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Multi-component nanorods for vaccination applications

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Abstract
Immune responses from Au/Ni nanorods prepared by electrochemical deposition in alumina templates are evaluated in C57BL/6 mice. When the nanorods are bombarded into skin, they generate a strong CD8 T-cell and antibody response. When pcDNA3 is bound to the Ni segment of the nanorod, it provides a strong immunostimulatory adjuvant effect to the antigen bound on the Au segment.

The goal in genetic vaccinations is to encode cells to transiently manufacture antigens that are subsequently taken up by macrophages or dendritic cells (key antigen presenting cells or APCs). APCs process these antigens via class I or class II pathways where they bind to major histocompatibility complexes that present the antigen on the surface of the APCs. These APCs then traffic themselves to the lymphoid organs where T lymphocytes that scavenge the surfaces of the APCs become stimulated to respond against the antigen presented [1]. When the encoded antigen is tumour specific, for example, a strong CD8+ T-cell and antibody response can be generated for protection and prevention against that tumour [2, 3]. We have recently described a new inorganic nanorod vector that can generate transient transgene expression when bombarded into skin, which has a natural abundance of antigen presenting cells [4]. These nanorods therefore have potential for vaccination applications. In contrast to other inorganic non-viral vectors, these nanorods can be engineered with different functionalities in spatially defined regions, which offers precise control of antigen: adjuvant ratios and the possibility of stimulating multiple immune responses [4]. However, before these unique nanorod properties can be exploited for further development, it is essential to ensure that the nanorods can generate a strong immune response *in vivo*.

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In this study, we evaluate the antibody and CD8+ T-cell responses from particle bombardment of nanorods delivering the model antigen ovalbumin or plasmids encoding ovalbumin. Ovalbumin is involved in a number of conditions related to children. For example, children with cystic fibrosis display higher anti-ovalbumin antibodies. Ovalbumin antibodies are also observed in kidney diseases such as nephropathy. Children with insulin-dependent diabetes mellitus show elevated immune responses to both β-lactoglobulin and ovalbumin, which may be associated with the progression of the disease [5–7].

The nanorods were fabricated by electrodeposition into an Al_2O_3 template (Anodisc, Whatman) with a nominal pore diameter of 100 nm [4, 8]. An evaporated silver film on one side of the template served as the working electrode in a three-electrode configuration. A thin layer of silver was electrodeposited into the template to ensure easy release of the nanorods from the template. Gold segments were deposited prior to nickel segments to prevent erosion of the nickel layers during silver removal. The silver layers were dissolved in 70 vol% nitric acid and the alumina template was then dissolved in 2 M potassium hydroxide. The nanorods were 1.6 μm in length by 170 nm in diameter with 800 nm length gold segments and 800 nm length nickel segments.

Confirmation of deposition of the nickel and gold segments was seen by back-scattering SEM. Using chemical moieties that bind selectively to either gold or nickel, we

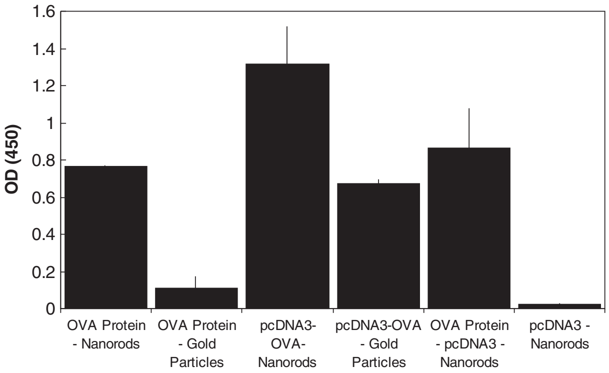

Figure 1. Ovalbumin-specific antibody responses in C57BL/6 mice immunized with various antigen or plasmid nanorod and gold particle formulations. C57BL/6 mice were immunized with control plasmid (no insert) bound to nanorods, ovalbumin antigen–nanorod formulation, ovalbumin antigen–gold particle formulation, pcDNA3–OVA–nanorod formulation, pcDNA3–OVA–gold particle formulation and ovalbumin antigen/control pcDNA3 (no insert)–nanorod formulation via a gene gun. Serum samples were obtained from immunized mice 21 days after the initial vaccination. The presence of the ovalbumin-specific antibody was detected by ELISA using serial dilution of sera. The results from the 1:1000 dilutions are presented showing the mean absorbance (A450 nm) $\pm$ SE.

