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Type 1 and 2 Immunity Following Vaccination Is Influenced by Nanoparticle Size: Formulation of a Model Vaccine for Respiratory Syncytial Virus

Patricia L. Mottram,[†] David Leong,[†] Blessing Crimeen-Irwin,[†] Simone Gloster,[†] Sue D. Xiang,[†] Jayesh Meanger,[‡] Reena Ghildyal,[‡] Nicholas Vardaxis,[§] and Magdalena Plebanski*,[†]

The Burnet Institute at Austin (Austin Research Institute), Studley Road, Heidelberg 3084, VIC, Australia, The Burnet Institute, Commercial Road, Prahran, 3181, VIC, Australia, and RMIT University, Bundoora West Campus, GPO Box 2476V, Melbourne, 3001, VIC, Australia

Received September 10, 2006; Revised Manuscript Received October 29, 2006; Accepted November 6, 2006

Abstract: Previous studies compared uptake by dendritic cells (DC) of 20, 40, 100, 200, 500, 1000, and 2000 nm beads in vivo. When beads were used as antigen carriers, bead size influenced antibody responses and induction of IFN-γ-producing CD4 and CD8 T cells. Beads of 40−50 nm were taken up preferentially by DC and induced particularly strong immunity. Herein, we examine immunity induced by minute differences in nanobead size, specifically within a narrow viral-sized range (20, 40, 49, 67, 93, 101, and 123 nm), to see if bead carrier size influenced the induction of type 1 or type 2 cells as demonstrated by the production of IFN-γ or IL-4. In vivo uptake by DC was assessed for selected sizes in this range. Responses to whole ovalbumin (OVA) or the OVA-derived CD8 T cell peptide epitope (SIINFEKL) were tested. After one immunization with beads−OVA, IFN-γ responses to both OVA and SIINFEKL were significantly better with 40 and 49 nm beads than other sizes, while, in contrast, IL-4 responses to OVA were higher after immunization with OVA conjugated to larger beads (93, 101, and 123 nm). Thus IFN-γ induction from CD8 T cells was limited to 40−49 nm beads, while CD4 T cell activation and IL-4 were induced by 93−123 nm beads−OVA. After two immunizations, there were comparable high levels of IFN-γ produced with 40 and 49 beads and IL-4 reactivity was still higher for larger beads (93, 101, 123 nm). Production of IgG1 was seen across the full range of bead sizes, increasing after two immunizations. Since protection against respiratory syncytial virus (RSV) depends on strong IFN responses, while IL-4 responses are reported to cause asthma-like symptoms, immunization with RSV antigens on the 49 nm carrier beads could provide the basis for a suitable vaccine. When the 49 nm beads were conjugated to RSV proteins G88 (surface) or M2.1 (internal capsid), one immunization with G88 induced high levels of IFN-γ and low levels of IL-4. IL-4 increased with two immunizations. Beads−M2.1 induced only moderate levels of IFN-γ and low titer antibody after two immunizations. Mice vaccinated once with G88-conjugated 49 nm beads and challenged intranasally with RSV strain A2 subtype showed reduced viral titers and recovered from weight loss more rapidly than mice immunized with M2.1-conjugated 49 nm beads or naive control mice. These results show that precise selection of nanobead size for vaccination can influence the type 1/type 2 cytokine balance after one immunization, and this will be useful in the development of effective vaccines against common human pathogens such as RSV.

Keywords: Type 1 and 2 immunity; nanobead size; ovalbumin; RSV vaccine; mice

Introduction

The difference between type 1 and type 2 immunity was originally defined by functionally distinct subsets of CD4 T helper cells, with type 1 (Th1) cells producing IFN-γ and IL-2 and type 2 (Th2) cells secreting IL-4 and IL-10. This definition has since been modified in recognition of the other cell types involved in the response.[1,2] This polarization of responses is particularly important in vaccine development, where the most effective antiviral responses are type 1, with

* Author to whom correspondence should be addressed. Mailing address: Vaccines and Infectious Diseases Unit, The Burnet Institute, Austin Campus, Austin Hospital, Studley Road, Heidelberg, Victoria 3084, Australia. Tel: 61-3-92870643. Fax: 61-3-92870643. E-mail: mplebans@burnet.edu.au.

[†] The Burnet Institute at Austin (Austin Research Institute), Heidelberg.

[‡] The Burnet Institute, Prahran.

[§] RMIT University.

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10.1021/mp060096p CCC: $37.00
Published on Web 11/30/2006

IFN-γ production and activation of CD8 T cells, while allergic reactions (asthma, rhinitis) are type 2.[3,4] Thus optimal vaccine formulations would favor induction of primary type 1 responses, leading to long-term CD4 and CD8 T cell memory. At present, the most effective antiviral vaccines contain attenuated or heat killed viral particles,[3] with recently available viral peptides and recombinant proteins being less effective. Coupling these new reagents to viral-sized particles[5] may well provide vaccine formulations of greater consistency and with fewer side effects, such as unwanted type 2 allergic responses.

Endocytic uptake mechanisms vary depending on the size of the particle engulfed, thus larger beads (>500 nm) enter via phagocytosis and localize to macrophages, while virus-sized particles (20−200 nm) enter either by receptor-mediated endocytosis into clathrin-coated pits (particles <150 nm)[6] or through caveolae (particles within 50−80 nm).[7,8] Smaller particles can enter via pathways used by viral pathogens, such as influenza and respiratory syncytial viruses.[9,10] Although solid polystyrene, gold, or silica beads have previously been used as carriers for surface-absorbed antigen to stimulate CD8 T-cell responses, bead diameters of 500−1000 nm were commonly used.[11] Smaller antigen-carrying particles such as ISCOMS[12] and engineered virus-like particles (VLP) of 30−200 nm diameter usually also provide "danger" signals, often through stimulation via Toll-like receptors (TLR), to induce activation of antigen presenting cells.[13,14] Solid, synthetic, particulate vaccines, in contrast, can induce immune responses without added classical "danger signals",[5,15,16] and our novel nanovaccine approach, using 40−50 nm polystyrene beads with covalently coupled tumor-specific antigen, has been shown to clear large established tumor masses in mice within two weeks after a single injection.[17]

Thus, the size of the carrier bead could play a role in determining the type of response induced, with virus-like particles inducing IFN-γ cell mediated type 1 responses and larger beads inducing type 2 responses.[18] In vaccine development, IFN-γ responses are more likely to be protective against systemic intracellular pathogens such as viruses,[19,20] so 40−49 nm beads may be more effective antigen carriers than beads of other sizes for antiviral vaccines. To test this hypothesis in a model viral infection in mice, 49 nm beads were used in the second section of the study to formulate a vaccine against respiratory syncytial virus, RSV. This virus is a major cause of lower respiratory tract disease in infants and small children, infecting almost all children under the age of two. While most children recover and develop immunity as they mature, naturally acquired protection is "neither complete nor durable",[21] and premature infants and those with cardiovascular disease are at risk of fatal lung

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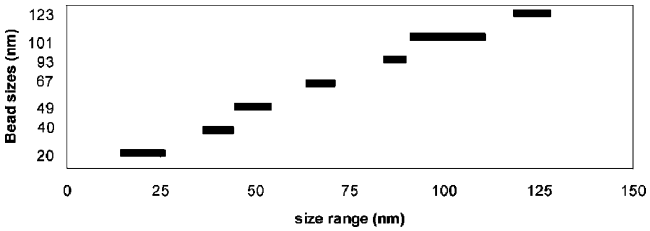
disease following infection.[21] Vaccine development has been hampered by the failure of initial clinical trials using formalin-inactivated virus, where induction of a type 2, IL-4 response resulted in eosinophilia and acute respiratory failure.[21] More recently the availability of purified RSV protein subunits (G, M, and F) has provided antigens that, with adjuvants, have entered clinical trials. Although these vaccines have not produced IL-4 and adverse side effects, they have also not provided long lasting protection: annual vaccination will be required even with the most effective of these formulations, now in phase III trials.[21] There is a need therefore to improve RSV vaccines, and designing suitable conjugates of recombinant antigens with nanobead-based vaccine carriers that stimulate type 1 rather than type 2 response may be useful in that context.

