Published in final edited form as:

Biomaterials. 2013 November ; 34(34): . doi:10.1016/j.biomaterials.2013.07.063.

The use of self-adjuvanting nanofiber vaccines to elicit high-affinity B cell responses to peptide antigens without inflammation

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Abstract

Balancing immunogenicity with inflammation is a central tenet of vaccine design, especially for subunit vaccines that utilize traditional pro-inflammatory adjuvants. Here we report that by using a nanoparticulate peptide-based vaccine, immunogenicity and local inflammation could be decoupled. Self-assembled β -sheet-rich peptide nanofibers, previously shown to elicit potent antibody responses in mice, were found to be non-cytotoxic *in vitro* and, remarkably, elicited no measurable inflammation *in vivo*—with none of the swelling at the injection site, accumulation of inflammatory cells or cytokines, or production of allergic IgE that were elicited by an alum-adjuvanted vaccine. Nanofibers were internalized by dendritic cells and macrophages at the injection site, and only dendritic cells that acquired the material increased their expression of the activation markers CD80 and CD86. Immunization with epitope-bearing nanofibers elicited antigen-specific differentiation of T cells into T follicular helper cells and B cells into germinal center cells, as well as high-titer, high-affinity IgG that cross-reacted with the native protein antigen and was neutralizing in an *in vitro* influenza hemagglutination inhibition assay. These responses were superior to those induced by alum and comparable to those induced by complete Freund's adjuvant. Thus, nanoparticulate assemblies may provide a new route to non-inflammatory immunotherapies and vaccines.

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The authors have no conflicting financial interests.

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Keywords

nanoparticle vaccines; non-reactogenic; OVA(323–339) peptide; self assembly; alum adjuvant

1. Introduction

A central strategy for the design of modern subunit vaccines has been the incorporation of adjuvants or immunostimulants to increase the response to otherwise poorly immunogenic subunit antigens [1, 2]. These adjuvants and stimulants target cells of the innate immune system in order to initiate a productive adaptive immune response, acting first on antigen-presenting cells (APCs) to promote their acquisition of antigen, migration to draining lymph nodes, and stimulation of antigen-specific T cells. These T cells differentiate into effector cells, including T follicular helper (Tfh) cells that promote the differentiation of germinal center (GC) B cells into plasma cells, which produce high-affinity antibodies that ultimately afford protection. The obligate use of adjuvants or immunostimulants complicates the design and regulatory approval of subunit vaccines, because their efficacy has traditionally been correlated with increased reactogenicity [3]. Even the modestly effective adjuvant alum (aluminum hydroxide or aluminum phosphate microparticles), which was until recently the only adjuvant used in US Food and Drug Administration-approved vaccines, is associated with local reactions that generally restrict it to intramuscular use [1]. It is also associated with occasional systemic reactions such as allergic IgE production [1, 4], and a recent study of three formulations of alum-based adjuvants found that their immunogenicity correlated with the levels of inflammation they elicited [5]. More powerful adjuvants, such as those incorporating nucleic acid-based or endotoxin-based agonists for Toll-like receptors (TLRs), face high regulatory hurdles due to similar issues of local reactogenicity [2]. This is particularly unfortunate for vaccines that use peptide antigens, which are otherwise chemically defined and relatively simple to manufacture [6]. Thus, there is a critical need for new vaccine strategies that can elicit protective antibody responses without inflammation.

One promising strategy for avoiding inflammation is to use self-adjuvanting vaccines that do not require addition of TLR ligands to elicit a productive immune response. In recent years, several types of antigen-conjugated nanomaterials, including virus-like particles [7, 8], non-viral protein self-assemblies [9, 10], synthetic nanoparticles [11–14], and peptide-based nanofibers [15–17], have been shown to raise strong antibody and/or T cell responses without additional adjuvants [18]. Like natural viruses, these materials feature particulate presentation of antigen to increase uptake by APCs, high-density antigen display to elicit high-avidity interactions with antibodies and B cell receptors, and nano-scale size in at least one dimension to facilitate uptake and/or lymphatic drainage [19]. Similarly, nano- and micron-sized particles also have been engineered specifically to interact with DCs and enhance presentation of antigens without the need for additional immunostimulators [20–25]. By avoiding the need for TLR ligation or other explicitly inflammatory signals, it is possible that self-adjuvanting materials may provide a new means to decouple immunogenicity from inflammation for vaccine development [26].