attached plasmids or the antigen ovalbumin to the different segments, as described previously [4]. A small proportion of the primary amine groups of ovalbumin were converted to sulfhydryl groups. The ovalbumin was then bound to the gold segments of the nanorods through a thiolate linkage [9]. Electrostatic interactions were used to bind DNA to the nickel segments by suspending the dual component nanorods in a 0.1 M solution of 3-[(2-aminoethyl) dithio] propionic acid (AEDP). The carboxylic acid terminus of AEDP binds to the native oxide on the nickel segments. This results in the surface presentation of primary amine groups spaced by a reducible disulfide linkage. In the reducing environment of the cell, the disulfide linkage between the plasmid and the nanowire is cleavable to enhance release of the plasmid. In this study, plasmids encoding ovalbumin (pcDNA3–OVA) or control plasmids with blank inserts (pcDNA3) were utilized. Previous UV–visible spectroscopy calibration measurements (260 nm) of DNA binding to the nanowires provided an average surface coverage of 4×10^{12} molecules cm^{-2} [4]. For condensation of the plasmids bound to the nanowires, a $CaCl_2$ solution was added to the nanowire–plasmid formulations. Ca^{2+} has a high affinity to DNA (K_d of 1.1×10^{-3} M^{-1}), forming $CaPO_4$ complexes with the nucleic backbone to provide stabilization and compaction to the DNA structure.

To evaluate the genetic vaccination potential of these nanorods, antibody responses from the bloodstream and CD8+ T-cell responses from the spleen were measured from C57BL/6 mice vaccinated with the nanorod/plasmid or nanorod/antigen formulations. In addition, we compared these responses to those generated by the industrially optimized gold particle formulations, the standard in the field of gene gun-mediated genetic immunization. For antigen/microcarrier formulations, the gold particles generated a sevenfold higher CD8+ T-cell response than the nanorods. In contrast, for the antibody response, the nanorods produced a sevenfold higher response in comparison with the 1.6 μm gold particles (figures 1 and 2). To evaluate the benefit of the nanorods' multifunctionality, pcDNA3, the blank molecular construct without the antigen gene, was bound to the nickel segments of the nanorods in conjunction with the ovalbumin–SH antigen on the gold segments. In control experiments, pcDNA3 bound to the nanorods alone generated very low or no antibody and CD8+ T-cell responses. However, co-addition of pcDNA3 and the ovalbumin antigen on the nanorods generated a significant eightfold increase in the CD8 response in comparison to the nanorods bound to the ovalbumin alone. This increase is likely due to a role of the CpG motif in the pcDNA3 acting as a strong immunostimulatory adjuvant to the ovalbumin antigen, thus enhancing the overall CTL immune response [10–12]. The ability of nanorods to deliver the CpG motif and the antigen to the same cell is essential for generating a stronger immune response. For example, Babuik and colleagues have shown that, in pigs, administration of CpG ODN and HBsAg vaccine in separate sites of the same muscle did not show an enhanced antibody response compared to administration of the HBsAg vaccine alone, whereas administration of CpG ODN with the HBsAg vaccine significantly enhanced the antibody responses [13].

Delivering plasmids encoding ovalbumin by both nanorods and gold particles generated stronger antibody and CD8 T-cell responses than the ovalbumin antigen alone. Gene gun delivery of antigens can directly enter and prime dendritic cells, but the delivery of plasmids encoding the antigen probably enhances the overall response because in addition to directly priming the dendritic cells, it may also transfect keratinocytes. The keratinocytes then produce antigens that once released can cross-prime more dendritic cells to further the overall immune response.

In summary, we have shown that these versatile nanorods generate strong antibody and CD8+ T-cell responses and therefore have significant potential for further development in vaccination applications. In future studies, we anticipate that aligning the nanorods within the cartridges to produce 'arrow'-like delivery may allow us a much more favourable penetration depth–pressure relationship in particle bombardment than the gold particles. Advantages to this would include transfecting both skin and the subcutaneous tissues for pressure modulated control over sustained or transient expression of genes and greater depths of penetration at lower pressures [14]. The ability to add new components to the nanorods such as adjuvants and/or cytokines in controlled ratios will allow us to generate stronger immune responses than single component particles as demonstrated in this study using the CpG motif from the pcDNA3 as an immunostimulatory adjuvant to the antigen. In addition, the ability to engineer and add extra segments [15, 16] to the nanorods will allow for the possibility of delivering multiple agents such as RNA, antigens and DNA to the same cell for the stimulation of multiple immune responses.