Experimental Methods

Protein and Peptide Antigens. Ovalbumin (OVA) was purchased from Sigma (grade III, Sigma-Aldrich, St Louis, MO). The minimum OVA CD8 T cell epitope, (SIINFEKL), was synthesized without flanking amino acids by Auspep (Melbourne, Australia). M2.1 proteins and pET-30a plasmids containing the coding sequence for RSV G88, tagged with poly-histidine residues, were obtained from Jayesh Meanger and Reena Ghildyal, Burnet Institute, Melbourne, Australia.[22,23] Small-scale production of recombinant RSV G88 was carried out within our laboratory using standard expression, solubilization, and purification methods. Briefly, the plasmids were induced to express in *Escherichia coli* BL-21 cells by the addition of 1 mM IPTG. The cells were harvested by centrifugation 3−4 h after IPTG induction. The cell pellets were lysed by sonication in lysis buffer (50 mM Tris-Cl/2 mM EDTA pH 8.0 + 1 mg/mL lysozyme and 0.1% Triton X-100). The resulting insoluble pellet was then solubilized in buffer (8 M urea/0.1 mM Tris-Cl/0.1 M sodium phosphate buffer), and the His-tagged recombinant proteins were purified using a column of Ni^{2+}NTA beads (Qiagen, www.qiagen.com) and solubilization buffer at a lower pH. The purified proteins were then transferred into 2 M urea/PBS and centrifuged in Centricon concentration devices (Amicon, from Millipore, www.millipore.com). Routinely, recombinant RSV proteins of >95% purity were produced at approximately 5 mg per liter of starting culture.

Nanoparticle−Antigen Conjugation. Carboxyl-modified 20, 40, 49, 67, 93, 101, and 123 nm polystyrene beads from Molecular Probes (Invitrogen), IDC/Polysciences (Polysciences, Inc., Warrington, PA), Sigma (St. Louis, MO) or PolyMicrospheres (PolyMicrospheres, Indianapolis) were used (Table 1). There were no significant differences between bead activity as assessed in side-by-side experiments using 20, 40, or 49 nm formulations from different manufacturers (not shown). Further, the standard error around the given mean particle diameter size was in all cases (and for all sizes tested) between 5% and 30%, as specified by the manufacturers, as determined by Transmission Electron Microscopy and shown in Table 1.

Table 1. Nanobead Specifications



bead size (nm) ± SD	bead source
20 ± 6	Molecular Probes, Polysciences, PolyMicrospheres
40 ± 4	PolyMicrospheres, Sigma, Molecular Probes
49 ± 5	Polysciences, Sigma
67 ± 4	PolyMicrospheres
93 ± 7	PolyMicrospheres
101 ± 10	PolyMicrospheres
123 ± 6	PolyMicrospheres

Conjugation to OVA was performed as previously described.[24] Briefly, carboxyl-modified polystyrene beads were diluted to a 2% slurry (wt:vol) and combined 1:1 (vol:vol) with 1 mg/mL OVA (grade III, Sigma-Aldrich, St. Louis, MO) to provide a 1% wt/vol bead preparation containing 50 μg of OVA/100 μL. Conjugation was for 15 min with EDAC (4 mg/mL, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; Sigma) and 0.05 M MES ([2-*N*-morpholino] ethane sulfonic acid; Sigma) added; the pH was adjusted to 6.5 with NaOH, and the mixture was rocked at room temperature for 2 h. Glycine (7 mg/mL; Sigma) was added for 30 min before overnight dialysis (12−14 kDa membrane pore) against PBS. This did not remove unconjugated OVA from the conjugation mix. The volume was adjusted to 1% wt:vol solids. Conjugated particles were stored at 4 °C for no longer than 3 days and sonicated for >5 min in ice water before use. To assess the amount of protein conjugated to beads, 25 μL from each conjugation was sampled and spun at 20000*g* in an ultracentrifuge for 20−30 min at 4−8 °C, and the concentration of unconjugated protein in the supernatant was tested using a BCA assay kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. This measured the amount of antigen not conjugated to beads and, with the known amount of antigen in the conjugation mix, allowed calculation of the % of antigen conjugated to the beads. At 500 μg/mL of OVA with 1% wt:vol beads, 96−100% of OVA was coupled to the beads, regardless of bead size. In previous studies we have

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also measured the amount of radioactive antigen coupled to different-sized beads, with similar results.[24]

For RSV protein conjugation, carboxylated polystyrene nanobeads from Polysciences (49 nm) were conjugated as described above to provide 1% wt:vol final solids with 20−80 μg of RSV protein/mouse. Again, unconjugated protein was not removed from the immunizing dose of bead−antigen. BCA assays showed 100% of OVA conjugated to beads at 20 μg of protein and 80−90% at 40 and 80 μg. In preparing injections of conjugates, all beads and soluble antigens were prepared in identical test tubes and syringes. For stored soluble antigen, protein concentrations were routinely checked by spectrophotometer and showed no significant decrease, at the concentrations used, due to absorption to container surfaces.

Animals and Immunizations. H-2K^b C57BL/6 and H-2K^d BALB/c 6−8 week old mice were obtained from the Walter and Eliza Hall Institute, Melbourne, Australia. Antigen-conjugated beads, beads alone, or soluble protein alone was administered intradermally (ID) to the hind footpads of mice in a final volume of 100 μL (50 μL in each footpad). For one immunization experiments, mice were sacrificed 10−14 days after vaccination. For two immunization experiments, two vaccinations at the same dose were given 14 days apart and mice were sacrificed at 10−14 days after the last immunization. Spleen cells were collected for cellular assays at that time. In all of the experiments, regardless of the bead size being tested, animals were immunized with 1% wt:vol of polystyrene, i.e., the same total dose of polystyrene material regardless of bead size (1 μg in 100 μL).

ELISPOT IFN-γ and IL-4 Assays. Spleen cells (0.2−1 $\times$ 10^6) were suspended in RPMI-1640 medium supplemented with 5% heat inactivated fetal calf serum (JRH Biosciences, Lenexa, KS), 2 mM glutamine, 100 μg/mL streptomycin and 100 units/mL penicillin (all Gibco/Invitrogen,), or with SIINFEKL at 2.5 μg/mL, OVA, 25 μg/mL, or RSV recombinant proteins G88 or M2.1, 20−25 μg/mL, final concentrations, for 18 h in 96-well plates (MAHA, Millipore, Billerica, MA) coated with anti-murine IFN-γ or anti-murine IL-4 mAb (551216 and 554387, respectively, Pharmingen, CA). Duplicate or triplicate wells were set up for each condition. Cells were then discarded and plates incubated for 2−4 h with anti-murine IFN-γ mAb-biotin (554410, Pharmingen, CA), or anti-IL-4 mAb-biotin (554390, Pharmingen, CA), followed by extravidin-alkaline phosphatase (AP) (Sigma). Spots of activity were detected using a colorimetric AP kit (Biorad, Hercules, CA) and counted using an automated ELISPOT reader with manual checking. Extensive washing (5×) in PBS was performed between each of the incubation steps. Data are presented as mean spot forming units (SFU) per million cells ± standard deviation (SD, for single experiments) or error (for pooled experiments) of the mean (SE).

