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Biomimetic Approaches To Modulating The T Cell Immune Response With Nano- and Micro- particles.

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Abstract— **Modulating immune responses to pathogen invasion and even tumors is a major goal in immunotherapy. T cells play a central role in these responses. Progress towards that goal is accomplished by stimulating the antigen-specific T cell immune response *in vivo* through active immunization, or by re-transfer of large numbers of T cells expanded outside the body in a process called adoptive immunotherapy. In both vaccination and adoptive cellular therapy, there is a critical need for a reliable and effective antigen-presentation strategy that stimulates T cells in a specific and efficient manner. Biodegradable nanoparticles can be engineered with bacterial lipopolysaccharides coating thus priming dendritic cells for improved immunization. Alternatively, micron-sized particles can be made to approximate the natural ability of dendritic cells in stimulating T cells by surface modification with the appropriate T cell antigens. Here we show how both of these approaches can be employed to produce safe and effective vaccine and cellular therapeutics.**

I. Introduction

THe immune system is comprised of a number of cell types which when stimulated appropriately can function to efficiently clear harmful pathogens and even cancerous cells. T cells are critical in this process of cell-mediated immunity and their efficient activation is a prerequisite to a successful immune response. T cell activation depends on its interaction with antigen-presenting cells (APCs) such as macrophages, B cells and dendritic cells. These cells are on constant patrol in the body capturing foreign matter (antigens), processing the captured material, and displaying fragment on the suface for recognition by T cells(1, 2). Upon successful recognition, T cells are induced to differentiate and proliferate into daughter effector T cells which are now antigen-specific. These cells migrate to peripheral tissue with effector functions that seek to clear the foreign antigen from the body.

Thus control over T cell stimulation in response to antigen is a major goal in immunotherapy against infectious disease and cancer. Manipulation of this response can be potentially achieved in vivo by vaccination (i.e priming the APC to present the appropriate antigens), or by infusion of antigen-specific T cell populations expanded ex vivo with APCs (adoptive immunotherapy). Although both vaccination and adoptive therapy are promising approaches with a historical and well-documented track record in immunotherapy for different disease states there are critical limitations to both approaches.

Limitations with Active Immunization:

Traditional methods for increasing the effectiveness of vaccines have focused on co-administration of adjuvants or use of a delivery system. While the adjuvant role is critical, there are obvious risks, costs and limitations associated with this traditional approach. For example, current adjuvants, represented predominately by colloidal alum (aluminum sulfate or aluminum hydroxide) or montanide polymers, have a limited capacity to adsorb many antigens and have greatly limited immunostimulatory properties(3, 4). There are also risks associated with using live attenuated vaccines and allergic side effects associated with aluminum salts(4, 5). Additionally, because of the historical emphasis on eliciting humoral immune responses, most adjuvants are optimized for effective induction of high antibody serum titers, but are ineffective at eliciting a strong cellular, T cell-mediated immune response or strong mucosal immune response. T cell responses are essential for inducing lasting viral immunity (or immune responses to cancer); mucosal immunity is essential for protective responses to cellular and viral pathogens that are transmitted through mucosal surfaces (e.g. human immunodeficiency virus, HIV; herpes simplex virus, HSV; enteric pathogens). These factors, coupled with the difficulties of manufacture, storage, and transport have together greatly limited the utility of current approaches in the clinic and in the field(6-8).