1. Methods and materials

1.1. Preparation of 200 nm dual component Au/Ni nanowires

Nanowires were fabricated by electrodeposition into an Al_2O_3 template (Anodisc, Whatman) with a nominal pore diameter

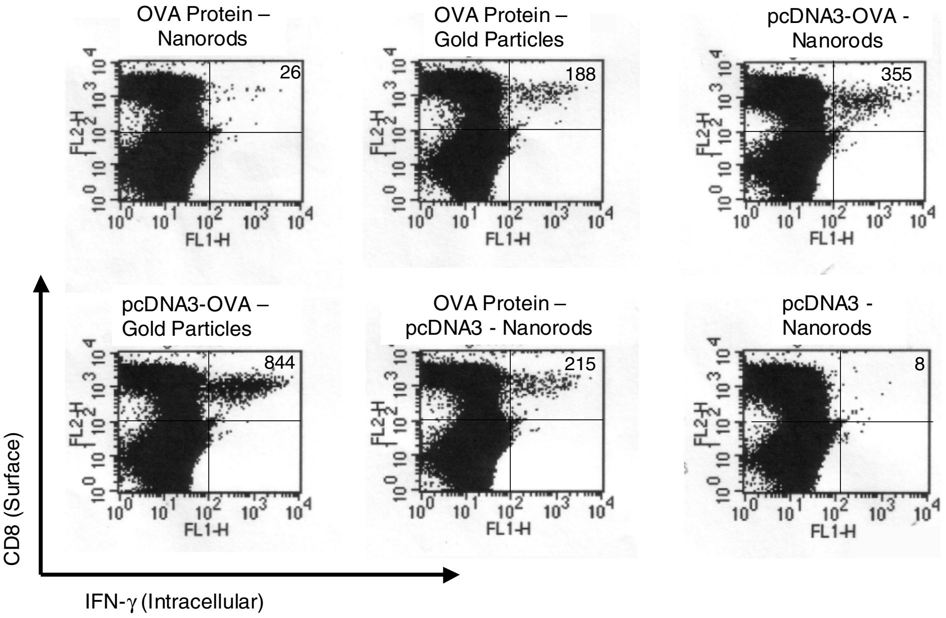

Figure 2. Ovalbumin-specific CD8+ T-cell precursors in C57BL/6 mice immunized with various antigen or plasmid–nanorod and gold particle formulations. C57BL/6 mice were immunized with control plasmid (no insert) bound to nanorods, ovalbumin antigen–nanorod formulation, ovalbumin antigen–gold particle formulation, pcDNA3–OVA-nanorod formulation, pcDNA3–OVA–gold particle formulation and ovalbumin antigen/control pcDNA3 (no insert)–nanorod formulation via a gene gun. For vaccinated mice, 2 μg of DNA or antigen/mouse were given twice at week 0 and 2. Splenocytes were harvested seven days after the last DNA/antigen vaccination. *Flow cytometry analysis*: splenocytes from vaccinated mice were cultured *in vitro* with the ovalbumin antigen overnight and were stained for both CD8 and intracellular IFN-γ. The number of IFN-γ secreting CD8+ T-cell precursors in mice immunized with antigen or plasmid–nanorod and gold particle formulations were analysed by flow cytometry. The number of CD8 + IFN-γ + double-positive T cells in 3×10^5 splenocytes are represented by the quadrant in the upper right corner.

of 100 nm. An evaporated silver film on one side of the template served as the working electrode in a three-electrode configuration. A thin layer of silver was first electrodeposited from 50 mM $KAg(CN)_2$ and 0.25 M Na_2CO_3 buffered to pH 13 at a potential of -1.0 V (Ag/AgCl) in order to ensure easy release of the nanowires from the template. The Au segments were deposited from a commercial gold plating solution (Technic) at a potential of -1.0 V (Ag/AgCl) and the Ni segments were deposited from a solution of 20 g l^{-1} $NiCl_2{\cdot}6H_2O$, 515 g l^{-1} $Ni(H_2NSO_3)_2{\cdot}4H_2O$), 20 g $l^{-1}$$H_3BO_3$ buffered to pH 3.4 at a potential of -1.0 V (Ag/AgCl). The silver layers were dissolved in 70 vol% nitric acid and the alumina template was then dissolved in 2 M KOH. The nanowires were washed repeatedly using 2 M KOH, deionized water, and ethanol.

1.2. Plasmid DNA constructs and DNA preparation

The generation of pcDNA3–OVA has been described previously [17]. Briefly, the DNA fragment encoding OVA was amplified by a set of primers, 5′-CCCGAATTCATGGGCTCCATCGGCGCAGC-3′ and 5′-CCCGGATCCAAATTCTTCAGAGACGCTTGC-3′, and OVA cDNA. The amplified product was further cloned into the EcoRI/BamHI sites of pcDNA3.

1.3. Functionalization of Au/Ni nanorods

DNA binding. A 150 μl of 0.1 M AEDP (Pierce) solution was added to 200 μl aliquots of nanorods ($\sim 1 \times 10^6$) suspended in distilled water. Following incubation for 24 h and washing, 2 μg of plasmid was added to each aliquot of nanorods, (pH 5.7) and incubated at 4 °C for 24 h. After washing, 2 μl of a 2 M $CaCl_2$ solution was added to each aliquot and then incubated for 24 h at 4 °C.