ELISA. Polyvinyl chloride microtiter plates precoated with OVA or recombinant RSV proteins G88 or M2.1 (10 μg/mL in 0.2 M $NaHCO_3$ buffer, pH 9.6) were blocked with 2% (w/w) bovine serum albumin (BSA) in PBS for 1 h at 37 °C, washed 5 times with PBS containing 0.2% (w/w) Tween-20 (Sigma) and incubated for 2 h at room temperature (RT) with mouse sera, washed and then incubated with horseradish-peroxidase (HRP)-conjugated sheep anti-mouse IgG or antibodies detecting mouse IgG1, 2a, or 2b (BD, Pharmingen) for 1 h at RT, and washed again before addition of developing buffer containing substrate (ABTS or TMB) for 30 min. Development was stopped by addition of 1 M HCl, and plates were read on an EL312e microplate reader (BMG Labtechnologies, Offenberg, Germany) at 450 nm. Data is presented as mean ± standard deviation for each dilution of sample, or as antibody titer, determined by the mean and SD of all the naive wells (negative controls). Thus the mean of naives + 3 × SD was a reference number specific for an ELISA. The experimental titer for test samples was the dilution with a mean value higher than the reference number.

FACScan Analysis of Bead Localization. Popliteal lymph node cells were prepared as single cell suspensions and incubated with rat anti-mouse mAb hybridoma supernatants to MHC Class II, F4/80, or CD11c, with binding detected with PE-mouse anti-rat (Pharmingen, www.pharmingen.com) by FACScan (Becton & Dickinson, www.bdbiosciences.com). Beads used (40 and 67 nm from Molecular Probes) were fluorescent yellow-green and conformed to excitation and emission spectra suitable for analysis through channel 1 by flow cytometry.

RSV Infection of Mice. BALB/c mice were immunized once with beads conjugated to protein (M2.1 or G88). After 12 days, mice were challenged intranasally with 50 μL (approximately 10^6 plaque forming units, pfu) of RSV strain A2 subtype (obtained from Greg Tannock, RMIT, Bundoora, VIC, Australia). Mice were anesthetized with ketamine/xylazine prior to intranasal administration of RSV, weighed daily from the day before challenge to the end of the experiment, and sacrificed at day 4 post RSV infection. Weight loss is commonly used to assess the effects of respiratory viral load in mice.[25,26] The lungs were dissected into portions and either fixed in formalin or frozen and stored at −70 °C. Blood samples were taken prior to immunization and at sacrifice.

Viral titers were measured using standard techniques.[27] Briefly, frozen lungs were homogenized in sterile PBS, centrifuged at 10000*g* for 5 min at 4 °C and the supernatants collected. For the plaque assay, HEp-2 cells (2 × 10^5/well)

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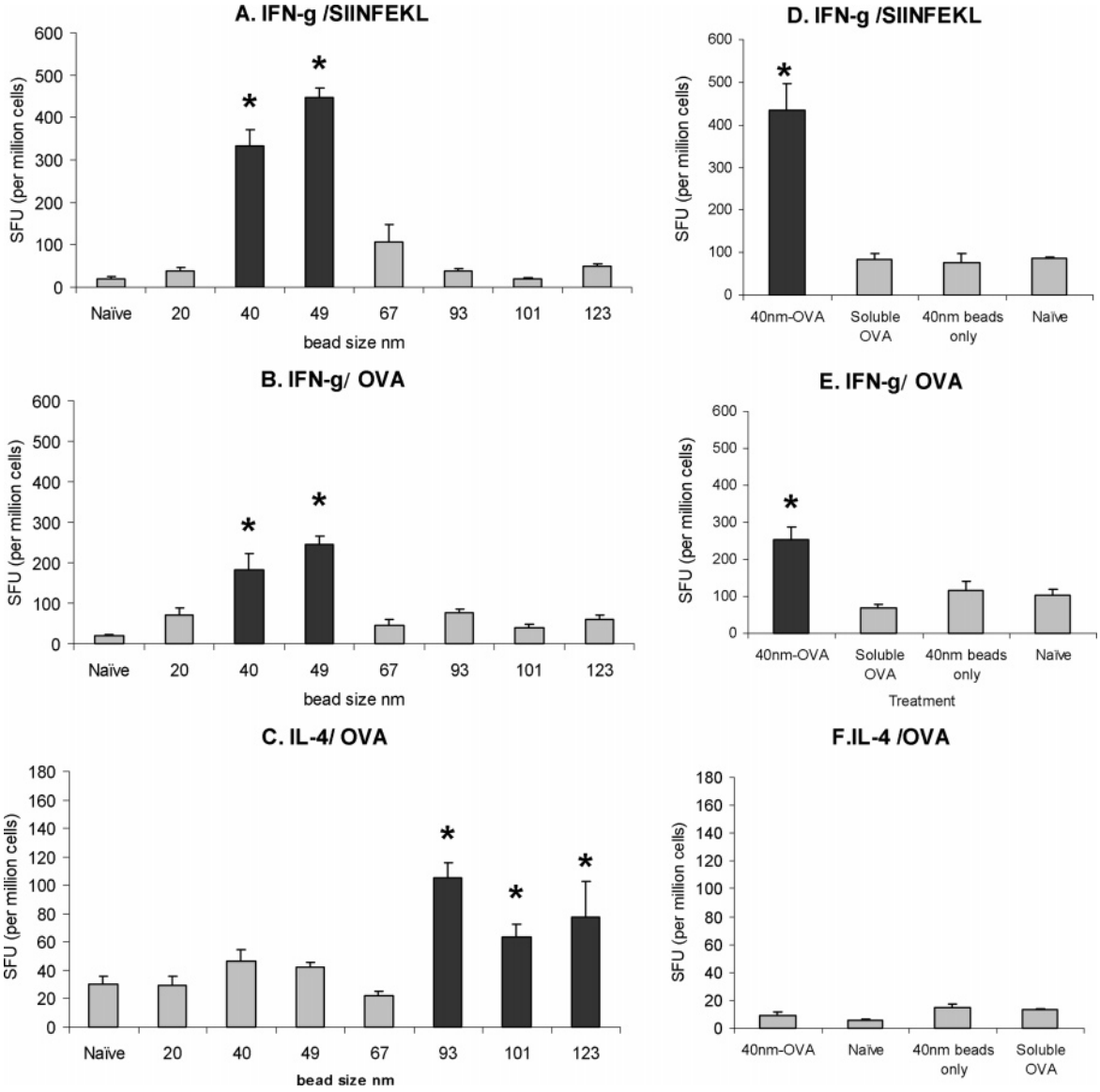

Figure 1. One immunization, spleen cells assayed at 10 days, after immunization with 50 μg of OVA/mouse on beads of different sizes: (A) IFN-γ responses to SIINFEKL stimulation. (B) IFN-γ response to OVA. (C) IL-4 response to OVA. (D−F) Response from spleen cells of mice immunized with soluble OVA, 50 μg/mouse or 40 nm beads alone (100 μL of 1% vol/vol beads) compared with OVA (50 μg) conjugated with 40 nm beads (40 nm−OVA, 100 μL of 1% vol/vol beads) and naive controls: (D) IFN-γ responses to SIINFEKL stimulation; (E) IFN-γ responses to OVA stimulation; (F) IL-4 response to OVA. Darker gray bars and * indicate significant responses ($P < 0.05$) compared with naive controls: 4−12 mice/group, mean ± SE.

were plated in 2 mL/well of DM10 into 24-well plates and incubated overnight to 80% confluence. To each well, 200 μL of virus samples was added for 2 h at 37 °C. The RSV supernatants were then removed and the wells overlaid with 1 mL of DMEM/F12 media (Gibco/Invitrogen, Carlsbad, CA). The cells were then incubated at 37 °C for 5 days, the overlay was removed, and the cells were fixed in 80% methanol for 30 min, blocked with 5% powdered milk in PBS at 37 °C for 1 h, rinsed with 0.05% Tween/PBS, then incubated with goat-anti-RSV monoclonal antibody (Biodesign, www.biodesign.com), and detected using rabbit anti-goat-HRP (P0449, Dako,Glostrup, Denmark). Plaques were counted by microscopy.