Limitations with Adoptive Therapy:

Dendritic cells are the most potent in initiating immune responses but their uses for *ex vivo* stimulation of the antigen specific immune response in clinical applications has been limited because of issues related to quality of isolated cells, quantity, labor, time and cost associated with their isolation(9-11). Moreover custom isolation is often needed per for individual patient cases limiting the generalization of therapy. Therefore, artificial antigen-presenting cells (aAPC) based on cellular or acellular systems have been proposed and tested in the expansion of a number of specific T cells for the treatment of a variety of disease states(9, 11). The acellular approaches, which use micron size beads or lipid-based vesicles with immobilized ligands, are attractive

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because of the flexibility in tailoring the composition and density of ligand presentation. However, these systems carry safety concerns if accidentally injected. Additionally, sustained release of cytokines, which are an essential requirement for T cell homeostasis, is not currently feasible with present technologies. A further limitation of all current systems is that they are mainly applied for *ex vivo* T cell expansion. It would be desirable if the same aAPC can be administered *in vivo* expanding the range of use of such systems in therapy, eliminating safety issues with accidental injections of aAPC and potentially promoting further T cell stimulation *in vivo*.

To overcome issues associated with active immunization and adoptive therapy we have focused on design of artificial biodegradable particles that can prime dendritic cells for antigen-presentation, or that can be used to mimic the APC itself by presenting antigens and releasing cytokines in the vicinity of T cells. Both of these approaches are shown in Figure 1.

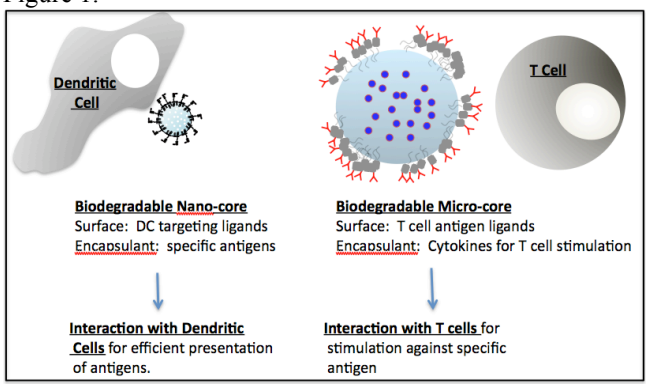

Figure 1. Two approaches to modulating immune responses with biodegradable nanoparticles and microparticles. Both approaches aim to stimulate the T cell response through A) Indirectly by priming dendritic cells and uptake of antigen-loaded nanoparticles displaying ligands that interact with DCs or B) Directly by interaction with T cells via T cell antigen-coated particles encapsulating cytokines.

Active Immunization with nanoparticles: To establish a design-oriented paradigm for vaccines, it is helpful to note that viruses and pathogens that elicit or subvert immune responses are, in essence, small particles endowed with the ability to interact with cells of the immune system in a variety of ways. Much has been learned about their individual strategies. For example, Lipopolysaccarides (LPS) a principal component of the cell wall of gram-negative bacteria and a ligand for Toll like receptor 4 induces DC maturation and thus T cell responsiveness(12, 13). We hypothesized that nanoparticles fabricated from poly(lactic-co-glycolic) (PLGA), a biodegradable and biocompatible, FDA-approved polymer and surface modified with LPS, encapsulating a model antigen can function as potent vaccine carriers. Unlike traditional adjuvants, PLGA particles allow for targeted delivery, protection, and sustained release of antigen during vaccination. Here we show that LPS-modified nanoparticles effectively enter antigen-presenting cells (APCs) and elicit both humoral and cellular immunity against encapsulated antigens in mice. We also demonstrate the modularity of this system for vaccine development by encapsulation of a West Nile virus envelope protein antigen (rWNVE) and the induction of protection in a murine model of West Nile Encephalitis.

Artificial antigen-presentation on Biodegradable Microparticles (aAPC):

T cell responses are mediated by the signals received from antigen-presenting cells. Efficient stimulation of antigen-specific T cells depends on the interaction of the T cell antigen receptor (TCR) with specific antigen in the form of a peptide/major histocompatibility complex (pMHC) on antigen-presenting cells. In addition to this recognition signal, co-stimulation through the B7 family of receptors on APCs, which engage the CD28 receptor on T cells, is known to amplify antigen-specific T cell responses . Thus, current approaches for engineering artificial antigen-presenting cells (aAPCs) exploit this co-stimulatory signal and either specific pMHC complexes or non-specific T cell antigens such as antibodies that crosslink the T cell CD3 complex.

