group, $p < 0.05$

containing only one BAK homolog found in BTC 50® NF, which also retains comparable physical characteristics and immunogenicity, would be analytically preferable. Physical characterization showed that the CRL1005/C_{14} BAK homolog formulation yielded particles with size and polydispersity closest to that of the CRL1005/BTC 50® NF formulation (Table 1). The influence of C_{12} and C_{16} BAK homologs on particle size and polydispersity was smaller than that observed with the C_{14} BAK homolog, and visible particulates were observed below the cloud point in C_{18} BAK homolog formulations. Thus the C_{14} BAK homolog was chosen for *in vivo* evaluation. The results showed that there were no statistically significant differences in antigen-specific cellular and humoral immune responses between mice immunized with formulations prepared using 0.3 mM BTC 50® NF and those immunized with formulations prepared using 0.3 mM C_{14} BAK homolog (Table 2).

Table 2. Immunogenicity of a C_{14} BAK homolog formulation

	Anti-pp65 responses SFU/10^6 cells[a]	Anti-gB responses EU/ml[a]
0.30 mM BTC 50® NF	134 ± 28	41480 ± 6203
0.30 mM C_{14} BAK	206 ± 41	46444 ± 6132

[a]The data represent the mean ± s.e.m. of combined data from two studies (n = 8–10 spleen pools/test group for T-cell data, n = 20 serum samples/test group for antibody data).

Effect of CRL1005/BAK formulation on transgene expression

To elucidate the mechanism by which the CRL1005/BAK formulation improves the immunogenicity of a pDNA-based vaccine, antigen expression was evaluated *in vivo*. In these studies, luciferase was used as a model cytoplasmic antigen, and EPO as a model secreted

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antigen. In muscles transfected with CRL1005/pDNA or CRL1005/BAK/pDNA formulations, luciferase expression was approximately 3-fold higher than in muscles transfected with unformulated pDNA (Figure 7A). When CRL1005/BAK was formulated with pDNA encoding EPO, using the same CRL1005/BAK/pDNA ratios that produced a significant enhancement in antigen-specific cellular and humoral immune responses (Figure 6), EPO levels in muscle (Figure 7B) and serum (Figure 7C) were approximately 5-fold higher than with unformulated pDNA.

Discussion

In the polymer-based vaccine formulation described in this report, pDNA was formulated with the nonionic triblock POE-POP-POE copolymer, poloxamer CRL1005, and the cationic surfactant BAK, to produce a thermodynamically stable, self-assembling system in PBS. Above the polymer cloud point, CRL1005 suspension spontaneously assembled into particles, and multiple small micelles quite likely fused to form micron-sized particles with a variable diameter and a slightly negative surface charge. While it is apparent that the CRL1005 polymer is a neutral molecule, its small negative surface charge can be attributed to

