

Optimization of Stability, Encapsulation, Release, and Cross-Priming of Tumor Antigen-Containing PLGA Nanoparticles

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Received: 27 September 2011 / Accepted: 15 May 2012 / Published online: 14 July 2012
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ABSTRACT

Purpose In order to investigate Poly (lactic-co-glycolic acid) (PLGA) nanoparticles (NP) as potential vehicles for efficient tumor antigen (TA) delivery to dendritic cells (DC), this study aimed to optimize encapsulation/release kinetics before determining immunogenicity of antigen-containing NP.

Methods Various techniques were used to liberate TA from cell lines. Single (gp100) and multiple (B16-tumor lysate containing gp100) antigens were encapsulated within differing molecular weight PLGA co-polymers. Differences in morphology, encapsulation/release and biologic potency were studied. Findings were adopted to encapsulate fresh tumor lysate from patients with advanced tumors and compare stimulation of tumor infiltrating lymphocytes (TIL) against that achieved by soluble lysate.

Results Four cycles of freeze-thaw + 15 s sonication resulted in antigen-rich lysates without the need for toxic detergents or protease inhibitors. The 80KDa polymer resulted in maximal release of payload and favorable production of immunostimulatory IL-2 and IFN- γ . NP-mediated antigen delivery led to increased IFN- γ and decreased immunoinhibitory IL-10 synthesis when compared to soluble lysate.

Conclusions Four cycles of freeze-thaw followed by 15 s sonication is the ideal technique to obtain complex TA for encapsulation. The 80KDa polymer has the most promising combination of release kinetics and biologic potency. Encapsulated antigens are immunogenic and evoke favorable TIL-mediated anti-tumor responses.

KEY WORDS antigen delivery · dendritic cell · immunotherapy · molecular weight · nanoparticles

ABBREVIATIONS

Ags	antigens
APC	antigen presenting cell
ATCC	American Type Culture Collection
BCA	bicinchonic acid
BMDC	bone marrow dendritic cells
BSA	bovine serum albumin
CBA	cytometric bead array
CD	cluster of differentiation
CTL	cytotoxic T cells
DC	dendritic cell
FT + S	freeze-thaw + sonication
GM-CSF	granulocyte macrophage-colony stimulating factor
HNSCC	head and neck squamous cell carcinoma
IFN- γ	interferon- γ
IL	interleukin
LPS	lipopolysaccharide
MP	microparticles
MW	molecular weight
NP	nanoparticles
PBS	phosphate buffered saline
PLGA	poly (lactic-co-glycolic acid)

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PVA	polyvinyl alcohol
RT	room temperature
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
SPSS	Statistical Package for the Social Sciences
TA	tumor associated antigen
Th	T-helper
TIL	tumor infiltrating lymphocytes
TNF- α	tumor necrosis factor- α
WB	Western blot

INTRODUCTION

Efficient delivery of tumor antigens (TA) is a major challenge facing dendritic cell (DC)-based immunotherapy for many solid organ malignancies like malignant melanoma and head and neck squamous cell carcinoma (HNSCC). One option to achieve this is by using the inherent advantages of micro- or nano-particles (MP/NP) made from poly (lactic-co-glycolic acid) (PLGA) (1–3). By selecting the appropriate polymer composition with a known rate of degradation, MP/NP can be exploited to produce a delivery system that releases an active agent at a predetermined rate (4,5). Release rate depends on particle properties such as PLGA composition (lactide: glycolide ratio), loading, size, porosity and ultrastructure (6,7). An additional factor of importance is the polymer molecular weight (MW). This is crucial because it alters hydrophilicity, diffusion and degradation via non-enzymatic autocatalysis. Moreover it affects the viscosity of polymer solution and sol–gel phase inversion induced by precipitation following solvent removal, both of which have a bearing on release rates of encapsulated payload (8,9). It is therefore important to evaluate and define the influence of polymer MW on delivery of either single, or multiple TA. While there have been isolated reports of encapsulation of cellular material (10,11), only one study has so far investigated the role of polymer MW on encapsulation and *in vitro* release of complex mixture of TA (12). However it did not evaluate the impact of MW on extent of DC-mediated antigen cross-presentation to cytolytic T cells (CTL), something to consider when using PLGA polymers as antigen delivery vehicles.

Stability of encapsulated material is key to the quality and magnitude of immune responses generated by particulate-based vaccine delivery systems (13,14). When fabricating NP/MP using the double emulsion and solvent evaporation technique, TA are exposed to a range of chemical and physical insults like excess heat (15), high speed homogenization-induced shearing forces and organic solvents (16). These can result in denaturation, aggregation, adsorption, hydrolysis, oxidation, β -elimination, racemization or deamidation (17,18), each of which could adversely affect their potency.

Concerns about antigen stability have led to modifications in polymer particle fabrication like using poly-D,L-lactide-poly (ethylene glycol) (PELA) instead of PLGA, incorporating protein stabilizers to shield antigens from organic solvents at the water/oil interface (19) and coupling cationic amphiphile molecules with encapsulation targets (20). It is therefore important to confirm the stability and immunogenicity of encapsulated material when considering this technology in anti-tumor immunotherapy. While there have been earlier attempts at evaluating single TA containing-NP (15,16), encapsulation and release of mixture of TA have only infrequently been studied. In an earlier study, our group reported that encapsulated melanoma antigens loaded onto C57BL/6 mice derived- bone marrow DC (BMDC) resulted in greater T helper (Th)-1-type responses when compared to soluble lysates (12). But the delivery of patient-derived TA has not been fully evaluated underscoring the importance of obtaining additional information regarding polymer composition and stability.

As part of ongoing efforts to develop a PLGA-based anti-tumor DC vaccination protocol, we herein report our experience with *in vitro* evaluation of encapsulated TA derived from a variety of sources. We hypothesized that: a) PLGA MW influenced downstream biological activity of NP and b) by using optimal techniques to generate and then encapsulate whole tumor lysates, TA could efficiently be delivered to DC for cross-presentation to CTL. The aims of the study were to: (i) improve antigen stability and potency during encapsulation by attempting to increase antigen concentration within tumor lysates, (ii) identify the PLGA formulation that resulted in favorable encapsulation and release of both single and multiple TA and (iii) compare immunostimulatory capacity of various formulations utilizing bonafide murine and human T-cell readouts. A number of techniques were initially used to solubilize HNSCC lines. The intention was to enhance antigens within the lysate and adopt the clinically compatible technique consistently resulting in maximal release of TA from a finite amount of cellular material. TA were then encapsulated within differing MW of 50:50 PLGA polymers using a double emulsion solvent evaporation method. The physico-chemical characteristics, *in vitro* controlled release profile and differences in biological activity of these formulations were evaluated. Using naïve tumor-specific CD8⁺ T cells derived from transgenic mice, we measured NP potency through DC-based cross-presentation of encapsulated antigen (s). Our observations suggest that PLGA MW could influence this phenomenon. Fresh samples from patients with advanced tumors were then employed in similar experiments. Cytokine profile of autologous tumor infiltrating lymphocytes (TIL) stimulated by DC loaded with TA contained within either PLGA NP or soluble tumor lysates were compared. The findings indicate the potency of encapsulated TA and represent a critical step forward in the development of PLGA-based reagents for tumor immunotherapy.