Ovalbumin binding. 5 mg of ovalbumin in PBS with 5 mM EDTA was reacted with 120 μl of 5 mg ml^{-1} iminothiolane (Pierce) for 30 min at room temperature. The protein was purified by dialysis at 4 °C. 20 μl of 5 mg ml^{-1} ovalbumin–SH was added to each aliquot of nanorods and incubated for 24 h at 4 °C.

1.4. Immunization experiments

We purchased 6- to 8-week-old male C57BL/6 mice from the National Cancer Institute (Frederick, MD) and kept them in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, MD). All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

Gene gun particle-mediated DNA vaccination was performed using a helium-driven gene gun (Bio-Rad, Hercules, CA) according to the protocol provided by the manufacturer. Briefly, DNA-coated gold particles were prepared by combining 25 mg of 1.6 μm of gold microcarriers (Bio-Rad, Hercules, CA) and 100 μl of 0.05 M spermidine (Sigma, St Louis, MO). Plasmid DNA (50 μg) and 1.0 M $CaCl_2$ (100 μl) were added sequentially to the microcarriers while mixing by vortex. Antigen-coated gold particles were prepared using the same protocol with the exception of the addition of $CaCl_2$. This mixture was allowed to precipitate at room temperature for 10 min. The microcarrier/DNA suspension was then centrifuged (10 000 rpm for 5 s) and washed three times in fresh absolute ethanol before resuspending in 3 ml of polyvinylpyrrolidone (0.1 mg ml^{-1}; Bio-Rad, Hercules, CA) in absolute ethanol. At this stage, Nanowire–pcDNA$_3$–OVA formulations and nanowire–ovalbumin formulations at the appropriate concentrations were also resuspended in 3 ml of polyvinylpyrrolidone (0.1 mg ml^{-1}; Bio-Rad, Hercules, CA) in absolute ethanol. The solutions were then loaded into tubing and allowed to settle for 4 min. The ethanol was gently removed, and the microcarrier/DNA suspension was evenly attached to the inside surface of the tubing by rotating the tube. The tube was then dried by 0.4 l min^{-1} of flowing nitrogen gas. The dried tubing coated with microcarrier/DNA was then cut to 0.5 inch cartridges and stored in a capped dry bottle at 4 °C. As a result, each cartridge contained 1 μg of plasmid DNA and 0.5 mg of gold particles or nanowires. The DNA-coated gold particles (1 μg of DNA/bullet), the antigen-coated gold particles, the DNA-coated nanowires and the antigen-coated nanowires (each 1 μg of DNA or antigen/bullet) were delivered to the shaved abdominal region of the mice using a helium-driven gene gun (Bio-Rad, Hercules, CA) with a discharge pressure of 400 psi. Mice were given a booster two weeks after the initial shots.

1.5. Anti-OVA ELISA

The anti-OVA antibodies in the sera were determined by a direct ELISA. A 96-microwell plate was coated with 100 μl of 10 μg ml^{-1} ovalbumin antigen and incubated at 4 °C overnight. The wells were then blocked with PBS containing 20% fetal bovine serum. Sera were prepared from the mice on day 21 after the initial vaccination, serially diluted in PBS, added to the ELISA wells, and incubated at 37 °C for 2 h. After washing with PBS containing 0.05% Tween-20, the plate was incubated with 1/2000 dilution of a peroxidase-conjugated rabbit antimouse IgG antibody (Zymed, San Francisco, CA) at room temperature for 1 h. The plate was washed six times, developed with 1-Step Turbo TMB-ELISA (Pierce, Rockford, IL), and stopped with 1 M H_2SO_4. The ELISA plate was read with a standard ELISA reader at 450 nm.

1.6. Intracytoplasmic cytokine staining and flow cytometry analysis

Splenocytes from the vaccinated mice were incubated with ovalbumin. The ovalbumin was added at a concentration of 2 μg ml^{-1} for 20 h. To detect ovalbumin-specific CD8+ T-cell precursors, ovalbumin peptide (SIINFEKL) was used. Golgistop (PharMingen, San Diego, CA) was added 6 h before harvesting the cells from the culture. Cells were then washed once in FACScan buffer and stained with phycoerythrin-conjugated monoclonal rat antimouse CD8 antibody (PharMingen, San Diego, CA). Cells were subjected to intracellular cytokine staining using the Cytofix/Cytoperm kit according to the manufacturer's instructions (PharMingen). FITC-conjugated anti-IFN-γ and the immunoglobulin isotype control antibody (rat IgG1) were all purchased from PharMingen. Analysis was done on a Becton Dickinson FACScan with CELLQuest software (Becton Dickinson Immunocytometry System, Mountain View, CA).

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