Statistics. *P* values for differences between the mean SFU by ELISPOT or mean of the inverse of titer by ELISA for diversely immunized animal groups were calculated using the unpaired two-tailed equal variance Student's *t* test.

Results

Immunogenicity of 20−123 nm OVA-Conjugated Beads. After one immunization with nanobeads conjugated to OVA, IFN-γ responses to OVA were measured by ELISPOT using spleen cells from immunized mice stimulated in vitro with either the OVA CD8 T cell epitope, SIINFEKL (Figure 1A) or whole OVA protein (Figure 1B). IFN-γ responses were sharply localized to a narrow bead size range, with 40 and

49 nm beads giving significantly ($P < 0.05$) greater responses than naive controls or animals immunized with the other bead sizes (20, 67, 93, 101, and 123 nm). In contrast, significant IL-4 responses were induced with the larger 93, 101, and 123 nm beads (Figure 1C). IFN-γ responses to SIINFEKL (Figure 1D) or OVA (Figure 1E) in spleens of mice immunized with soluble OVA (50 μg in 100 μL of PBS) or unconjugated 40 nm beads (100 μL of 1% wt/vol in PBS) remained low compared with mice immunized with 40 nm beads−OVA (50 μg of OVA plus 1% wt/vol beads in PBS), and IL-4 responses in these treatment groups were not above background levels (Figure 1F).

Antibody responses to OVA after one immunization, as observed previously, were relatively low (titers consistently between 1/600 and 1/4000) for all bead sizes ($P < 0.05$ compared with naive controls at 1/600 dilution for all groups, Figure 2A). Mice immunized once with 40 nm beads−OVA showed significantly higher antibody responses at the 1/3200 dilution compared with mice given an equivalent dose of beads alone or 50 μg of soluble OVA ($P < 0.05$, Figure 2B). After two immunizations all bead sizes induced high titer IgG responses ($P < 0.05$ compared with naive controls at 1/5400 dilution for all groups, Figure 2C). In all cases, IgG1 was the dominant isotype seen (data not shown).

After two immunizations, IFN-γ T cell responses following in vitro stimulation with SIINFEKL or OVA increased and again OVA-conjugated 40 nm and 49 nm beads inducing significantly higher effector T cell responses than naive controls ($P < 0.05$) and than larger beads (67, 93, 101, 123 nm) (Figure 3A,B). In other studies, two immunizations with higher doses of OVA, conjugated using different conjugation chemistry, could also induce significant IFN-γ responses with 20−67 nm beads, but not larger particles (not shown). IL-4 production in response to OVA did not increase significantly after the second immunization, but larger beads−OVA (93, 101, 123 nm) still induced significantly higher responses in mice compared to that seen in spleen cells from naive animals ($P < 0.05$) (Figure 3C).

In Vivo Localization and Uptake of 40 nm Compared to 67 nm Beads. Given the differences in the immune response to OVA or its CD8 T cell epitope, SIINFEKL, in mice immunized once with OVA-conjugated 40 nm or 67 nm nanobeads, we compared the in vivo uptake of these nanobeads by cells in the draining lymph node. In naive C57BL/6 mice, FACS analysis of large granular cells of the popliteal lymph node cells, back-gated on high FSC vs SSC to enrich for MHC Class II positive cells, showed that 22.7% of naive cells were CD11c+^{high}. Cells from the 40 nm group included significantly more CD11c^{high} cells (37%, $P = 0.044$) while the 67 nm group showed an increase in CD11c^{high} cells (29% of gated cells), but this was not significant compared with naive controls ($P = 0.18$). The gated cells from 40 nm treated mice also included more bead positive cells (50.8%) than the 67 nm treated group (41.6%), but this was not a significant difference ($P = 0.14$) (Figure 4A).

In comparing bead positive cells only in the gated populations (Figure 4B), significantly more cells carrying

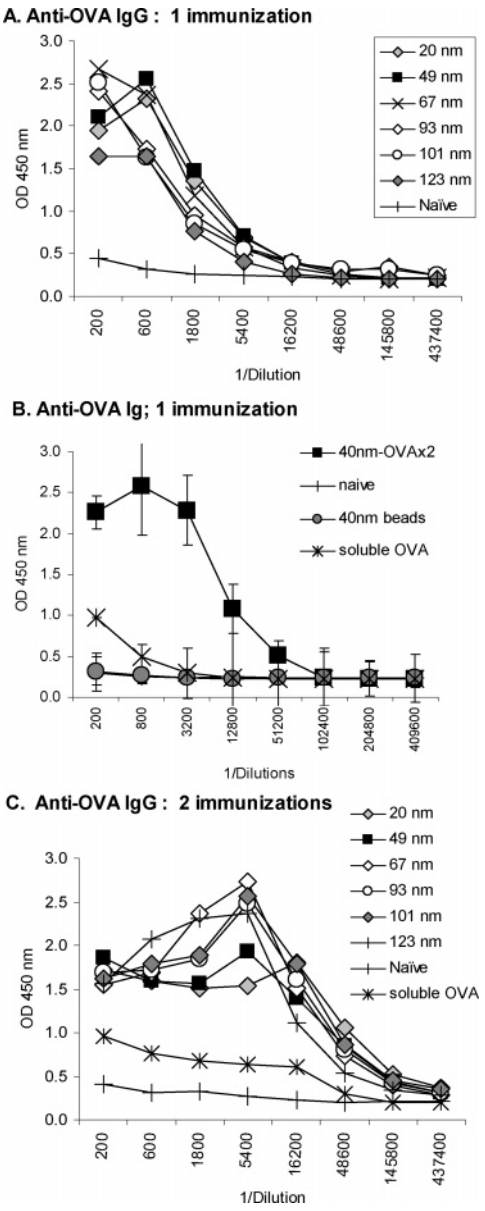

Figure 2. Anti-OVA IgG responses after the following: (A) One immunization, 50 μg of OVA/mouse, beads−OVA, 4−8 mice/group, mean shown (SD was less than 15%, $P < 0.05$ compared with naive controls at 1/600 dilution for all groups in A). (B) Mice immunized once with 50 μg of 40 nm−OVA beads (100 μL 1% solids wt/vol), an equivalent dose of beads alone (100 μL of 1% solids wt/vol), 50 μg of soluble OVA/mouse in 100 μL of PBS or 100 μL of PBS alone (naive). Mice were bled and serum was assayed 10 days after injection, $n = 4-11$ mice/group (mean ± SD), the OVA−40 nm beads group was significantly different from all other groups at 1/3200 dilution, $P < 0.05$). (C) Mice immunized twice, days 0 and 14, with 50 μg of OVA-conjugated beads/mouse, 50 μg of soluble OVA, or naive, and serum assayed 10 days after the second injection (SD was less than 15% for all groups, $P < 0.05$ for all bead immunized groups compared with naive controls at 1/5400 dilution, except for soluble OVA, $P > 0.05$): 4−6 mice/group.