Finally, cytokines, the largest class of immunoregulatory molecules, are secreted by activated antigen presenting cells after T cell encounters and impact expansion, survival, effector function, and memory of stimulated T cells . None of the current aAPC platforms take advantage of this paracrine mode of cytokine delivery although it is a central component of physiological T cell-APC signaling. This is a clear limitation in design that is addressed by the approach we report here.

We demonstrate that the features of an ideal APC discussed above can be incorporated into a biodegradable microparticle. Multivalent contacts between natural APCs and T cells are necessary to facilitate avid interactions leading to efficient stimulation of T cells, and we have found that recapitulation of this presentation on an artificial polymeric particle incorporating immunologically relevant ligands facilitates efficient stimulation of T cells. The biodegradability of this system allows for encapsulation and local release of cytokines, thus mimicking the natural mode of action of this important third signal. The ability of this system to release cytokines in a controlled manner, coupled with the ease of ligand attachment, results in an ideal, tunable antigen-presenting cell capable of stimulating primary T cells(14).

II. MATERIALS AND METHODS

Preparation of nano and microparticles: 50:50 PLGA with an inherent viscosity of 0.59 dL/g, was purchased from Lactel Polymers, Inc. (Pelham, AL, USA). Polyvinyl alcohol (PVA) (M_w average 30-70 kD), LPS (*Escherichia coli* strain 0111:B4), LPS-FITC, chicken egg ovalbumin (OVA), rhodamine B, and Nile red were all obtained from Sigma-Aldrich. Methylene chloride was of chromatography grade and supplied by Fisher Scientific. All other reagents were of reagent grade and used as received. Recombinant

West Nile virus envelope protein antigen (rWNVE) was made in Drosophila S2 cells as described previously (16). We used a modified water-in-oil-in-water (W/O/W) emulsion method for preparation of LPS-modified PLGA particles. In the first emulsion (W/O), concentrated OVA (100 mg/ml) or rWNVE (20 mg/ml) in phosphate-buffered saline (PBS) was added drop-wise to a vortexing PLGA solution (2 ml) dissolved in methylene chloride. Polymer and encapsulant were added drop-wise to 5% PVA in the second emulsion (W/O/W). After each emulsion, the samples were sonicated for 30 s on ice using a Tekmar Sonic Distributor fitted with a model CV26 sonicator – amplitude set at 38%. The second emulsion was rapidly added to 0.3% PVA. This external phase underwent vigorous stirring for 3 hr at constant room temperature to evaporate methylene chloride. LPS-modified particles were prepared with LPS (20 mg/ml in de-ionized (DI) water) added to the second emulsion containing 5% PVA. Particles were collected at 12,000 rpm for 15 min and washed with DI water three times. The particles were freeze dried and stored at –20˚C for later use. All animals received 5 mg of particles per mouse.

Microparticles were fabricated using a single emulsion solid-in-oil-in-water technique or a double emulsion water-in-oil-in-water, while nanoparticles were created using a double emulsion water-in-oil-in-water technique (17). These were surface modified with avidin-palmitate conjugate as described previously (18). For cytokine encapsulation, 100 μg of rhIL-2 (obtained as a generous gift from Maria Parkhurst, NCI) was lyophilized with or without a 10-fold excess by mass of trehalose (Sigma Aldrich, St. Louis, MO) and incorporated as a solid during fabrication (solid-in-oil-in-water single emulsion technique) or added in 100 μl of PBS (water-in-oil-in-water emulsion technique). Particles were lyophilized and stored at -20°C until use.

Biotinylated anti-mouse CD3ε and anti-mouse CD28 (BD Biosciences, San Jose, CA) were added at 10 μg/mL to a 10 mg/mL solution of PLGA particles in PBS and rotated at room temperature for 20 minutes. Particles were washed with PBS + 1% FBS and resuspended in complete RPMI-10. Anti-CD28, when present, was added at a 1:1 molar ratio to anti-CD3.