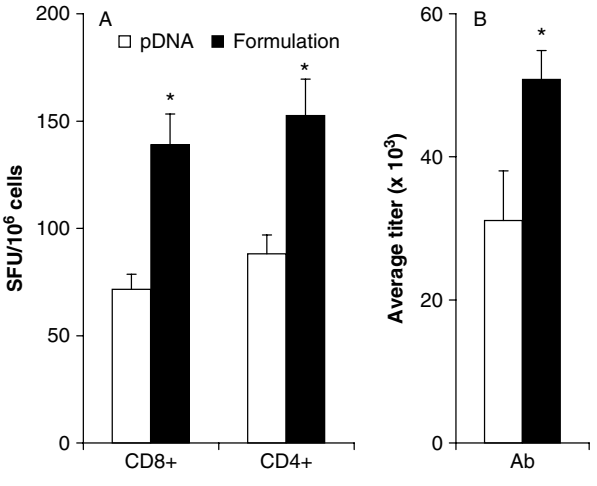

Figure 6. CRL1005/BAK/pDNA formulation significantly enhances cellular and humoral immune responses. CRL1005/BAK/pDNA formulation was prepared at concentrations of 7.5 mg/ml CRL1005, 0.3 mM BAK and 5 mg/ml pDNA. Prior to injections, the formulation was diluted with PBS to final concentrations of 0.15 mg/ml CRL1005, 6 μM BAK and 0.1 mg/ml pDNA. In nine separate experiments, mice (n = 9 per group per study) were vaccinated with CRL1005/BAK/pDNA formulation (10 μg pDNA/mouse/injection) on days 0, 20 and 48 (black bars). Control mice were vaccinated with an identical dose of unformulated pDNA (white bars). On days 62 through 64, spleens from three mice per test group were harvested, spleens were pooled, and the pools were assayed for NP-specific IFN-γ-secreting $CD8^+$ or $CD4^+$ T-cells using an ELISPOT assay (A). NP-specific antibodies were analyzed by ELISA (B). The bars represent the mean ± s.e.m. of combined data from 63–81 mice. *Significantly different from the unformulated pDNA group, $p < 0.01$

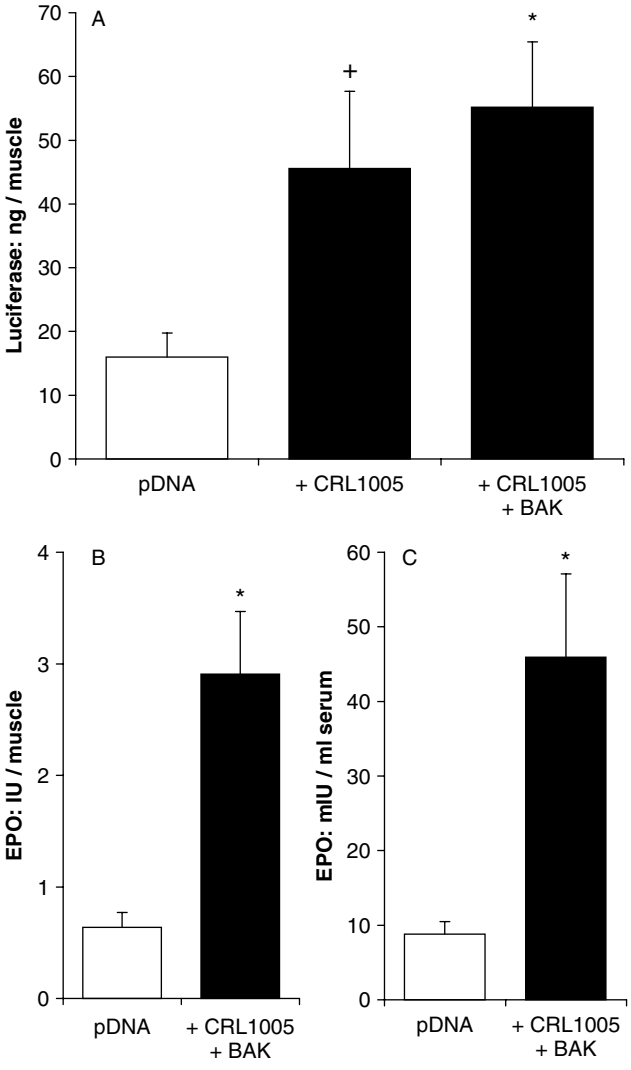

Figure 7. CRL1005/pDNA and CRL1005/BAK/pDNA formulations increase transgene expression. Plasmid DNA encoding luciferase was formulated with CRL1005 with or without BAK at final concentrations of 1 mg/ml CRL1005, 6 μM BAK, and 0.1 mg/ml pDNA. Formulations were injected into mouse *rectus femoris* in 50 μl of PBS/muscle, and transgene expression was measured 7 days postinjection (A, n = 16–20 muscles/group). Plasmid encoding EPO was formulated with CRL1005/BAK at concentrations of 7.5 mg/ml CRL1005, 0.3 mM BAK and 5 mg/ml pDNA. The formulation was stored at −80 °C, thawed at ambient temperature, diluted to a final concentration of 1.5 mg/ml CRL1005, 60 μM BAK and 1 mg/ml pDNA, and injected into mouse *rectus femoris* in 50 μl of PBS/muscle. Transgene expression in muscle (B, n = 10) and serum (C, n = 10) was measured 7 days postinjection. Control mice received an identical dose of unformulated pDNA (white bars). +Significantly different from the unformulated pDNA group, $p < 0.05$. *Significantly different from the unformulated pDNA group, $p < 0.01$

the overall partial negative character induced by the highly electronegative oxygen atoms of each ethylene oxide and propylene oxide subunit. In contrast, formulating CRL1005 in the presence of BAK produced more uniform-sized submicron particles with a mean hydrodynamic diameter of approximately 200 nm. This is most likely the result of the cationic surfactant being anchored

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onto the surface of the poloxamer particles, giving them a positive surface charge which prevents the fusion of CRL1005/BAK particles due to charge repulsion.