MATERIALS AND METHODS

Antigen Encapsulation Procedures

Individual Antigen

gp100 conjugated to myelin basic protein (MBP) (MBP-gp100; a kind gift of the Rothberg Institute for Childhood Diseases, Guilford, CT, USA) was concentrated on YM-10 Ultracel YM membrane Microcon filtration units (Millipore Corporation, Billerica, MA, USA) by centrifugation at 12000 rpm for 30 min at 4°C to attain a final concentration of 10 µg/µl. Recombinant gp100 protein was also spiked (5% w/w) into B16 murine melanoma cell lysates prepared from freshly excised tumors as described below.

Cell Lines

Cells from FaDu and FAT7 (HNSCC) cell lines [American Type Culture Collection (ATCC), Manassas, VA, USA] were grown to more than 90% confluence in monolayers in ATCC recommended media in a humidified atmosphere containing 5% CO₂. FaDu was grown in 1% sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA) + Eagle's Minimal Essential Medium + 10% fetal bovine serum + 1% non-essential amino acids + 1.5 mg/ml sodium bicarbonate + 1% penicillin/streptomycin (all reagents from Invitrogen, Carlsbad, CA, USA). FAT7 cells were grown using Ham's F12K medium + 10% fetal bovine serum (non-heat inactivated) + 1% penicillin/streptomycin (all reagents from Invitrogen, Carlsbad, CA, USA) + 0.1% insulin-HEPES + 0.5% hydrocortisone (both from Sigma-Aldrich, St. Louis, MO, USA) and 0.5% Transferrin (Calbiochem, San Diego, CA, USA). These served as sources of TA for the initial set of experiments aimed at enhancing stability of antigens.

Determination of Optimal Lysate Generation Technique

Confluent cells from FAT 7 and FaDu lines were re-suspended in ice cold PBS at concentrations ranging from 2–4 × 10⁸/ml. In order to maximize release of protein antigens from cells and thus increase concentration of the lysate used for encapsulation, cell suspensions were converted into lysates using different techniques and compared. To alleviate the need for additional (cytotoxic) protease inhibitors, cell solutions were maintained on ice prior to and during lysate preparation. Methods screened initially included: Freeze-thaw (four cycles of alternating liquid nitrogen and 37°C water bath treatment) (F/T) alone, sonication (Ultrasonic Processor, Tekmar, Cincinnati, OH, USA) (S) for 60 s on ice at 38% amplitude, F/T + S for 15 s and F/T + Dounce homogenization (DH). DH involves disruption of cultured cells by forcing the cell suspension through a

narrow space causing shearing of cell membranes. This is done using a Dounce homogenizer which consists of a round glass pestle that is manually driven into a glass tube. In all instances, cell death and lysis were confirmed by trypan blue exclusion. Lysates were initially spun at 12000 rpm for 20 min at 4°C to remove cellular debris and supernatants collected. These were either used immediately or stored at –20°C for future assays. Protein content of lysate preparations was measured by the standard test tube procedure using the Bicinchonic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL, USA) as per the manufacturer's protocol. Absorbance at 562 nm was measured using a spectrophotometer and protein concentrations determined by comparing absorbance readings to a standard calibration curve of bovine serum albumin (BSA) in the concentration range between 5 µg/ml and 250 µg/ml.

Differences in protein content of selected lysates were also evaluated by Western Blot (WB) analysis and Coomassie blue staining. Samples were run on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) mini gel system (Pre-cast 8–16% Tris-HCl 10-well, Jule Inc, CT, USA) @ 30–35 mA per gel before being transferred to nitrocellulose membranes (0.2 µm, Invitrogen, Carlsbad, CA, USA). Rainbow™ coloured protein molecular weight (MW) marker (Amersham Life Science, Amersham, Buckinghamshire, England) was used to determine the relative molecular weights of the proteins. WB was performed to detect HNSCC-associated protein p53. This was done using a combination of polyclonal goat anti-p53 antibody (AF1355, R&D Systems, Minneapolis, MN, USA) and horse radish peroxidase-conjugated donkey anti-goat immunoglobulin (Ig) (HAF 109, R&D Systems, Minneapolis, MN, USA) before detection by the enhanced chemiluminescence method (GE Healthcare, Amersham, Buckinghamshire, U.K.). Differences in intensity of bands served as indirect indicators of differences in antigen content of lysates. The spectrum and intensity of proteins released by various techniques were further evaluated by Coomassie blue staining. Membranes were placed in a staining jar containing Coomassie stain solution (0.2% Brilliant Blue R250 in 20% methanol) for 60 min. The stain solution was discarded before briefly rinsing the membranes in Milli-Q water followed by destaining over a further 60 min using 30% methanol. Based on the results of these experiments, the method favoring optimal protein release was used to make antigen rich-lysates for purposes of assays or nanoparticle fabrication.

Whole Tumor Isolates

The above results were adopted when generating lysates from freshly isolated murine tumor material. Melanoma tumors were initially induced by subcutaneous injection of cells from B16.F10 (murine melanoma; ATCC, Manassas, VA, USA) line into the flanks of female syngeneic C57BL/6

mice (Jackson Laboratory, Bar Harbor, ME, USA). Institutional Animal Care and Use Committee (IACUC) granted approval for rodent usage. Animals were housed and cared for as per national guidelines specified in the *Guide for the Care and Use of Laboratory Animals*, Institute for Laboratory Animal Research (National Academies Press). Tumors were surgically resected and dissected under sterile conditions prior to admixing with a tumor digest solution (collagenase, dispase and hyaluronidase) for 2 h in an incubator at 37°C. Following removal of the undigested tumor material by filtering through a cell strainer (BD Falcon™ 70 µm filter; BD, Franklin Lakes, NJ, USA), cell solution was washed twice in ATCC-recommended B16.F10 medium, counted and re-suspended in sterile ice cold PBS.

Nanoparticle Fabrication

PLGA NP were fabricated employing the solvent evaporation method from a water/oil/water ($W_2/O/W_1$) emulsion as described in an earlier publication from our group (12). Encapsulated material included MBP-gp100 @10 µg/µl or typically lysates of 80×10^6 cells (@ 1×10^7 per 30 µL of sterile PBS) from either cultured FaDu or FAT7 lines or B16.F10 murine melanoma explants described above. To evaluate the ideal MW of PLGA co-polymers (50% lactide: 50% glycolide), differing preparations with inherent viscosity (i.v.) of 0.39 dL/g (45 KDa), 0.59dL/g (80 KDa) or 0.67dL/g (105 KDa, carboxylated end group) (DURECT Corp., Pelham, AL, USA) were dissolved in methylene chloride (Fisher Scientific, Pittsburgh, PA) prior to being used for encapsulation with 0.5% Polyvinyl alcohol (PVA) (Sigma-Aldrich, St. Louis, MO, USA) acting as the stabilizer. Control NP (blanks) were synthesized by identical techniques utilizing sterile PBS instead of aqueous protein solutions.

Nanoparticle Characterization

Assessment of Morphology

Scanning Electron Microscopy. Scanning electron microscopy (SEM) was used to characterize individual NP formulations. A thin film of test sample was smeared onto a metal stub with double-sided adhesive carbon tape (Nisshin EM. Co. Ltd, Tokyo, Japan) and coated with gold (Cressington Sputter Coater 108 Auto, Redding, CA, USA). Imaging was completed using a XL-30 Environmental Scanning Electron Microscope-FEG (FEI, Hillsboro, OR, USA) with an acceleration voltage of 10 kV in a high vacuum mode to achieve magnifications between 9000x and 22,000x. Diameters from representative images of test samples ($n=150$ NP per image) were analyzed using Image J (image analysis software developed by National Institutes of Health, USA).