In this paper, we tested this hypothesis by comparing the immunogenicity and reactogenicity of alum- and emulsion-based adjuvants with those of a self-adjuvanting nanomaterial. We used a recently described peptide-based material in which peptide antigens are conjugated to a short peptide domain (QKQFQFQEQQ, Q11) that self-assembles into α -sheet-rich nanofibers in water or physiological buffers [15–17]. The assembly process is compatible with a wide variety of peptide antigens synthesized in tandem with Q11 or other similar self-assembly domains. When Q11 was coupled to a model peptide antigen from ovalbumin, OVA_{323–339} (designated pOVA herein to distinguish it from whole OVA protein), the

pOVA-bearing fibers (OVAQ11) elicited a strong antibody response in mice. This antibody response is CD4 T cell-dependent and similar in strength and longevity to the response raised by pOVA in complete Freund's adjuvant (CFA) [16], a potent adjuvant with strong local reactogenicity that precludes its use in humans. Here, we tested whether this nanomaterial-based approach to vaccination could decouple immunogenicity, as measured by the strength of the B-cell and CD4 T-cell responses, from the reactogenicity that characterizes other adjuvant-based approaches.

2. Materials and methods

2.1. Peptide and vaccine preparation

The peptides OVAQ11 (H₂N-ISQAVHAAHAEINEAGR-SGSG-QQKFQFQFEQQ-amide), pOVA (H₂N-ISQAVHAAHAEINEAGR-COOH or H₂N-ISQAVHAAHAEINEAGR-amide), and Q11 (acetyl-QQKFQFQFEQQ-amide) were synthesized using standard Fmoc solid-phase chemistry, purified by HPLC and MALDI-MS, and lyophilized as previously reported.[27, 28] Biotinylated and fluorescein-labeled peptides were prepared by reacting Biotin-ONp (Novabiochem) or NHS-fluorescein (Pierce) in 1:1 DMSO/DMF overnight in a 2 – 4-fold molar excess with amine-terminated peptides before their cleavage from the resin; 100 mM triethylamine was added during the fluorescein reaction.

To prepare immunization solutions, lyophilized OVAQ11 or pOVA peptides were dissolved in sterile water at 8 mM and stored overnight at 4 °C. Then, sterile, endotoxin-free 1x PBS (HyClone SH30028.02) was added to bring the peptide to the working concentration (2 mM), and the solution was incubated 3 – 5 hr at room temperature before immunization. For pOVA with Imject alum or Imject CFA/IFA, a 4 mM working solution of pOVA was mixed 1:1 v/v with Imject Alum (Pierce) and vortexed for 30 – 60 min to allow adsorption. CFA/IFA emulsions were prepared similarly. Endotoxin measurements were conducted on the same peptide solutions that were used for immunization (e.g. 2 mM OVAQ11 or 2 mM pOVA), using the Limulus Amebocyte Lysate chromogenic endpoint assay (Lonza). Endotoxin levels of all peptide solutions used for immunization were < 0.1 EU/mL (< 0.01 EU per 100 µL dose).

2.2. TEM analysis

Sample solutions were deposited on 400 mesh carbon grids for analysis by a FEI Tecnai F30. OVAQ11 nanofibers were prepared as for immunization, diluted to 0.4 mM in 1x PBS, deposited on the grid, stained with 1% uranyl acetate, and allowed to dry. Imject Alum was diluted 1:1 v/v in 1x PBS, vortexed 30 min, diluted 40-fold in water (to 1 mg/mL), sonicated for 10 min to disrupt aggregates, deposited on the grid (unstained), washed twice with water, and allowed to dry.

2.3. In vitro cytotoxicity assay

Q11 peptide was dissolved in water at 8 mM concentration and incubated overnight at 4 °C, then diluted to 2 mM in 1x PBS and incubated at room temperature for 4 hr. A stock solution of Imject alum was prepared by mixing the commercial solution (which contains 80 mg/mL total aluminum and magnesium salts) 1:1 v/v with cell culture medium, thus producing a 40 mg/mL stock solution. The culture medium was DMEM (HiClone), supplemented with 10 % FCS (Gibco), 2 mM L-glutamine (Corning Cellgro), and 1X Penicillin/Streptomycin (Corning Cellgro). J774.1 macrophages were plated in a 96-well culture plate (70,000 cells/well) and allowed to adhere for 2 hr at 37°C. Working solutions of Q11 peptide or Imject Alum were prepared by diluting the 2 mM Q11 or 40 mg/mL Alum solutions by 10, 100, or 1000-fold with culture medium, and then the culture medium in each well was replaced with one of these solutions (100 µL). After 4 hr incubation at