In vitro analysis with internalization and lysosomal destabilization inhibitors. Bone marrow derived DCs (BMDCs) were matured with 12.5 ng/ml GM-CSF until day 8 when they were incubated in unsupplemented media with or without 10 μM of inhibitors for 1 hour. Cytochalasin D (CytoD) or CA-074 Me were used to inhibit the actin cytoskeleton or cathepsin B, respectively. Cells were then incubated with nanoparticles at 25 μg/ml OVA, soluble or encapsulated in LPS/OVA or -/OVA particles for 1.5 hours. Cells were washed 3X and then co-cultured with 1 x 10^6 splenocytes/well for 48 hour. Supernatant was analyzed for IFN-γ by ELISA.

Animal immunization with antigen-loaded nanoparticles. For west nile nasal vaccinations female C57Bl/6 mice at 6-8 weeks of age were vaccinated intranasally with unmodified nanoparticles encapsulating recombinant West Nile Virus envelope protein (L2 Diagnostics, New Haven, CT) (-/rWNVE); LPS-modified nanoparticles loaded with rWNVE (LPS/rWNVE); or PBS alone. Mice were administered 25 μg of rWNVE in particles suspended in 20 μl of PBS. Mice were boosted with an identical dose at 2 weeks and then challenged 2 weeks later with 1000 PFU of West Nile Virus isolate 2741.

Antibody analysis. Blood was collected retro-orbitally at week 2 for OVA vaccinations and s.c. rWNVE vaccinations and at week 4 for nasal and oral rWNVE vaccinations. Samples were incubated at 4°C overnight and centrifuged at 3000 rpm for 10 min. Serum was isolated and stored at -80°C for later analysis. Antigen-specific IgG titers were analyzed by ELISA. End-point antibody titer was the reciprocal dilution that corresponded to an absorbance two standard deviations above the control.

Tumor studies with aAPC. Mice (C57BL/6) were injected with 1 x 10^5 B16-luciferase cells (Caliper Life Sciences Hopkinton, MA) subcutaneously on day 0, treated with PLGA microparticles on day 10, and were euthanized when tumors reached 2 cm^2. On Day 10 tumors were treated with a single intratumoral injection of 2 mg of 8±2 micron PLGA particles. Tumor areas were calculated by taking the product of the cross perpendicular diameters which were obtained using tumor calipers. Control particles did not display antibodies or release cytokine.

III. Results and Discussion

A. APCs primed with LPS-modified nanoparticles induce both cellular and humoral immune responses.

Dendritic cells were incubated with LPS modified nanoparticles encapsulating the model antigen Ovalbumin (OVA), LPS/OVA, -/OVA nanoparticles, soluble OVA and LPS with blank nanoparticles, soluble OVA and LPS, and OVA alone; and then co-cultured with splenocytes from an animal with T cells that specifically recognize the OVA antigen presented in the context of MHC. Previously we showed that while incubation with both modified and unmodified nanoparticles yielded higher T cell responses than soluble antigen as assessed by IFN-γ secretion, LPS/OVA nanoparticles were more effective(15). Here we treated cells with cytochalasin D, an inhibitor of actin polymerization, or CA-074 Me, an inhibitor cathepsin B (a lysosomal protease thought to play a role in lysosomal destabilization), to see if intracellular uptake and lysosomal processing of particles is responsible for the antigen presentation and subsequent T cell responses. Both inhibitors abated, but did not eliminate, the IFN gamma response to LPS-modified nanoparticles (Figure 2). Furthermore, the addition of soluble LPS, unmodified particles and soluble antigen did not instigate a similar

response as modified particles highlighting the importance of the surface modification step. Thus LPS presented on the surface of biodegradable particles encapsulating antigen illicts a cellular immune response and is dependent on active uptake and lysosomal processing of the nanoparticles.