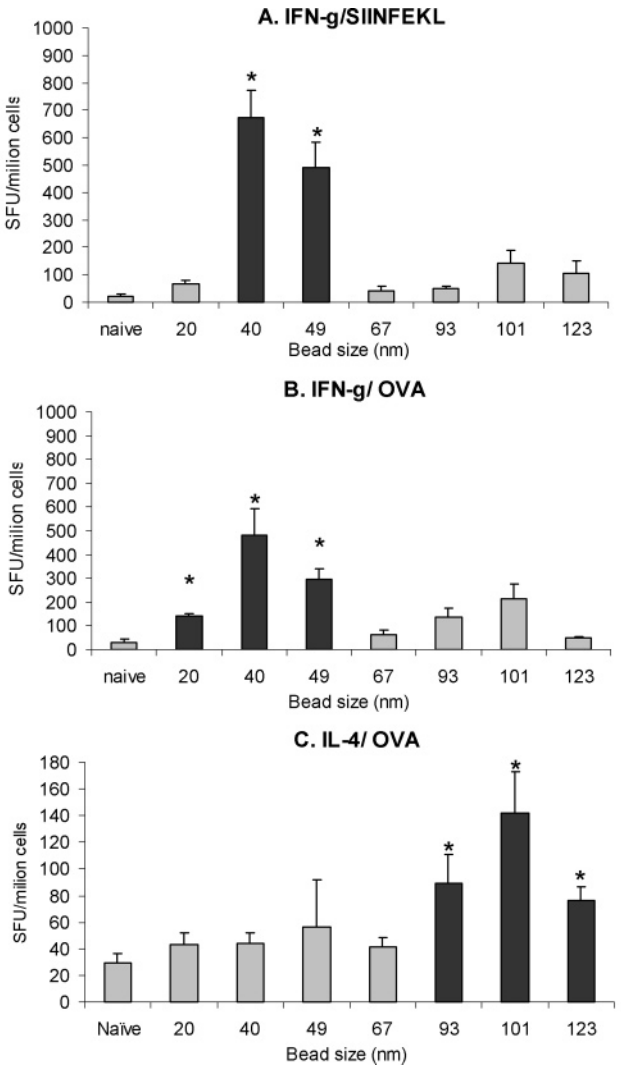

Figure 3. IFN-γ or IL-4 ELISPOT, two immunizations with 50 μg/mouse OVA conjugated to different bead sizes, or not immunized (naive). Spleen cells were stimulated in vitro with the OVA CD8 peptide, SIINFEKL, or OVA. Mean SFU/million cells $\pm$ SD per group (n = 4−8 mice/group) are shown. Concanavalin A responses were used as quality control for cell viability and are not shown. Bar graphs show (A) IFN-γ SIINFEKL, (B) IFN-γ OVA, (C) IL-4 response to OVA. Darker gray bars and * indicate significant responses compared with naive controls ($P < 0.05$).

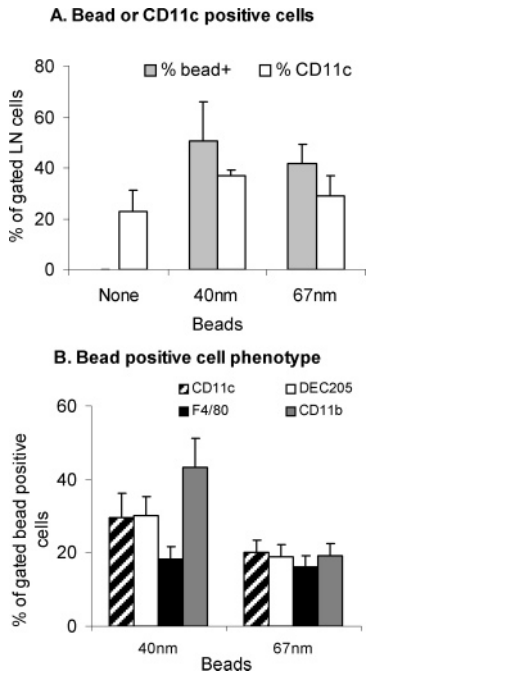

Figure 4. Bead localization. FACS data summary showing the following: (A) percentage of bead positive and CD11c^{high} positive cells in a gated population (large granular cells, back-gated on high FSC vs SSC to enrich for MHC Class II positive cells) from the popliteal lymph nodes of naive mice, or mice injected in the hind footpads with fluorescent 40 or 67 nm beads. Cells were harvested 48 h after injection of beads. (B) Selected phenotypic markers of bead positive cells within the above gated population (percent of bead positive cells n = 4 mice/group, mean $\pm$ SD).

40 nm beads expressed CD11b (43%) or DEC 205 (30%) than cells carrying 67 nm beads (19% CD11b and 19% DEC205, both $P < 0.05$ compared with 40 nm) while expression of F4/80 and CD11c^{high} was not significantly different ($P > 0.05$), with 30 $\pm$ 7% of 40 nm and 20 $\pm$ 3.4% of 67 nm bead positive cells expressing CD11c^{high}. Thus a substantial and comparable proportion of both 40 and 67 nm beads localized to CD11c^{high} positive cells in the draining lymph node (LN). However, the population of cells taking up 40 nm beads had higher expression levels of the markers CD11b and DEC205, implying an additional population of antigen presenting cells (APC). The limited T cell stimulatory capacity of 67 nm nanobead carriers thus was not due to a general lack of nanobead uptake by CD11c^{high} dendritic cells (DC) in the draining LN, but may still reflect subtle differences in uptake and/or processing by distinct APC subpopulations.

Type 1 and 2 Responses to RSV G88 Protein Conjugated to 49 nm Nanobeads. From the size studies above, it seemed that the 40 and 49 nm beads would be likely to induce type 1, protective immune responses against viral pathogens, rather than type 2 responses. This was tested in a mouse RSV model system, using RSV protein subunits as antigens. Splenocytes from mice immunized with 49 nm beads conjugated to G88 protein (beads−G88,) when stimulated in vitro with recombinant G88 protein, produced substantially more IFN-γ than splenocytes from G88 protein alone immunized mice, the latter being comparable to naive animals. The response observed in the mice immunized once (Figure 5A) was less than that of mice immunized twice (Figure 5B), in which the mice appeared to have reached maximal response at 40 μg of beads−G88. Thus administration of beads−G88 to mice induced IFN-γ production and, when the mice were immunized twice, a lower dose of G88 protein (e.g., 40 μg) could be used to induce a comparable type 1 immune response. We similarly analyzed IL-4