37°C, the cells were washed 3 times with PBS, stained with LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Invitrogen) for 30 min at room temperature, and washed again 3 times with PBS. Cells were imaged in PBS at room temperature using a Zeiss Axiovert 35 fluorescent microscope (CP-Achromat objective, 10X magnification, 0.25 NA), with 3 wells per condition and 3 images per well. The images were acquired using an AxioCam MRm camera (Carl Zeiss) and Zeiss Axiovision 4.6 software. Live and dead cells were counted using ImageJ software (Operations: Type 8-bit, Make Binary, Watershed, Analyze Particles).

2.4. Animal work

Wild-type C57BL/6 mice were purchased from Harlan Sprague Dawley or Taconic Farms; OTII mice (45.1 congenic, C57BL/6) were a kind gift from Jesse Williams (Anne Sperling's lab in University of Chicago); Toll Like Receptor 4-deficient mice (B6.B10ScN- *Tlr4^{ops-del}*/JthJ) were purchased from the Jackson Laboratory. Mice were housed in a centralized animal facility at the University of Chicago. All procedures were approved by the University of Chicago Institutional Animal Care and Use Committee (protocol #71-900).

2.5. Footpad inflammation assay

Anesthetized mice were immunized s.c. in the hind footpad with one 30- μ L injection of OVAQ11 or pOVA-Alum, and the thickness of the immunized and unimmunized hind feet was measured at the indicated times using digital calipers (Marathon, # CO 030150, 0.01 mm increments). Swelling was calculated as the thickness of the immunized foot minus the thickness of the unimmunized foot, which was approximately 2.2 mm in thickness. Mice were sacrificed and footpads were collected on day 8 after injection. Intact feet were fixed in 10 % formalin for 4 days, rinsed with water, and placed in decalcification solution (Surgipath Decalcifier II, from Leica, < 15 % HCl, < 5 % EDTA) until they were flexible (7 days). Then, the feet were rinsed with water and 70 % ethanol, sliced along with midline of the foot (sagittal plane from toes to ankle), and stored in 70 % ethanol. Samples were paraffin-embedded, sliced (5 μ m), and stained with hematoxylin & eosin by the University of Chicago Tissue Resource Center. Slides were analyzed for evidence of inflammation by a pathologist unfamiliar with the experiment. Images were collected using a Zeiss Axioskop equipped with a Zeiss Axiocam digital color camera and OpenLab software, using 4x/0.10 NA and 20x/0.5 NA objectives.

2.6. Antibodies and flow cytometry

Antibodies were purchased from Ebioscience unless otherwise specified. Flow cytometry was performed on a Fortessa or LSR-II Blue (BD) and analyzed by FlowJo (Threestar), using cells suspended in flow buffer (PBS containing 2 % FCS and 0.05 % sodium azide). Specific staining procedures and clone numbers are provided in the Supporting Information.

2.7. Intraperitoneal recruitment, uptake, and activation

To assess recruitment and inflammatory cytokine release, mice were immunized with the indicated solutions by peritoneal cavity injection ($2 \times 50 \mu$ L). After 4, 20, or 72 hr, the mice were sacrificed, 1 mL HBSS buffer was injected into the cavity, the area was massaged 30 times, and 0.7 mL of lavage fluid was collected. The fluid was centrifuged (400 g, 5 min) to pellet the cells, which were analyzed by flow cytometry, and the supernatant was collected and frozen immediately at -80 °C for subsequent analysis by a bead-based immunoassay (Milliplex). To assess uptake of the material and activation of antigen presenting cells, mice were immunized i.p. with pOVA-Fluorescein or OVAQ11-Fluorescein, and 20 hr later, cells were collected from peritoneal lavage fluid as above. For surface staining, the cells were stained with CD11C (N418), MHC II (M5/114.15.2), F4/80 (11-26C.2A, Biolegend), and

allophycocyanin-conjugated anti-FITC (fitc-9) antibody; for intracellular staining, cells were pre-blocked with anti-FITC antibody for 30 min at 4 °C, then washed, fixed, permeabilized with the reagents from a Foxp3 staining kit (00-5523-00, Ebioscience), and finally stained with allophycocyanin-conjugated anti-FITC antibody (fitc-9).