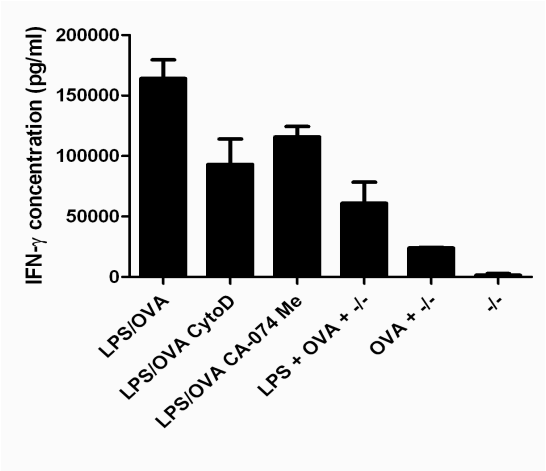

Figure 2. LPS/OVA particles are internalized by DCs and induce a cellular immune response in vitro. Soluble LPS, soluble OVA and blank nanoparticles (-/-) are not as efficient in inducing similar responses.

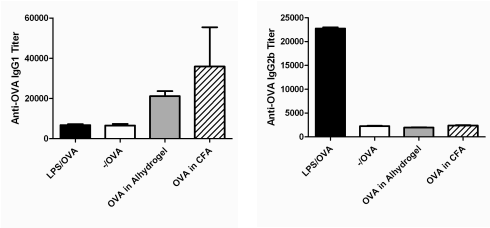

Figure 3. Antibody titers from vaccinated animals (5 mg per mouse) reveal a preferential skewing towards a Th1 immune response with LPS/OVA nanoparticles.

Anibody responses from Animals vaccinated with LPS/OVA, umodified particles, OVA in alum (a standard human vaccine adjuvant) and OVA in complete freunds adjuvant (a gold standard adjuvant used only in mouse studies) is shown in Figure 3. We measured the levels of two classical anibody isotypes, IgG2b and IgG1. IgG2b reports on Th1 mediated immune responses while Ig1 reports on the Th2 immune responses. The generation of either response confers protection and may lead to certain immunopathologies. For example, allergies are predominantly of the Th2 type. While pathogen protection is best elicited via Th1 responses. Strikingly, IgG2b (Th1) mouse titers were significantly higher with LPS/OVA group compared to all other groups while this trend was reversed with IgG1 mouse titers.

B. Vaccination with West Nile Viral antigen-loaded LPS modified nanoparticles provided protection to viral challenge.

After intranasal immunization with LPS particles loaded with recombinant West Nile virus antigen (LPS/E), 80 percent protection to viral infection was conferred to mice compared to 60% without LPS. Previously we noted,

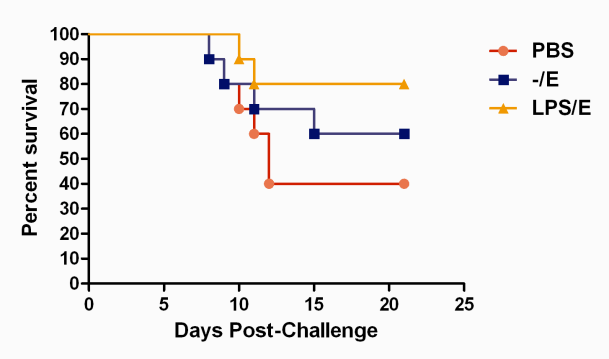

Figure 4. Nasal immunization against the West Nile antigen with LPS-modified nanoparticles loaded with the recombinant West Nile E protein (LPS/E) or umodified particles with E proteins (-/E) or buffered saline. Mice were administered 25 µg of rWNVE in particles suspended in 20 µl of PBS then boosted with an identical dose at 2 weeks and then challenged 2 weeks later with 1000 PFU of West Nile Virus isolate 2741.

antigen-specific IgG titers were more than 20 times higher from antigen delivered in particulate form, compared to soluble antigen when animals were vaccinated subcutaneously(15). Control of flavivirus infection is generally assumed to be primarily mediated by neutralizing antibodies. Interestingly, while titers were relatively low after intranasal and oral vaccination, significant protection was observed. This finding may highlight the importance of the cellular immune response reported here with the model antigen, ovalbumin, which may also play a significant role in survival rates.