The hydrophobic POP portion of each CRL1005 molecule is positioned internally and the hydrophilic POE component is exposed to the external aqueous environment in these structures [6,18,27–29]. Dissolving Nile Red into the hydrophobic domain of the polymeric micelles results in a dramatic increase in fluorescence, allowing the poloxamer particles to be visualized by fluorescence microscopy [22]. The diameter of the CRL1005 and CRL1005/BAK particles visualized with Nile Red was consistent with the particle size data obtained using photon correlation spectroscopy. Under the fluorescence microscope, the particles were stationary over time and appeared to have adsorbed to the surface of the glass slide, possibly via electrostatic interactions. CRL1005/BAK/pDNA particles, however, did not adsorb to the glass slides and were in constant motion. Hence it was not possible to obtain images of CRL1005/BAK/pDNA particles stained with Nile Red due to the long exposure times required for fluorescence photomicrographs.

Divalent cations like Ni^{2+} can form a bridge between the negative phosphate groups of DNA and the negatively charged mica surface in aqueous solution, enabling visualization of DNA in AFM. The AFM images suggested that pDNA does not form aggregates in PBS, but likely exist as individual supercoiled plasmid molecules. The pDNA molecule should have a similar width and height value. However, the measured width (20–30 nm) of pDNA immobilized on mica was much larger than the height (0.4–0.8 nm). The attractive capillary force due to the thin water layer on the sample surface, as well as compression of the sample by the AFM tip during the measurement, quite likely is responsible for the lower-than-expected height values.

In AFM images of the CRL1005/BAK/pDNA formulation, rounded particles with entangled pDNAs covering the entire mica surface were visible. This is consistent with observations reported in a previous publication demonstrating that, in a similar formulation, the amount of pDNA binding to CRL1005/BAK particles represents about 2–3% of the total pDNA [6]. The pDNA in the CRL1005/BAK/pDNA formulation is attached to the surface of positively charged microparticles made out of the hydrophobic cationic surfactant, BAK, anchored onto the surface of a poloxamer particle [6], giving the CRL1005/BAK/pDNA particles a negative surface charge that enables them to be immobilized on the mica surface using Ni^{2+} to form a bridge between the pDNA and mica. Round particles were visible in the CRL1005/BAK/pDNA formulations using AFM. The estimated diameter of the particles observed in AFM images (200–250 nm) was consistent with the particle size data obtained with photon correlation spectroscopy (approximately 260 nm).

A previous study [6] described a CRL1005/BAK/pDNA (7.5 mg/ml, 0.6 mM, 5 mg/ml, respectively) formulation with a much more negative zeta potential (−46.7 mV) than in the formulation characterized in this report (−11.6 V). Formulations in that study were prepared by adding CRL1005 to an aqueous solution of pDNA in PBS at room temperature, followed by the addition of BAK and several cycles of cooling on ice, vigorous vortexing, and, finally, warming the formulation above the cloud point with additional vortexing. The BAK solution used was obtained from Spectrum (New Brunswick, NJ, USA) and contained 67% C_{12} BAK and 33% C_{14}/C_{16} BAK [6]. The CRL1005/BAK/pDNA formulation described in this study was prepared using a different mixing procedure, and was made at a 2-fold lower BAK concentration using BAK 50® NF, consisting of a mixture of C_{12}, C_{14}, C_{16} and C_{18} BAK homologs in a percentage ratio of 50 : 30:17 : 3, respectively. These two factors may explain the differences in the measured surface charges.