Dynamic Light Scattering. Dynamic light scattering (DLS) and zeta potential measurements were performed on a Malvern Zetasizer NanoZS system (Worcestershire, UK) with illumination from a 633 nm He-Ne laser. Samples were introduced in a clear folded capillary cell at a concentration of 100 µg per ml of phosphate buffer saline (PBS). Zeta potential was determined from the electrophoretic mobility using the Smoluchowski approximation.

Assessment of Protein Encapsulation. Extent of protein encapsulation was calculated based on the amount of protein extracted from a fixed mass of NP. Five mg of NP were degraded in 1 mL of 100 mM NaOH + 0.05% SDS (Ricca Chemicals, Arlington, TX, USA) by incubation at 37°C and continuous agitation in an orbital shaker. Samples were centrifuged at 16,100 x g for 5 min at room temperature (RT). Supernatants were analyzed for total protein as described above. Encapsulation efficiency was calculated using the formula:

$$(\text{mass protein encapsulated} \div \text{mass protein used in encapsulation}) \times 100\%$$

Measurement of Protein Release Rate. The rate of release of encapsulated proteins by nanoparticle degradation was measured during incubation under controlled conditions. Five mg of NP were suspended in 1 ml of PBS and incubated at 37°C with continuous agitation in an orbital shaker. At designated time points, the suspension was centrifuged at 16,100 x g for 5 min at RT. Supernatants were collected and stored at -20°C for future studies. The particles were re-suspended in 1 ml of fresh PBS in the original tube for further incubation. To increase the extent of re-suspension and reduce clumping that could affect the release profile, a 1 ml pipette was used to pipette the samples as they were being vortexed. On occasions, NP were also briefly sonicated (≤ 15 s; Tekmar sonicator) to ensure re-suspension. Supernatant samples were analyzed for total protein as described above. Cumulative release was studied for at least eight days (the approximate functional lifespan of Ag-loaded DC), to reflect the amount of antigen available to DC for cross-presentation to CD8⁺ T cells. Percentage cumulative release was calculated using the formula:

$$(\text{cumulative protein released} \div \text{total protein content of NP}) \times 100\%$$

Cytometric Bead Array. Influence of PLGA MW on extent of BMDC-mediated cross-presentation to antigen-specific CD8⁺ T cells was determined utilizing a recombinant version of the melanoma-associated protein gp100 and naïve transgenic T cells specific for a gp100-derived epitope.

Syngeneic H-2K^d-restricted BMDC were obtained from C57BL/6 mice as previously described (21) and plated in 6-well polystyrene flat-bottom cell culture plates (BD, NJ, USA). Day 5 DC were loaded with differing MW PLGA NP (0.5 mg of NP per 1×10^6 DC, a ratio previously determined to minimize the presence of unincorporated NP following co-culture) containing recombinant gp100 protein on its own or spiked into B16 tumor lysates. Four to six hours later, Lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO, USA) at 10 ng/ml was added to trigger DC maturation. Following overnight incubation cells were harvested, washed twice, re-suspended in complete RPMI and plated in 96-well polystyrene U-bottom cell culture plates. These were admixed with gp100-specific CD8⁺ T cells isolated from the spleens of *pmel* transgenic mice [TCR transgenic mice with 95% gp100^(25–33)-specific, H-2K^d-restricted splenic CD8⁺ T cells (22); experiments were done in triplicate. After a 24-hour co-culture, the supernatants were removed and analyzed for production of cytokines associated with DC/T cell stimulation [such as Interleukin (IL)-2, Interferon (IFN)- γ and Tumor Necrosis Factor (TNF)- α]. T cell stimulation was quantified using a mouse Th1/Th2 cytometric bead array (CBA) cytokine kit (BD Biosciences, San Diego, CA, USA) as previously described by our group (12). Blank NP served as a control to rule out the possibility of non-specific stimulation by molecules such as endotoxins or the particles themselves.

Evaluation of Immunogenicity of Encapsulated TA from Patient Tumor-Derived Material. The results of the above experiments were adopted for the assessment of the immunogenicity of human tumor-derived material as TA in parallel experiments utilizing autologous TIL as T cell stimulation targets. Ethical permission from the Yale School of Medicine Human Investigation Committee was obtained to use freshly excised tumor and blood from patients with advanced tumors. Patients signed an informed consent and procedures followed were in accordance with institutional guidelines and the tenets of the Declaration of Helsinki promulgated in 1964. Tumor tissue served as source of TA as well as TIL while peripheral blood (drawn on the same day) was used to obtain DC from blood monocyte precursors.

Generation of Tumor Lysate and Isolation of TIL. Fresh tumor isolates were dissociated to generate both a soluble lysate as well as to isolate tumor-derived TIL. Tumor tissue (collected from surgical specimens with minimum delay, directly from surgical suites) was washed three times in modified FaDu medium (Eagle's Minimal Essential Medium + 10% fetal bovine serum + 1% sodium pyruvate + 1% non-essential amino acids + 1.5 mg/ml sodium bicarbonate + 10% penicillin/streptomycin), dissected under sterile conditions prior to admixing with tumor digest solution as described above.

Single cell suspensions were washed twice in modified FaDu medium and a portion, re-suspended in sterile ice cold PBS for lysate-NP production (as mentioned below). Cell solutions were brought up in minimum volumes of PBS (depending on original tumor volume and number of cells isolated) to ensure that tumor lysate concentrations were as high as possible. Protein content was determined using the BCA assay as described above. Remainder of the single cell suspension referred to above was suspended in complete TIL medium [(DMEM + 5% FBS + 1% L-Glutamine + 0.2% Gentamicin) (all Invitrogen, Carlsbad, CA, USA) + 100 IU/ml IL-2 (Peprotech, Rocky Hill, NJ, USA)]. TIL were isolated and expanded using CD3/CD28 Dynabeads (DynaL Prod No: 111.31) as per manufacturer's instructions.

Nanoparticle Fabrication and Characterization. A portion of the single cell suspension obtained from tumor tissue (mentioned above), was encapsulated within 50:50 PLGA copolymers by employing the solvent evaporation method (12). Rate of tumor antigen release was evaluated by nanoparticle degradation during incubation under controlled conditions.

Generation of DC from Peripheral Blood Monocyte (PBMC) Precursors. With a goal of generating DC from PBMC for use in co-culture with TIL, whole blood was collected at the time of surgery in heparin-containing 50 ml conical tubes (BD FalconTM). This was diluted 1:1 in sterile 1x PBS, layered over IsolympH gradient solution (CTL Scientific Supply Corp, Deer Park, NY, USA) and centrifuged at 1500 x g for 30 min at RT. The interface was collected and washed twice with sterile Hanks' BSS (Invitrogen, Carlsbad, CA, USA). Cells were counted and viability checked by Trypan blue exclusion prior to suspension in complete RPMI (RPMI 1640 plus 10% FBS, 2% Pen/Strep and 25 mM HEPES) @ 2×10^6 cells/ml. Cells were plated in 5 ml aliquots in 6-well polystyrene flat-bottom cell culture plates and monocytes were allowed to adhere for 1 h at 37°C with 5% CO₂. Adherent cells were incubated with complete RPMI + 0.25 ng/ml of IL-4 and 0.8 ng/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF; Peprotech, Rocky Hill, NJ, USA) with media changes on days 1, 3 and 5. On day 6, the cells were harvested by gentle scraping, washed and counted. A portion of the DC was used immediately in antigen presentation assays. The remainder was re-suspended in 90% medium (complete RPMI)/10% Dimethyl sulphoxide (DMSO) prior to storage in cryovials at -80°C for subsequent use.