C. *Artifical antigen-presenting cells*

Artificial antigen-presenting cells were constructed as previously reported(14). Briefly, these are micro-sized PLGA particles surface modified with avidin for attachment of biontinylated recognition and co-stimulatory ligands. Particles are loaded with cytokines such as IL-2 to enhance

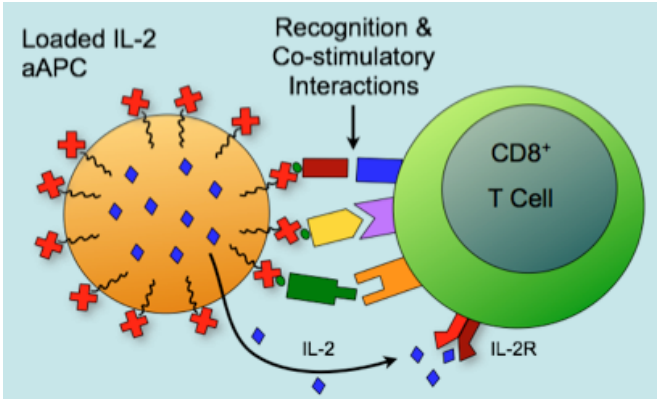

Figure 5. Schematic of an artificial antigen-presenting cell (aAPC) interacting with T cells. Three signals are required for efficient stimulation: Recognition, costimulation and cytokine delivery.

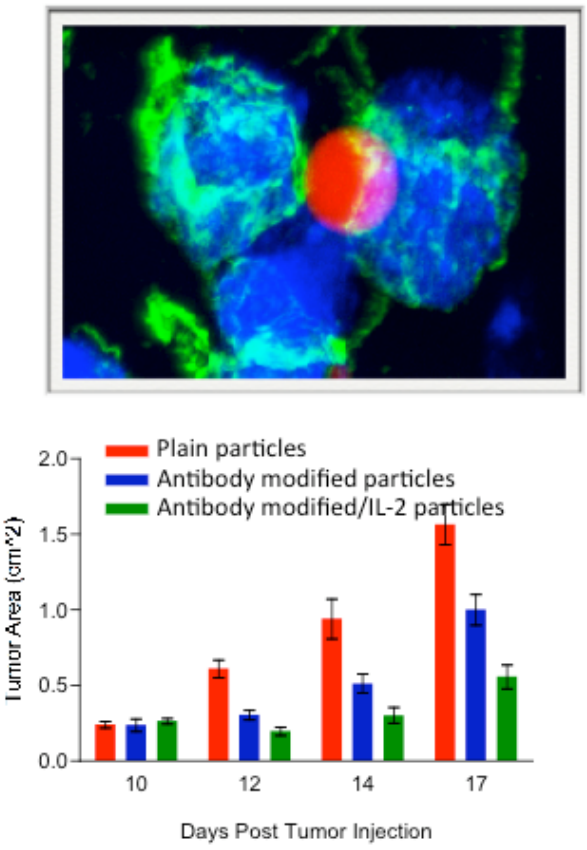

Figure 6. Top: aAPC-T cell binding was visualized by immobilizing B3Z cells to poly-L-lysine-coated coverslips, which were washed 3 times and blocked before incubation with a 5 mg/mL solution of targeted microparticle aAPCs containing encapsulated Rhodamine B and surface-bound anti-CD3 and anti-CD28 for 1 hour at 4°C. Following incubation, the coverslips were rinsed to remove unbound particles, and cells were stained with phalloidin-FITC and DAPI. Bottom: Delayed B16 tumor kinetics in animals intratumorally injected with biodegradable particles surface modified with anti-CD3 and anti-CD28 and encapsulating IL-2. Day 10 tumors were treated with a single intratumoral injection of 2 mg of 8±2 micron PLGA particles. Tumor areas were calculated by taking the product of the cross perpendicular diameters which were obtained using tumor calipers.