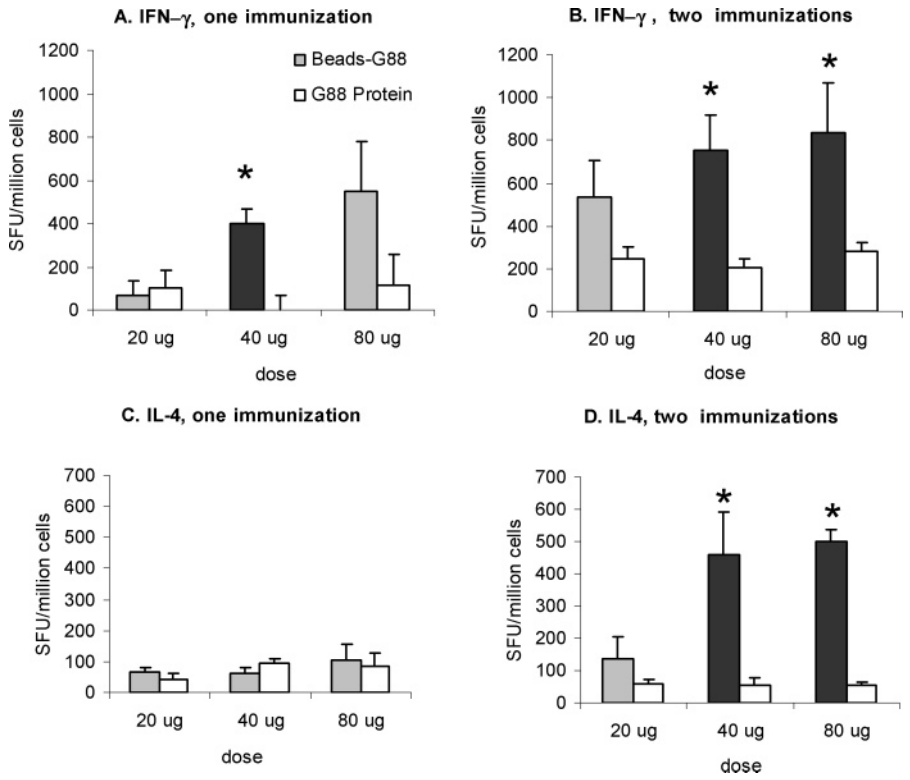

Figure 5. IFN-γ and IL-4 responses to G88 in ELISPOT assays: (A) IFN-γ production from BALB/c spleen cells from mice immunized once with 40 μg or 80 μg of G88 protein, compared with beads−G88 ($P < 0.05$ for 40 μg of beads−G88). (B) After two immunizations, the 40 μg and 80 μg beads−G88 groups produced significantly more IFN-γ than those immunized with G88 protein ($P < 0.05$ for both groups). The response by naive splenocytes incubated with media (mean 309 SFU/million) was subtracted. (C) IL-4 after one immunization of nanobeads−G88, or G88 protein alone. There was no significant increase in IL-4. (D) In mice immunized twice, the 40 μg and 80 μg beads−G88 groups produced significantly more IL-4 than those immunized with G88 protein ($P < 0.05$ for both groups). Naive mice gave <10 IL-4 SFU/million cells. Results are shown as the mean spot forming units (SFU) per million splenocytes, 3 mice per group ± standard deviation of the mean. Darker gray bars and * indicate significant responses ($P < 0.05$) compared with naive controls.

production after one or two immunizations with different antigen doses (Figure 5C,D). After one immunization, mice did not produce IL-4 at levels significantly higher than naive controls. However, mice immunized twice with nanobeads−G88, could produce a moderate amount of IL-4 at both the 40 and 80 μg injection doses, while mice immunized with the same amount of protein G88 alone did not produce detectable IL-4.

The amount of anti-G88 antibody produced in mice immunized with beads−G88 and G88 protein alone (at 100 μg/mouse) was measured by ELISA after 1 or 2 immunizations. Mice immunized once with G88 protein alone or beads−G88 produced low titer antibodies (titers of less than 1/1280, data not shown). After two immunizations (Figure 6C), nanobeads−G88 produced high titer IgG antibodies (out to >1/40 960), significantly greater than G88 alone ($P < 0.05$). Isotype studies showed that IgG1, 2a, and 2b were equally induced after two immunizations with nanobeads−G88 (data not shown).

Responses in Mice Immunized with RSV M2.1 Protein. The RSV protein, M2.1, normally requires additional adjuvants to be immunogenic. Immunogenicity was tested for bead conjugates (beads−M2.1) and compared with M2.1 protein alone, or M2.1 with adjuvants. In the latter treatment, M2.1 was mixed with complete Freund's adjuvant (CFA) for the first immunization, with a boost 14 days later of M2.1 mixed with incomplete Freund's Adjuvant (IFA). Splenocytes from mice immunized twice with beads−M2.1, incubated with the immunizing protein in ELISPOT assays, produced significantly more IFN-γ than mice immunized with M2.1 alone ($P = 0.02$), with responses from beads−M2.1 also significantly greater than CFA/IFA/M2.1 ($P = 0.013$) (Figure 6A). None of the immunizations induced IL-4 responses above background levels (data not shown).

The production of anti-M2.1 antibody in mice immunized with beads−M2.1, CFA/IFA/M2.1, or M2.1 protein alone (at 40 μg of M2.1/mouse) was measured after 2 immunizations (Figure 6B). Beads−M2.1 and CFA/IFA/M2.1 responses were equivalent and significantly greater (both $P < 0.05$ for end titer) than for M2.1 protein alone immunizations. Thus, beads−M2.1 induced antibody of higher titer than immunization with M2.1 protein alone and equal to immunization with CFA/IFA/M2.1. Titers after two beads−M2.1 immunizations were however significantly lower than for beads−G88 given at the same 40 μg dose ($P < 0.05$ for end titers, Figure 6C).

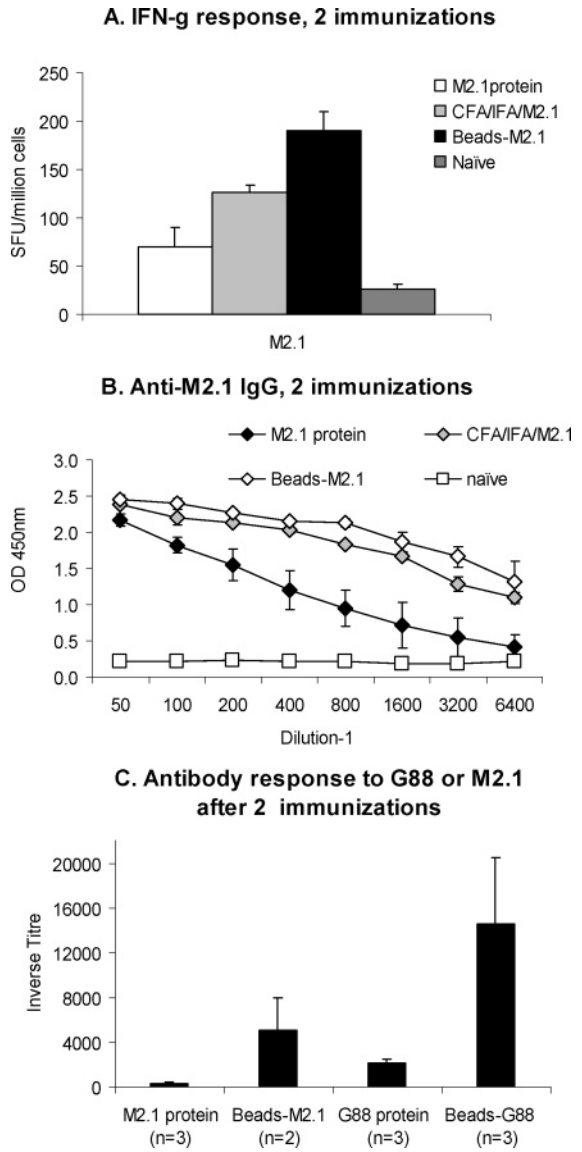

Figure 6. Responses to M2.1 (A) IFN-γ produced by beads−M2.1, CFA/IFA/M2.1, or M2.1 protein alone, 40 μg/mouse, 2 $\times$ immunized BALB/c mice. IFN-γ was measured by ELISPOT assay. Results are shown as the mean spot forming units (SFU) per million splenocytes of 3 mice per group $\pm$ standard deviation from the mean. The background value (naive cells with media alone < 50 SFC) was subtracted. (B) Anti-M2.1 antibody produced in mice immunized twice with beads−M2.1, CFA/IFA/M2.1, or M2.1 protein alone, 40 μg/mouse. Results are shown as the mean OD at each dilution $\pm$ standard deviation, $n = 3$ mice/group. (C) Comparison of end titers for IgG produced by M2.1 and G88 as protein alone or conjugated to beads (mean titer $\pm$ standard deviation, $n = 3$ mice/group).