Assessing Immunogenicity Using DC/TIL Co-Culture

Stability and immunogenicity of encapsulated TA from human tumor samples were assessed utilizing TIL stimulation by antigen-loaded DC. Cultured DC were plated in 6-

well plates and incubated with equimolar quantities of antigen in either of two forms –soluble lysate (conventional antigen delivery) or NP lysate (novel antigen delivery) as described above. TIL (@ 0.5×10^6 cells per ml of TIL medium) were added to the wells containing DC in a ratio between 1:3 and 2:3. The cell medium was changed on days 3 and 5. On day 6, autologous frozen DC were thawed, counted and re-plated as above for a second round of TIL stimulation. In order to compare the immunogenicity of encapsulated material as well as to confirm the increased effectiveness of NP as antigen delivery vehicles, for this round of stimulation, DC were loaded with a 5-fold molar excess of soluble lysate for comparison to NP-mediated Ag delivery. DC and pre-stimulated TIL were co-incubated in IL-2-free medium at a ratio of 1:2; experiments were done in triplicate. After 24 h, the supernatants were harvested and used immediately or stored at -20°C for future assays. Cytokine profiling of DC/T cell stimulation was performed on supernatants using a human Th1/Th2 CBA kit (BD Biosciences, San Diego, CA, USA) as described above.

Statistical Analysis

Statistical analysis was done using Statistical Package for the Social Sciences (SPSS) version 17.0 (SPSS, Surrey, UK). Differences were assessed using the independent samples *T*-test. Pearson's coefficient of correlation was used to assess relationship between MW and extent of encapsulation. Significance was accepted at the 5% level.

RESULTS

Optimal Method for Lysate Preparation

A series of preliminary experiments were performed to determine the ideal technique for generating cellular lysate from cultured tumor cell lines. Different methodologies were evaluated in order to maximize protein (antigen) liberation. An initial screen showed that F/T (freeze/thaw) and F/T + S (sonication)

yielded relatively high concentrations of lysates as measured by the BCA assay (Table I-column 1). In addition, Trypan Blue exclusion study revealed maximum cell lysis and fragmentation of cellular membranes following F/T + S (Table I- columns 2 and 3). During repeated treatments, F/T + S consistently yielded greater protein than F/T alone (mean \pm SD= 47 ± 21.5 versus 35.8 ± 22.1 ; $p > 0.05$). Additional characterization of lysates made by these two techniques through SDS/PAGE and western blot confirmed that F/T + S resulted in the greatest overall release of a mixture of TA as well as the specific release of p53. This was noted as increased intensity on Coomassie Blue stained gels (Fig. 1a) as well as a significantly larger p53-associated band by western blot (Fig. 1b). Taken together, the results indicated that F/T + S was an effective, reproducible and non-toxic/chemical-free methodology for the generation of tumor lysates for NP fabrication.

Effect of Differing PLGA MW

NP Morphology

Tissue culture lysates were encapsulated within PLGA copolymers of differing MW (45KDa, 80KDa and 105KDa), using the double emulsion procedure. SEM of resultant NP demonstrated a broad distribution in size and moderate differences in shape. They were largely spherical, non-porous in appearance and within the size range favoring efficient phagocytosis by DC ($\leq 1 \mu\text{m}$) (data not shown). Mean diameters (nm) \pm S.D for 45KDa, 80KDa and 105KDa NP were 204 ± 17 , 252 ± 22 and 337 ± 18 respectively. NP made using 105KDa were larger than 80KDa ($p=0.007$) which in turn was larger than 45KDa particles ($p=0.044$), indicating that polymer had a proportional effect on final NP size (longer polymer = larger NP). DLS analysis showed a similar pattern (Fig. 2) where an increase in NP size was associated with increase in PLGA MW. Mean diameters (nm) were 342, 439 and 477 for the 45 KDa, 80KDa and 105 KDa formulations ($p < 0.05$ for 80 and 105 KDa vs. 45 KDa formulations), with a large size polydispersion observed (Fig. 2b, c). The proportionally increased NP size determinations by DLS vs SEM may have

Table I Profile of Tumor Antigen Lysates made by different Techniques. Protein Content was measured using the Bicinchonic acid assay while Cell Death and Extent of Cellular Fragmentation were Assessed by Light Microscopy. Despite Starting with Similar Amounts of Cellular Material, there were Identifiable differences in the Final Lysate Content and Viability of Cells. Freeze-thaw Plus Sonication for 15 s proved to be Highly effective in Triggering Cellular Fragmentation and release of Antigens from Suspensions, making it the Preferred Method to Liberate Antigens from whole Tumor Cells. Data represent mean and Standard Deviation of Pooled Observations from repeat Experiments

Lysate preparation technique	Protein (antigen) (mg/ml)	Trypan Blue positivity (%)	Cellular fragmentation (%)
F/T + S	50 ± 28.3	98.3 ± 1.2100	98 ± 1.9100
F/T alone	35 ± 23	93.7 ± 5.890	93 ± 5.870
F/T + DH	10.5 ± 7.3	93 ± 2.995	45 ± 750
Sonication for 60 s alone	26 ± 19.3	91.7 ± 2.895	93 ± 5.8100

F/T + S, Freeze-thaw + sonication for 15 s; F/T, Freeze-thaw alone; F/T + DH, Freeze-thaw + Dounce homogenization

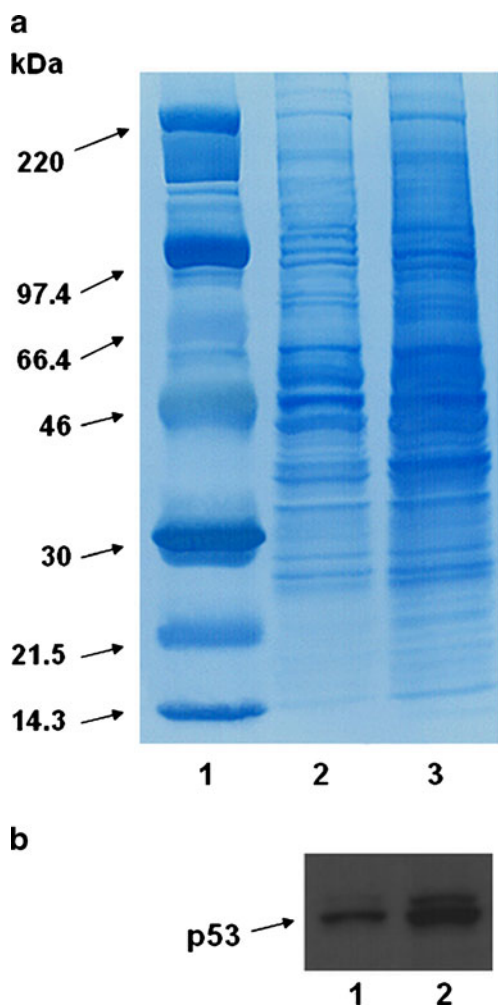


Fig. 1 Differences in antigen release from tumor cells by freeze thaw (F/T) alone and freeze-thaw + 15 sonication (F/T + S). In order to determine differences in protein content, lysate samples from identical numbers of starting cells were electrophoresed on a SDS-PAGE mini-gel system and transferred onto nitrocellulose membranes. After an initial screen, two techniques (F/T and F/T + S) resulting in relatively greater protein liberation from cellular lysates were compared against one another. Greater intensity of staining in the lanes loaded with lysate subject to F/T + S implied greater antigen release by this method, an observation confirmed by increased release of the defined tumor Ag (p53). **(a)** Coomassie Blue staining: Lane 1: Molecular weight ladder; lane 2: F/T alone; lane 3: F/T + S. **(b)** Western blot utilizing an anti-p53 antibody shows increased release of this protein: Lane 1: F/T alone; lane 2: F/T + S.

resulted from DLS sizes representing a hydrodynamic radius. In addition, small differences in zeta potential were observed for the three MW formulations (Fig. 2a).