stimulation of cells (Figure 5). Previously, we discovered that T cells were vigorously stimulated when incubated with loaded IL-2 aAPCs presenting T cell antigens such as anti-CD3 or peptide/MHC. Compared to unloaded aAPCs or equal amounts of IL-2 cultures did not produce a response either in proliferation or cytokine response equal to that of loaded IL-2 aAPCs(14). Increasing the concentration of exogenous IL-2 improved stimulation, but expansion was less than stimulation by loaded IL-2 aAPCs. Only with a 10-fold increase in the concentration of soluble T cell stimulus in addition to a 10-fold increase in IL-2 did we begin to observe comparable effects with loaded IL-2 aAPCs. These results highlighted the utility and effectiveness of this approach for induction of T cell responses(14).

To examine the efficacy of aAPCs for tumor immunotherapy in vivo we used an established murine B16 melanoma tumor model. Here animals were engrafted with B16 tumors expressing luciferase enabling for bioluminescent imaging to ensure uniformity of tumor size prior to treatment (Figure 6). Mice received intratumoral injections of aAPCs consisting of PLGA particles encapsulating IL-2 and displaying anti-CD3 and anti-CD28 on their surface. Compared to particles without cytokine, paracrine delivery of IL-2 from aAPCs significantly delayed tumor growth kinetics with a single injection at day 10 after tumor implantation (Figure 6). Our Current studies are focusing on boosting this effect with multiple administrations of aAPCs and on elucidating the mechanism of this enhanced delay as it may be tied to local delivery of IL-2 affecting $CD8^+$ T cell responses at the tumor site[54].

This in vivo efficiacy data maybe partially explained by our previous results that showed that unsorted murine splenocytes, upon stimulation with PLGA particles (10 ug aAPCs per 10^5 cells) and presenting anti-CD3 and anti-CD28 encapsulating IL-2, resulted in a pronounced expansion of $CD8^+$ T cells(14). In vitro, this population expanded 45-fold over one week and expressed levels of the IL-2 receptor alpha subunit, CD25 approximately twice as high as other methods(14). These effects were not observed in the absence of encapsulated IL-2. Compared to soluble antibodies or magnetic beads in cultures supplemented with exogenous IL-2, the system also stimulated cells significantly better even in the absence of encapsulated cytokine. This confirms the feasibility and promise of this approach as an APC replacement for in vivo induction of therapeutic responses to tumors for both ex vivo expansion of T cells and in vivo stimulation.

IV. CONCLUSIONS

Biomimicry on artificial nano and microsystems is a powerful methodology to modulate the immune response for both active immunization and adoptive immunotherapy. Two examples were demonstrated: Nanoparticles loaded with antigen and surface modified with bacterial components were shown to prime a vigorous immune response against encapsulated antigen. These vaccine systems are attractive because they are modular in nature, allowing flexible addition and subtraction of antigen, adjuvant, immune potentiators and molecular recognition elements. The appeal of nanoparticles for vaccine delivery is that they allow control over many of the variables that are important in optimizing an effective vaccine delivery system.

Similarly, artificial antigen-presentation on micron sized particles is a potentially useful strategy for stimulation and expansion of T cells primarily because it offers the flexibility over assembly of different combinations and ratios of ligands enabling the investigation of a wide range of T cell activation conditions. These conditions can

potentially affect the quantity and quality of expanded cells. Additionally, such systems are not subject to genetic variability of ligand expression or culture conditions that may alter their function, thus they offer savings in time and labor.

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