2.8. Immunization and monitoring of antibody and T cell responses

Anesthetized mice were immunized subcutaneously with the indicated solutions ($2 \times 50 \mu\text{L}$, 200 nmol total peptide) and boosted where indicated with half-doses ($2 \times 25 \mu\text{L}$, 100 nmol total peptide) after 4 weeks and 8 weeks. In adoptive transfer experiments, each recipient mouse intravenously received 0.5 – 1 million CFSE-labeled OTII T cells one day prior to immunization (supporting methods). Mice receiving OTII cells by adoptive transfer were not boosted. Serum was collected from the submandibular (cheek) vein and analyzed by ELISA for pOVA-specific IgG, IgM or IgE. Mice were sacrificed 7 days after the final immunization, and the cells from the draining lymph nodes were collected. ELISPOT plates were prepared for IL-4 or IFN- γ analysis, and 0.5×10^6 cells were plated in each well (200 μL volume) and stimulated with 10 $\mu\text{g/mL}$ pOVA for 48 hours before developing the plates. Detailed ELISA and ELISPOT procedures are provided in the supporting methods. Proliferation was determined by CFSE dilution, and Tfh development by expression of CXCR5 and PD-1.

2.9. Characterization of antibody affinity and neutralization

Serum samples collected one week after the second booster immunization were analyzed for antibody binding and desorption kinetics on a pOVA-coated surface, using a BioRad ProteOn XPR36. Please refer to supporting methods for details. To assess neutralization, a hemagglutinin inhibition (HAI) assay was performed according to standard procedures (described in supporting methods), using serum samples collected one week after the second booster immunization and testing against wildtype or pOVA-expressing A/HK/X31 influenza viruses. The pOVA-expressing strain [29] was a kind gift from R. Webby and P. Doherty at St. Jude Children's Research Hospital, and the wild-type virus was prepared in house at the University of Rochester.

2.10. Statistical analysis

Statistical comparisons between groups were made using 1- or 2-way ANOVA with Tukey post-hoc tests to determine p values, as indicated in figure legends.

3. Results

3.1. Contrasting cytotoxicities of adjuvants towards antigen-presenting cells

One proposed mechanism for the adjuvant activity of particulate adjuvants such as aluminum salts is that they induce cell death at the injection site, which in turn causes the release of damage-associated molecular patterns such as uric acid or host DNA in less than 6 hours [30, 31]. We tested whether the nanofiber adjuvant functioned in a similar manner, by comparing the cytotoxicity of Q11 fibers (5 – 10-nm wide and 100 – 1000-nm or longer when conjugated to antigens such as pOVA; Figure 1a) to that of a commercial aluminum adjuvant, Imject Alum (referred to throughout the paper as simply Alum). Imject Alum is a mixture of aluminum and magnesium hydroxides and their derivatives, and it is commonly used as an adjuvant for animal studies, though its immunogenicity and associated reactogenicity are actually slightly less than that of related alum-based salts that are used in humans [5]. In electron microscopy studies, we found that Imject Alum took a physical form similar that of other alum-based salts [32], consisting of both large plate-like structures and needle-like particles (Figure 1b). To assess cytotoxicity, we incubated J774.1, a macrophage cell line, with Q11 or Alum for 4 hr, at concentrations ranging from 0.1 to 10 % of the

concentrations used for immunizations. The percent of cells that was non-viable was quantified according to a fluorescent Live/Dead assay. Consistent with its known *in vivo* toxicity, alum particles elicited dose-dependent cell death at all concentrations tested, whereas Q11 nanofibers were indistinguishable from buffer controls. Their total lack of cytotoxicity in this assay led us to compare the levels of inflammation after *in vivo* injection of these adjuvants.

3.2. Absence of nanofiber-induced inflammation at the injection site

We compared the inflammatory responses raised by Q11-adjuvanted peptide with those raised by alum-adjuvanted peptide without a carrier protein (Figure 2). After footpad injection, OVAQ11-immunized footpads remained similar in color and thickness to the contralateral (unimmunized) footpad, whereas pOVA-Alum-immunized footpads reddened and swelled to as much as 140 % their normal thickness, remaining swollen for greater than one week (Figure 2a). In histological cross-sections obtained at day 8 (Figure 2a and SI Figure 1), OVAQ11-immunized footpads were indistinguishable from unimmunized controls, whereas pOVA-Alum-immunized footpads exhibited acute inflammation located primarily in the muscle, along with myocyte cell death [33]. Thus in these tissues, the local inflammatory response to OVAQ11 was strikingly diminished compared to alum.