Encapsulation Profile

To determine the overall effects of polymer MW on protein encapsulation, TA were encapsulated within the three different PLGA MW polymer formulations. NP were rapidly degraded and total protein content quantified. In a reproducibly observed pattern, total protein encapsulated and efficiency of

encapsulation increased with higher polymer MW (Table II). Irrespective of protein (antigen) concentration of lysate used for encapsulation, Pearson's coefficient of correlation was ≥ 0.9 suggesting a strong linear relationship between MW and extent of encapsulation.

Release Kinetics

To determine which PLGA formulation was most likely to release TA in a clinically-relevant eight day period (the approximate function lifespan of an Ag-loaded DC), cumulative release was estimated for this duration. There was a biphasic pattern of protein release: a "burst release" - within the first 24 h and a "plateau phase" - a more sustained release over the subsequent days. Initial burst ($\mu\text{g}/5$ mg of NP; mean \pm SD) for 45KDa, 80KDa and 105KDa were: 8.8 ± 0.8 , 44.5 ± 4 and $27.4 \pm 4.4\%$, respectively. This was greatest with 80KDa PLGA ($p=0.008$ versus 105KDa and $p=0.003$ versus 45KDa). Total release ($\mu\text{g}/5$ mg of NP) was 8.85, 61.4 and 35.3%, respectively. This was highest with 80KDa MW polymer ($p=0.002$ versus 105KDa and $p=0.001$ versus 45 KDa) (Fig. 3). Percentage cumulative release for 45KDa, 80KDa and 105KDa were 11, 39 and 25%, respectively. Similar results were observed in repeat experiments.

Biological Activity of Differing Formulations of TA-Loaded NP

To determine whether a particular MW formulation was associated with superior TA delivery and cross-presentation by DC, melanoma-associated antigen gp100 (either in isolation or contained within a murine melanoma B16.F10 tumor lysate) was evaluated in a CD8⁺-based antigen-specific stimulation assay. Equimolar amounts of each PLGA formulation were loaded onto syngeneic murine BMDC and differences in stimulation of naive gp100-specific CD8⁺ T cells assessed by characterizing profiles of anti-tumor cytokines. As shown in Fig. 4, delivery of gp100 (in isolation) by 80KDa polymer led to greatest immunostimulatory IL-2 production ($p < 0.001$ versus 45 KDa and $p > 0.05$ versus 105KDa) by antigen-specific T cells. Also, 105KDa MW PLGA resulted in greater IL-2 production when compared to 45KDa ($p=0.001$) (Fig. 4a). Similarly, 80KDa polymer resulted in maximal immunostimulatory IFN- γ production ($p < 0.001$ versus 45KDa and $p=0.014$ versus 105KDa). Once again, the 105KDa polymer associated with increased IFN- γ production as compared to 45KDa ($p < 0.001$) (Fig. 4b). When the gp100-specific stimulation was followed with the target Ag admixed with a large group of cellular proteins (i.e. B16 lysate), similar but not identical results were observed. Delivery of gp 100 within B16 tumor lysate utilizing the 45KDa polymer formulation led to greatest IL-2 production ($p=0.002$ versus 80KDa and $p=0.003$ versus 105KDa) (Fig. 4c). There were no significant differences when comparing 80KDa and 105 KDa

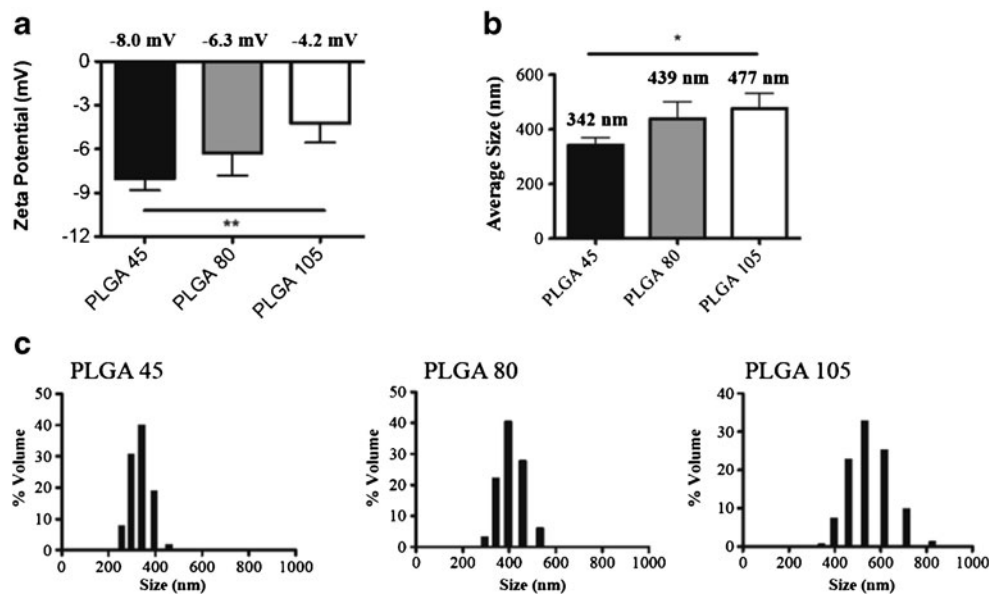


Fig. 2 DLS and charge measurements of nanoparticles synthesized using different molecular weights of PLGA 45, 80 and 105. **(a)** Average zeta potential values for PLGA 45, 80 and 105. **(b)** Average particle diameters for PLGA 45, 80 and 105 as measured by DLS. **(c)** Size distributions for PLGA 45, 80 and 105 as measured by DLS. Data are represented as mean \pm SD. In all experiments, statistical analysis was performed using student t-test where (*) represents $p < 0.05$, (**) represents $p < 0.01$.

polymers. However, IFN- γ synthesis was once again maximal with 80KDa ($p=0.001$ versus 105KDa and $p>0.05$ versus 45KDa); there were no significant differences when comparing 45KDa and 105KDa polymers (Fig. 4d). Taken together, this data suggest that the 80KDa polymer would be the best overall choice for delivery of encapsulated antigen to DC.