Response to Challenge with Live RSV. Mice were immunized with beads−G88 or beads−M2.1 proteins and challenged intranasally with approximately 10^6 pfu of RSV strain A2 subtype. Mice were weighed before challenge and each day thereafter until day 4. At this time point, lungs were removed and virus titer was determined by plaque assay. Challenge with RSV caused rapid weight loss on day 1 in all groups (there was no significant difference between the

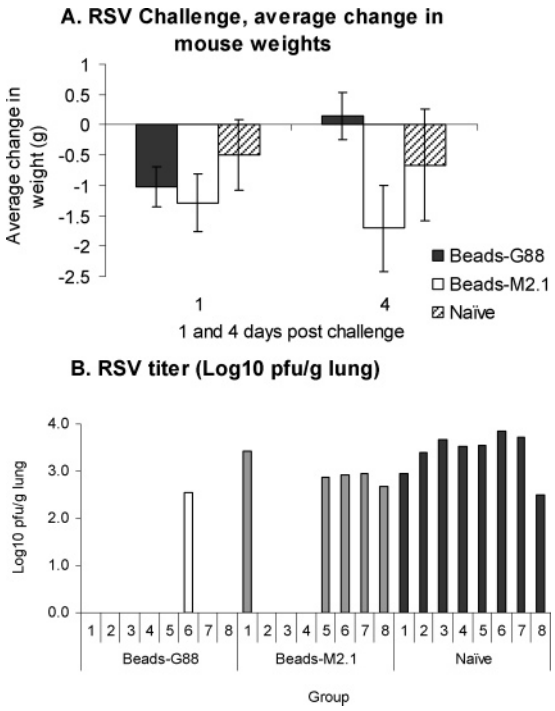

Figure 7. Response to RSV challenge. (A) BALB/c mice were immunized with beads−G88 or beads−M2.1 (50 μg/mouse) and then challenged intranasally with approximately 10^6 pfu of RSV strain A2 subtype. Mice were weighed prior to challenge and each day thereafter until the end of the experiment at day 4. Results are shown as the average change in weight of 8 mice per group $\pm$ standard deviation of the mean. A paired, 2-tailed Student's *t* test was used to compare the weights. On day 1 there was no significant difference between groups ($P > 0.05$). On day 4, the weight of the beads−G88 group was significantly different from the weight of the beads−M2.1 group ($P < 0.05$). Comparing day 1 weights with day 4, a significant difference was seen between these two times in mice treated with beads−G88 ($P = 0.006$). (B) Mice immunized once with nanobeads−G88 or nanobeads−M2.1 had reduced viral titers at day 4 postchallenge.

day 1 weight loss of the three groups, $P > 0.05$). Recovery to prechallenge weight by day 4 was achieved only by mice immunized once with beads−G88 (Figure 7A). Weights at day 4 compared with those at day 1 indicated highly significant differences in the group that received beads−G88 ($P = 0.006$). This was a promising result given that the mice were immunized only once. The RSV titer in the frozen lung tissue also showed a significant reduction in mice treated with beads−G88 compared with mice immunized with beads−M2.1 or naive mice ($P < 0.05$ for both groups) (Figure 7B). Weight recovery and viral titers for mice treated with beads−M2.1 were not significantly different from naive controls ($P > 0.05$), potentially reflecting the lower antibody and IFN-γ responses seen with this protein. In similar experiments, proteins G88 or M2.1 alone were unable to induce any significant changes in weight loss or viral titers (data not shown).

Discussion and Conclusions

The data herein describes preclinical studies of 40 nm nanobeads, and other beads of minute size variation on either side of 40 nm, conjugated with a model antigen (OVA) and, for the first time, the use of these nanobeads to immunize against an infectious agent, RSV. In other studies[17,24] we have shown that antigen covalently conjugated to synthetic, biocompatible polystyrene nanobeads in the viral size range can elicit both antibody and CD8 T cell immunity, with responses comparable to those of leading adjuvants.[17,24] These novel nanovaccines were protective against tumor challenge and cleared established tumor masses in three distinct mouse models[17] as well as being able to promote combined cellular and humoral immunity in sheep.[28] These studies also showed that conjugation of protein or peptide antigens did not destroy reactive epitopes and that covalently linked antigen was a more effective immunogen than antigen simply mixed with beads.[17,24] Thus, OVA covalently conjugated to particles induced comparable or higher antibody responses than OVA alone, or admixed with a range of standard adjuvants (Alum, MPL, QuilA) or methods (ex vivo antigen pulsed DC) that allow presentation and recognition of native OVA, demonstrating that particle conjugation has not "hidden" away reactive epitopes.[24]

Size was identified as a critical and fundamental parameter for the induction of specific immunity in our studies using particulate carriers, with bead sizes tested ranging from 20 to 2000 nm, covering viral and bacterial size equivalents, with the optimal size for immunogenicity located between 40 and 100 nm.[17,24] The size of the particle engulfed can determine the cellular entry mechanism, with phagocytosis of larger beads (>500 nm) and localization to macrophages, while virus-sized particles (20−200 nm) preferentially enter via endocytosis, localizing to clathrin-coated pits (particles <150 nm)[6] or caveolae (particles within 50−80 nm).[7,8] Influenza and respiratory syncytial viruses and other pathogens in this size range can enter via caveolae.[9,10] Herein, the nanobead viral size range between 20 and 123 nm was studied in detail, including fine mapping of an optimal reactivity size threshold between 40 and 100 nm, found to be at <67 nm. Moreover, given that the size of the carrier bead could play a role in determining the type of response induced, the initial aim of these investigations was to compare OVA-specific IFN-γ (type 1 associated) and IL-4 (type 2 associated) responses in mice immunized once or twice with OVA conjugated to beads within the size range 20−123 nm. We confirmed that OVA-conjugated 40−49 nm beads were very effective at inducing IFN-γ after one or two immunizations, and found, surprisingly, that larger nanobead carrier sizes in the viral range favored IL-4 production after one or two immunizations with OVA−beads.

In the first set of experiments, nanobead vaccines were shown to induce qualitatively (as well as quantitatively) different immune responses depending on minute size variations in the viral range size of the particles used as antigen carriers. Here the OVA concentration was kept constant, and the polystyrene bead content of the vaccine dose in the conjugation mix was also kept constant at 1% wt:vol; therefore the particle number varied. In our previous publications, we have shown that particle number and surface area do not determine immune reactivity, while concentration of protein and the total amount of particulate material are important.[17,24] In this study, after either single or double immunization, type 1 and type 2 cytokine responses were consistently induced by beads of different sizes (40−49 nm vs 93−101 nm, respectively). This finding offers the possibility of manipulating particulate vaccine design to promote specific type 1 or 2 responses. A very tight, narrow range was identified for maximal initial type 1 priming with 40−49 nm OVA-conjugated beads, while 67 nm beads−OVA were significantly less effective at inducing type 1 CD8 or CD4 T cells. Larger beads−OVA (93, 101, 123 nm) favored type 2, IL-4 induction. Thus, particle size restriction may be a critical factor that allows effective vaccine entry to specific APC and/or their subcellular compartments to promote rapid induction of type 1 or 2 cells.