A simplified vaccine formulation was prepared using only one of the BAK homologs found in the commercial BTC 50® NF. Particle size analysis showed that the 0.3 mM C_{14} BAK homolog yielded CRL1005/BAK particles with size and uniformity closest to that of 0.3 mM BTC 50® NF. *In vivo* studies demonstrated that the immunogenicity of the formulation prepared using the C_{14} BAK homolog was comparable to that obtained with CRL1005/BAK/pDNA formulations containing BTC 50® NF.

The adjuvant activity of poloxamer CRL1005 was first reported with protein antigens, such as ovalbumin, HIV-1 envelope gp120 and the trivalent influenza virus vaccine, Fluogen® [27–32]. Those studies showed elevated production of IFN-γ, IL-2, IL-5 and IL-10 by splenocytes of immunized mice or by peripheral blood lymphocytes obtained from human subjects [27,28,32]. Physical association of the protein and poloxamer CRL1005 was shown by particle size analysis and electron microscopy, suggesting that CRL1005 may exert its effect with protein vaccines by acting as a depot for the antigen [27–30]. Consistent with this observation, low molecular weight poloxamers L101 and L121 have been shown to delay the clearance of a protein from the site of injection, and there was a positive correlation between slow clearance and increased antibody responses [33].

The mechanism by which CRL1005/BAK enhances immune responses with pDNA-based vaccines has not been determined, although it has been postulated that CRL1005/BAK particles may act as a plasmid-delivery system and enhance pDNA uptake into APCs [6]. Poloxamers have been shown to decrease the viscosity and to increase the permeability of lipid bilayers, and to insert into lipid membranes forming channels and pores [34–36]. Such interactions of poloxamers with cell membranes could increase pDNA transport into cells. Consistent with this hypothesis, low molecular weight poloxamers, such as SP1017, P188 and PE6400, have previously been shown to increase intramuscular reporter gene expression when coinjected with pDNA [37–39]. This study showed that CRL1005/pDNA and CRL1005/BAK/pDNA formulations significantly increased transgene levels in muscle and serum, suggesting that one of the mechanisms by which these formulations improve immunogenicity involves improved delivery of the pDNA

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vaccine. Preliminary data show that coinjection of pDNA with CRL1005 increases expression of proinflammatory cytokines and chemokines in muscle, such as IL-1ß, IL-6, G-CSF, TNF-α, KC and MIP-2, suggesting that the CRL1005/BAK/pDNA formulation may also improve immunogenicity by stimulating innate immunity (data not shown).

Particulate vaccine formulations, such as PLG/CTAB microparticles, have been reported to enhance immunogenicity by several mechanisms, including enhanced transfection of phagocytic APCs with the antigen-encoding plasmid. Studies of PLG/CTAB/pDNA formulations have shown a correlation between particle size and enhanced immune responses. Particles with a diameter between 300 nm and 3 μm were the most potent in enhancing immune responses whereas particles with a diameter around 10–30 μm were significantly less immunogenic, possibly because they were too big for efficient phagocytosis [4,9,10]. Varying the size of CRL1005/BAK/pDNA particles from 250 nm to over 1000 nm had no significant effect on the immunogenicity of the CRL1005/BAK-based pDNA vaccine formulations in this study. Since these formulations could not be prepared at particle sizes >10 μm, we were unable to evaluate whether larger CRL1005/BAK/pDNA particles are ineffective in enhancing immune response as previously demonstrated for PLG/CTAB/pDNA formulations [4,10].

Poloxamers formulated with a pDNA vaccine, without BAK, have been shown to significantly increase cellular immune responses in nonhuman primates [6,11–13]. Furthermore, doses of CRL1005 adjuvant as high as 75 mg injected intramuscularly have been shown to be safe and well tolerated in humans [27,32]. Studies in rhesus macaques have demonstrated that adding BAK to the CRL1005/pDNA vaccine formulation further enhances T-cell responses [6,11]. In this report, we have characterized a CRL1005/BAK/pDNA vaccine formulation that significantly enhances the levels of antigen-specific cellular and humoral immune responses, possibly by improving the delivery of pDNA. This formulation is currently being evaluated for the prevention of CMV-associated disease in the allogeneic hematopoietic cell transplant population in a phase 2 clinical trial [14].

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