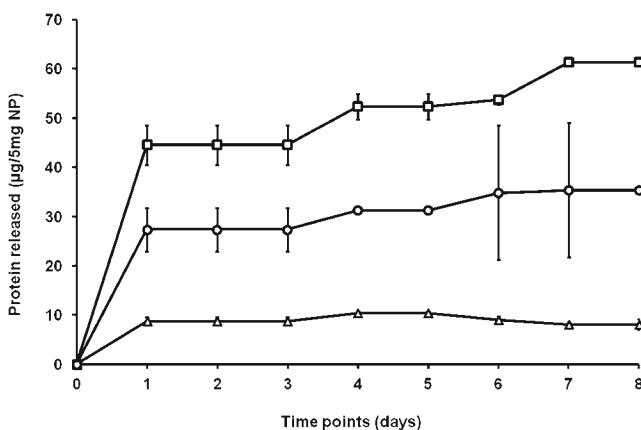


Fig. 3 Effect of PLGA molecular weight (MW) on release of encapsulated tumor material. Proteins were released under controlled conditions from 5 mg of the three PLGA formulations; samples taken at designated time points and analyzed for protein content by the BCA assay. The initial burst of protein release was increased in association with the 80KDa PLGA (open square); $p=0.008$ versus 105KDa (open circle) and $p=0.003$ versus 45KDa (open triangle). Cumulative protein release over 8 days was maximal with 80KDa MW polymer; $p=0.002$ versus 105KDa and $p=0.001$ versus 45KDa. Data refers to mean \pm SD of triplicates from a representative experiment.

Evaluation of Immunogenicity of Tumor Lysate-NP

To confirm whether the results observed with the model TA (gp100) were broadly applicable to lysates and T cell responses found in cancer patients, the above observations were extended to patient-derived whole tumor lysate containing NP. Since the 80KDa polymer appeared to have the most favorable overall release profile and immunostimulatory cytokine production, it was the polymer of choice for the experiments. NP were generated and loaded onto monocyte-derived autologous DC mobilized from patient PBMC. TA-containing DC were used as APC in stimulation assays which targeted tumor-derived TIL. Tumor material dissociated from fresh surgical samples acted as source of tumor NP (from pure tumor) as well as TIL (cultured from tumor interstitium and expanded by CD3/CD28 bead stimulation) (Fig. 5a). Autologous DC were loaded with either soluble tumor lysate (in 5-fold molar excess) or lysate NP (Fig. 5b). Stimulation of autologous TIL was monitored from DC/TIL co-cultures by phase contrast microscopy (Fig. 5c) and quantification of two cytokines known to be critical to anti-tumor immune responses. These were IFN- γ (emblematic of an active Th1 or inflammatory anti-tumor response) and IL-10 (associated with non-productive tumor-tolerized T cells). As shown in Fig. 6, NP-mediated antigen delivery led to increased immunostimulatory IFN- γ ($p=0.002$ versus lysate and $p=0.035$ versus blank-NP) (Fig. 6a). Critically, NP delivery was associated with decreased immunoinhibitory IL-10 production ($p=0.002$ versus lysate and $p=0.015$ versus blank-NP) when compared to soluble lysate (Fig. 6b).

Table II Effect of PLGA MW on Encapsulation Profile. Extent and Efficiency of Encapsulation Increased with Increasing MW. Rotein Content in Lysate (μg /Unit of Polymer (mg). Encapsulation Efficiency was Calculated as: (mass protein encapsulated \div mass protein used in encapsulation) \times 100% formula: Mass Protein Encapsulated/mass Protein loaded) \times 100%. Data represent mean and Standard Deviation of Pooled Observations from repeat Experiments and Values from a Representative Experiment

PLGA MW (KDa)	Protein content in lysate (μg)	Encapsulated protein ($\mu\text{g}/\text{mg}$ of NP)	Encapsulation Efficiency (%)
45	3600 \pm 2018 950	22 \pm 15 2	24 \pm 9.530
80	3600 \pm 2018 950	26 \pm 17 5.6	33 \pm 15.540
105	3600 \pm 2018 950	34 \pm 19.7 8	42 \pm 1746.2

DISCUSSION

Superior bioavailability and CTL responses associated with the PLGA system can help overcome many of the limitations of current DC-based adoptive immunotherapies (23,24). Key components that need to be optimized prior to employing this novel vaccine strategy in human clinical trials include evaluation of antigen loading and release kinetics, assessment of preclinical safety and estimation of biological potency (25,26). An ideal TA-NP formulation should satisfy three

main criteria: high encapsulation efficiency, sustained antigen delivery over 7–8 days (the approximate period of functional antigen presentation by APC) and heightened stimulation of cell-mediated immunity. Although polymer MW can influence encapsulation and release (27), its role in TA presentation has not been clearly elucidated to date (28). Additionally, it is important to establish that the encapsulated substrate retains its structural integrity and remains antigenic. This is a concern because of the well-documented degradation of payload both during NP synthesis and release (29,30). The

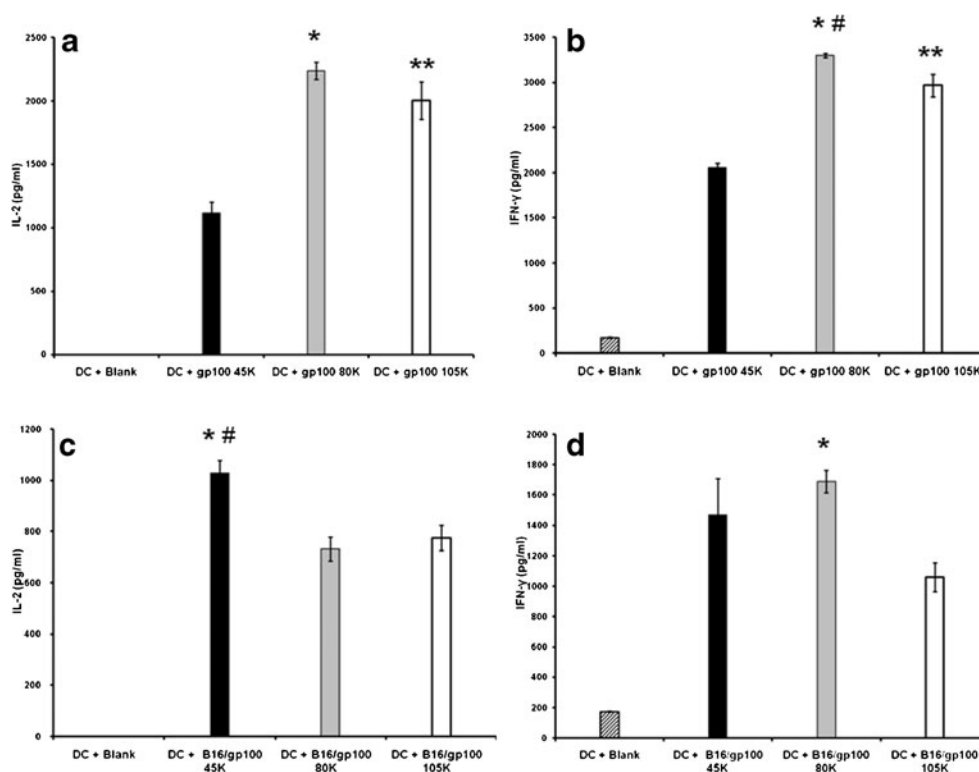


Fig. 4 Effects of differing PLGA formulations on DC-mediated antigen cross-presentation to gp100-specific CD8⁺ T cells. Experiments were performed with recombinant gp100 protein encapsulated alone (**a** and **b**) or after co-encapsulation with murine B16 tumor lysate (**c** and **d**). Differences in T cell stimulation was assessed by measuring production of the immunostimulatory cytokines IL-2 and IFN- γ . DC loaded with blank NP (diagonal bar) were used as negative controls for assays. Single Ag delivery: (**a**) 80KDa polymer (gray bar) led to greatest IL-2 production; * p < 0.001 versus 45KDa (black bar) and p > 0.05 versus 105KDa (open bar). Also, 105KDa MW PLGA was associated with increased IL-2 production when compared to 45KDa (** p = 0.001). (**b**) 80 K polymer resulted in significantly higher IFN- γ production; * p < 0.001 versus 45KDa and # p = 0.014 versus 105KDa. Again, 105KDa polymer caused greater IFN- γ synthesis as opposed to 45KDa (** p < 0.001). Ag in complex lysate: (**c**) IL-2 production was greatest with 45KDa; * p = 0.002 versus 80KDa; # p = 0.003 versus 105KDa. There were no significant differences when comparing 80KDa and 105KDa polymers. (**d**) IFN- γ synthesis was highest in association with 80KDa polymer; * p = 0.001 versus 105KDa and p > 0.05 versus 45KDa. There were no significant differences when comparing 45KDa and 105KDa polymers.