Although most sizes of OVA-conjugated beads were able to induce type 1 or type 2 immune reactivity, as seen in T cell ELISPOT assays after one immunization, the notable exceptions to this rule were 20 nm and 67 nm beads, which failed to induce either type 1 or type 2 cytokine responses (although both still stimulated antibody production). One possibility was that they simply were not taken up sufficiently by APC in vivo and thus could not prime T cells very effectively. Our previous studies demonstrated that uptake of 20 nm beads was indeed more limited than uptake of 40 nm beads, and that cells ingesting 20 nm particles in vivo failed to express DC markers such as DEC205, in contrast to cells that took up 40 nm beads.[24] However, in the studies shown herein, there was no significant difference in the percentage of cells taking up 40 or 67 nm beads, and within the bead positive populations, no significant difference in percentage of $CD11c^{high}$ cells (Figure 4). Although the 40 nm bead positive cells expressed increased levels of DEC205 and CD11b, substantial numbers of 67 nm bead positive cells also expressed these markers. Thus, the limited T cell stimulatory capacity by 67 nm nanobead carriers was not due to a general lack of nanobead uptake by APC in the draining LN.

The sharp difference in immune response seen with variation in bead sizes, after either one or two immunization protocols, was very surprising. There was a bimodal pattern, with immunogenicity of 20 nm bead conjugates low, then immune responses peaking with 40−49 nm beads, with these sizes consistently inducing type 1 responses, followed by low responses with 67 nm beads. Responses then peaked again, with 93−123 nm beads−OVA inducing type 2 reactivity. Thus with 67 nm beads−OVA, only limited CD4 or CD8 T cell responses are observed, although, as discussed above, different levels of uptake by LN cells cannot account

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for the observed differential ability of these nanobead−antigen carriers to stimulate type 1 and type 2 immunity. We hypothesize that there may be a link between nanobead uptake into APC promoting type 1 or type 2 responses and the subset of endocytic pathways being engaged. The specificity and size restrictions in this system will have to be extraordinarily subtle, to be able to promote the observed immunogenicity patterns.

An extensive body of literature shows that smaller particles (<150 nm) enter APC through caveolae or clathrin coated pits, a route also used by viruses like RSV and influenza virus.[7, 8,10] Our results show quantitative and qualitative differences in vivo between 20 and 40 nm[24] and 40 and 67 nm (results herein). Different subtypes of caveolae or clathrin coated pits have been reported,[29] and these may finely tune the size requirements for uptake and/or intracellular trafficking of particles into APC and DC. Indeed, the minute differences in size studied herein are representative of differences in size between many single/double and (when this exists) double/triple layer viruses, for example, rotavirus, hepatitis B and C, HIV, and dengue virus. Thus, the size-based differential in type 1/type 2 priming bias observed in our model (using synthetic nanobeads covalently linked to protein) may in fact mimic some of the initial type 1/type 2 induction biases observed with "real" virus particles, which access similar endocytic pathways in APC. Uptake and processing of nanoparticles and access to endosomes via caveolae is a poorly understood process, with debate continuing on the distinction between lipid rafts and caveolae, and the trafficking, signaling, and processing of particulate antigen entering cells.[30,31] It seems that caveloae may have less access to proteolytic pathways than other modes of antigen uptake, and are thus favored by some pathogens.[30] Conversely, particle uptake mechanisms that avoid proteolysis seem to induce better immunity, possibly by retaining antigens and maintaining presentation of peptide−MHC complexes.[32] The data presented here shows carrier size dependent variation in response for presentation of the same antigen, OVA, which has clearly defined CD4 and CD8 T cell epitopes. Epitopes in other antigens may promote IL-4 or IFN-γ intrinsically, and many produce balanced type 1/type 2 responses,[33] and this will vary with the immunization protocol used, as seen with our second immunization data for OVA and RSV G88.

RSV, a virus known to enter cells through caveolae[10] and to induce long-term immunity in adults, but not in infants,[21] provides a particular challenge for vaccine design, given that type 2/IL-4 responses can exacerbate disease, as shown by responses to the formalin fixed vaccines originally used in clinical trials.[34] Thus a vaccine formulation that preferentially induces type 1 protective immunity is required. As an alternative to whole virus vaccines, a number of protein subunit antigens have been identified, including viral surface G proteins, that mediate attachment to the cell membrane, and F proteins that cause fusion of infected cells, enhancing cell−cell transmission of the virus. Matrix M proteins have also been identified that encompass CTL epitopes.[11] Pathogenic type 2 responses are primarily due to reactivity to the RSV G-glycoprotein, leading to IL-4 and IL-10 secretion and subsequent cytokine dependent neutrophil activation.[34,35] Recombinant G88[22] and M2.1[23] subunit proteins were used in this study as they are immunogenic in the BALB/c mouse model.

Herein, mice immunized once with recombinant RSV protein G88 conjugated to 49 nm nanobeads showed significantly improved recovery from infectious live virus challenge-induced weight loss, and significantly reduced lung viral titers, indicating protection with this vaccine formulation. In this case the response was predominantly IFN-γ, confirming the observation of others that a systemic IFN response can be effective against both systemic and mucosal infections.[20,36] This *single* dose of antigen was associated with the induction of a strong cellular (type 1, IFN-γ) response, a low level antibody response, and the absence of a type 2 (IL-4) response. Others have used RSV antigens with inert particles for nasal delivery of vaccine, and shown immunogenicity, but not reduced viral titers or rapid recovery from challenge.[37] Currently, there is no licensed vaccine for

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RSV, although clinical trials of G, F, and M vaccines are ongoing.[21] In searching for suitable carriers for these antigens, evaluation of live attenuated virus, recombinant vectors, DNA vaccines, and subunit vaccines have been carried out.[38,39] To date, the best protective therapy for RSV is short-term passive immunity following treatment with a monoclonal antibody, palivizumab.[40] Simple, inert, size-controlled nanobead-based formulations could improve antigen delivery,[24,28] and may be effective as vaccines against both systemic and mucosal pathogens (as shown herein).

Nanobead formulations offer a highly flexible system for testing a wide range of potentially protective antigens including proteins and peptides,[17] alone or in combination. In our other studies, nanobead−antigen conjugates were also shown to be effective in large animals such as sheep,[28] indicating that the "size dependency" principle will translate across a range of species. Given that our study indicates that differences in size as small as 10 nm are the key to the generation of specific types of immunity, specifically CD4 or CD8 T cells, future studies can now focus on the threshold CD4/CD8 and type 1/2 T cell stimulatory sizes identified herein, to further explore differences in APC internal signaling and its relation to endocytic pathways. The use of specifically tailored and strictly size controlled viral range nanovaccines based on simple inert carriers capable of facilitating uptake by appropriate stimulatory APC in vivo provides the potential to investigate and optimize the induction of effective immunity, particularly for pathogens such as RSV where type 1, but not type 2, responses are required.

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Acknowledgment. M.P. is a Senior NHMRC Fellow and thanks the NHMRC for support. Thanks also to PRIMA Biomed Ltd (Australia) for financial support for this project. Thanks to Ms. Katie Adrdjipadja for technical assistance with ELISA assays and Ms. Cassandra David for recombinant protein production (Burnet Institute, Austin), Dr. Ben Shields (Burnet Institute) for providing the G88 plasmid, Anita Gamvrellis (University of NSW) for back up data for Figure 4, and Prof. Geoff Pietersz (Burnet Institute) for useful advice on conjugation chemistry.

MP060096P