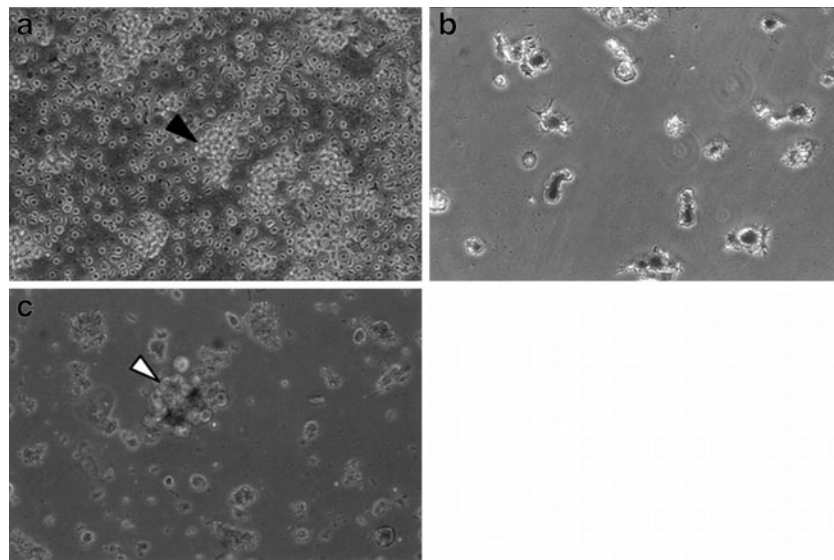


Fig. 5 Phase contrast microscopy of components of DC/TIL co-culture: **(a)** TIL were isolated and expanded using CD3/CD28 Dynabeads®; black arrow indicating conglomeration of expanded TIL. **(b)** PBMC-derived DC were loaded with TA containing-80kDa PLGA NP; cells demonstrating characteristic dendrites and visible NP associated with the cell surface and surface invaginations. **(c)** Co-culture of freshly obtained autologous antigen loaded-DC with TIL; white arrow depicting representative DC/TIL complex.

scope of the present study was to determine the best methodology to generate a cellular lysate, the PLGA MW formulation that was associated with efficient TA encapsulation and delivery to DC, and to assess whether such TA-containing NP could ultimately evoke superior anti-tumor cytolytic CD8⁺ T cell responses.

Different methods were compared when trying to maximize the spectrum and content of tumor antigens in the pre-encapsulation lysate. Since there have been reports of up to 20% antigen instability (31,32), we hypothesized that increasing the lysate concentration would improve encapsulation, provide a buffering effect and eventually increase the amount of stable antigen within PLGA particles. Increasing antigen content of the loading lysate is purported to reduce the extent of antigen adsorbed and denatured at the water-oil interface during particle fabrication. This is due to irreversible adsorption of proteins at the interface. At higher concentrations these adsorbed proteins act as “self-protectants” preventing further

degradation of remaining proteins present in the lysate and causing enhanced encapsulation (33). In a previous study, increasing the theoretical drug content from 20 to 50%, resulted in an increase in the mean amount of drug entrapped from 19.67 ± 3.21 to 51.53 ± 3.15 (34). Factors influencing the choice of a suitable protein isolation technique include safety (for clinical use), reproducibility and efficacy in liberating TA. It was observed that four cycles of freeze-thaw plus 15-second sonication (Fig. 1 and Table I) satisfied these criteria. In the only other report pertaining to encapsulation of a complex mixture of tumor antigens, our group employed four cycles of freeze-thaw and octyl β -glucoside (a dialyzable detergent) to generate lysates (12). The present study suggests that such detergents are not necessary for significant protein liberation. Our initial observations suggest that liberation of critical target proteins such as gp100 (17) and p53 (Fig. 1) by F/T + sonication adequately solubilizes critical target proteins.

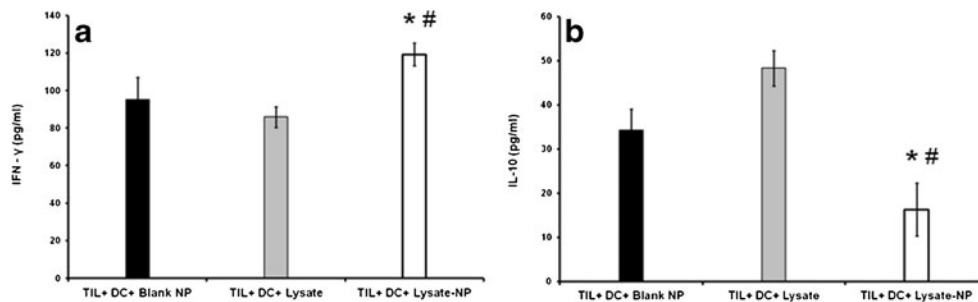


Fig. 6 Enhanced stimulation of TIL after cross-presentation of NP encapsulated tumor antigens by autologous DC. Antigen loaded-DC and TIL were co-cultured overnight and supernatants collected and analyzed for T cell production of IFN- γ and IL-10. NP-mediated antigen delivery led to: **(a)** increased immunostimulatory IFN- γ (* $p=0.002$ versus lysate and # $p=0.035$ versus blank-NP) and **(b)** decreased immunoinhibitory IL-10 (* $p=0.002$ versus lysate and # $p=0.015$ versus blank-NP).

The effects of PLGA polymer MW on the efficiency of encapsulation of both single and complex mixtures of TA within tumor lysate mixtures were provocative. While keeping the concentration of protein in the internal aqueous phase of the double emulsion constant, we observed that increase in polymer molecular weight resulted in concomitant increase in encapsulation rate and efficiency (Table II). This was in keeping with the previous report by our group (12). Increase in molecular mass of polymer increases viscosity of the polymer which in turn improves stability of the primary w/o emulsion. This results in enhanced encapsulation of hydrophilic molecules (proteins and peptides) present in aqueous droplets (35). Our observation is important when attempting to encapsulate tumor tissue as part of NP-based vaccine therapy. Should only meagre amounts of surgical tissue be available (as is often the case), the PLGA MW preparation associated with highest encapsulation should be used in an effort to maximize entrapment of TA. As will be discussed below, this should be considered in conjunction with other factors such as the rate of release and biological potency.

There was an increase in particle diameter associated with increasing polymer molecular weight as determined by both on SEM and DLS (Fig. 2). Similar observations were recorded previously; particle size increased from 98.3 nm to 155.4 nm with increase in polymer MW from 14.5 KDa to 213 KDa, during the encapsulation of estradiol (27). A possible explanation for this is that as polymer molecular weight increases, there is an increase in the viscosity of the emulsion leading to lower stirring efficiency at the same agitation speed. This makes it difficult to generate smaller organic droplets and in turn small sized spheres (27). Our findings confirmed that the even the largest NP were well within the size range that could be efficiently internalized by DC, something of importance when considering NP as antigen delivery vehicles in a DC-based anti-tumor immunotherapy (36).

As reported previously (12), we saw that the extent of release of protein encapsulates varied with alterations in the molecular weight of PLGA co-polymer. Total release over 8 days was greatest from NP made using 80KDa (Fig. 3). Percentage cumulative release for 45KDa, 80KDa and 105KDa were 11, 39 and 25%, respectively. These findings underline the fact that polymer molecular weight plays a role in rate of degradation and release kinetics of payload. Lower molecular weight polymers tend to have shorter chain lengths causing them to be less lipophilic. This favors rapid water hydration causing a drop in the glass transition temperature (T_g) (called plasticizing effect of the water) resulting in greater mobility of polymer chain segments in turn leading to increased degradation (9). Maximal release with the mid-range 80KDa polymer, could imply that additional factors like polymer shape and ionic interaction of encapsulated material with PLGA terminal end-groups

influence release (37). Nevertheless, the biological effect of this phenomenon has only infrequently been described. For instance, testosterone suppression in Sprague–Dawley rats following Leuprolide acetate delivered using differing PLGA (50:50) formulations indicated that 8.6 KDa demonstrated quicker but less lasting effect than 28.3 K (38). The present study reports differences in immunological potency attributable to alteration of polymer MW. In all but one instance (Fig. 4c), 80 KDa polymer was associated with greatest production of anti-tumor inflammatory/Th1 cytokines (Fig. 4 a, b and d) implying superior antigen cross-presentation. The overall antigen encapsulation,/release and T cell stimulation profile suggest that 80 KDa PLGA could be the formulation of choice for encapsulating fresh tumor material from patients. This could enhance CD8⁺ T cell stimulation and reduce chances of tumor immune escape by targeting multiple TA in a “personalized” vaccination strategy.

As part of a stepwise approach to generating important pre-clinical data prior to adoption of NP reagents in clinical trials, we conducted further *ex vivo* experiments aimed at assessing the immunogenicity of encapsulated complex TA derived from freshly isolated tumor. These involved samples from patients with loco-regionally advanced malignancies because this sub-group would most likely be the targets for early (Phase I/II) clinical trials testing novel therapies like NP-mediated DC-based immunotherapy. The experimental design paralleled a potential vaccination protocol; the two rounds of TIL stimulation by antigen loaded-DC mimicked a primary and a booster dose of antigen-loaded DC vaccine. To approximate the trial setting, fresh human tumor samples and TIL rather than established tumor or epitope-specific T cell lines were used. The results suggest that encapsulated material is not only immunogenic but also more efficient at T cell stimulation when compared to conventional lysate. NP-mediated antigen delivery led to significantly increased immunostimulatory IFN- γ (Fig. 6a) and decreased immunoinhibitory IL-10 (Fig. 6b). These phenomena could be critical in overcoming immune resistance that has become the hallmark of solid organ malignancies (39). The data reported in the present study suggests that whole cell lysates (a staple in DC-based immunotherapy outside of melanoma) may be a new and powerful source of antigenic reagents, provided they are delivered to DC through a NP intermediate, for potentially overcoming real-world tumor tolerance. This work is in agreement with earlier reports of favorable T cell stimulation evoked by single antigen-containing NP (40,41). But it extends these observations to encapsulation of multiple (unidentified) TA present within many solid tumors.

This paper reports novel data from encapsulation, release and biological activity of complex mixture of antigens derived from cell lines and whole tumor lysate. More specifically, various techniques were evaluated with a view to finding out

the best method to maximize release of mixture of tumor antigens and ensure production of an antigen rich lysate. Enhancing the release of tumour antigens would in turn ensure that relatively large amounts of antigens are available for encapsulation into and release from PLGA particles. This would then lead to greater delivery of antigens to dendritic cells and potentially cause broader and more intense stimulation of anti-tumor T cells. We present data on the differences in extent of DC-mediated cross-presentation of complex panoply of whole tumor antigens to cytolytic T cells brought about by varying PLGA molecular weight. This is a crucial factor to consider when using PLGA polymers as antigen delivery vehicles. Our results were derived following experiments involving naïve, tumor-specific CD8⁺ T cells derived from gp100-specific transgenic mice in a highly specific T cell assay. These observations were subsequently adopted in encapsulating patient derived tumor antigens and then delivering them via DC for stimulation of freshly isolated autologous T cells. The experimental setup closely mimicked a vaccination protocol that could be used in a clinical trial of NP-DC vaccine, and represents an incremental step forward in the development of PLGA-based reagents for tumor immunotherapy.

It is noteworthy that the NP reagents described here were fabricated without the inclusion of additional maturation stimuli (for example pathogen associated molecular patterns) co-encapsulated or decorating the NP surface. This was a conscious decision aimed at safely using these reagents clinically in the near-term. But our group is actively investigating the use of additional NP formulations utilizing DC-targeting pathogen associated molecules or direct DC ligands on the NP surface. We expect to use these reagents in the future as a step-wise approach to NP-based vaccination strategies leading to overall improvements in vaccine potency.

CONCLUSIONS

Four cycles of freeze-thaw followed by 15 s sonication of tumor cell suspension is an effective, reproducible and non-toxic methodology for the generation of rich tumor Ag containing-lysates for NP fabrication. Irrespective of polymer MW, resultant particles fabricated from a variety of PLGA MWs are within the size range favoring easy phagocytosis by antigen presenting cells. While extent of encapsulation increases with rising PLGA MW, the 80KDa PLGA formulation appears to have the most promising combination of release kinetics and biologic potency for TA delivery. This indicates that polymer MW may directly influence efficient access to antigen cross-presentation compartments found specifically within DC. Encapsulated antigens within such a formulation are immunogenic and evoke favorable TIL-mediated anti-tumor responses when compared to conventional soluble lysate. A favorable cytokine profile in the form of increased

immunostimulatory IFN- γ and reduced immunoinhibitory IL-10 could surmount the immune tolerance milieu prevalent in loco-regional advanced or recurrent solid tumours like HNSCC, thus supporting their use in novel DC-based adoptive solid tumor immunotherapy in the future.

ACKNOWLEDGMENTS AND DISCLOSURES

The authors thank Prof Mark Saltzman and Dr Camille Solbrig in Biomedical Engineering for assistance and supply of reagents associated with NP encapsulation, Dr Michael Girardi and Kacie Carlson in Dermatology for their help in animal handling, and members of the Girardi and Cresswell laboratory for helpful discussions. We also thank Prof Sasaki and Shelley Jolie in Otolaryngology Head and Neck Surgery, Dr Diane Kowalski and Lori Patruno in Surgical Pathology, and members of the team of anaesthetists involved in care of patients recruited to this study. This work was partially funded by intra mural grant available to the Department of Dermatology, Yale University School of Medicine and partially by a NCI/NTRAC grant managed by Cancer Research UK. There is no perceived, potential or real conflict of interest.

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