Alum, MF59, and CFA function in part by inducing the rapid secretion of inflammatory chemokines (MCP-1 ;CCL2, KC/CXCL1) and cytokines (G-CSF, IL-5, IL-6, IL-1) at the site of immunization, a process through which APCs are recruited and activated [30, 34, 35]. Thus, we examined cellular recruitment and inflammatory cytokine production following immunization, using the intraperitoneal (i.p.) space to facilitate sampling and first confirming that i.p. immunization raised antibody response profiles similar to s.c. injection (SI Figure 2). By 20 hr after injection, the OVAQ11-immunized peritoneal lavage fluid was indistinguishable from that of negative control pOVA- or PBS-immunized mice (Figure 2b); all three groups contained similar levels of macrophages and G-CSF and lacked other inflammatory cells and cytokines. The profile 4 hr after injection with OVAQ11 was also similar (SI Figure 3). In contrast, immunization with pOVA-Alum recruited significant numbers of inflammatory cells to the injection site and induced significantly higher levels of inflammatory chemokines and cytokines (Figure 2b), in agreement with previous reports [31, 34]. These differences suggested that OVAQ11 stimulated an antibody response via a non-inflammatory pathway distinct from current adjuvants.

3.3. Internalization of OVAQ11 and restricted DC activation

To test whether OVAQ11 was simply inert or if it interacted actively with antigen-presenting cells, we immunized mice i.p. with fluorescein-labelled pOVA or OVAQ11, collected peritoneal lavage fluid after 20 hr, and measured the uptake by dendritic cells and macrophages using flow cytometry (Figure 3a,b). The soluble pOVA was acquired by both cell types in relatively low quantities, with the cells having only moderately increased fluorescence relative to those from PBS-immunized mice. In contrast, OVAQ11 nanofibers were acquired by a larger fraction of the cells and in greater quantities, with many cells having over 10-fold brighter fluorescence. To test whether the fluorophore-conjugated peptide was located intracellularly or simply adsorbed on the surface of these cells, we stained cells from the lavage fluid of OVAQ11-fluorescein immunized mice with allophycocyanin-labelled anti-fluorescein to detect the labelled peptide, either with or without prior permeabilization of the cell membrane (Figure 3c). Without permeabilization (surface staining), no fluorescein-positive cells were labelled by the anti-fluorescein, indicating that the peptide was not present on the surface of the cells. With permeabilization (intracellular staining), all fluorescein-positive cells were labelled with the anti-fluorescein, thus confirming that the peptide was located inside dendritic cells after i.p. injection.

Finally, DCs and macrophages from OVAQ11- or PBS-immunized mice were tested for markers of activation (Figure 3d). Interestingly, DCs that were positive for uptake of fluorescent-OVAQ11 had significantly increased CD80 and CD86 expression levels compared to PBS-immunized mice, whereas DCs in the same sample that had not acquired fluorescent-OVAQ11 did not have increased expression levels. Macrophages, despite ready uptake of OVAQ11, had unchanged CD80 and CD86 expression. Thus, activation by the peptide nanofibers was highly specific, with none of the broad or bystander activation induced by inflammatory adjuvants such as alum or MF59 [36].

3.4. Comparison of T cell responses between adjuvants

Because adjuvants are commonly thought to require inflammation to raise productive T cell-dependent antibody responses to vaccines [2], we assessed the extent of functional CD4 T cell activation in OVAQ11-immunized mice. To track pOVA-specific CD4 T cells, CFSE-labelled pOVA-specific lymphocytes (OT-II cells) were adoptively transferred i.v. before immunizing mice s.c. with OVAQ11, pOVA-Alum, or pOVA in PBS. After adoptive transfer, OVAQ11 elicited serum IgM by day 3.5 (titer approx. 10^3) and IgG by day 7 (titer approx. 10^4), as well as significantly greater differentiation into T_{fh} cells and IFN- γ or IL-4-producing cells than was elicited by pOVA alone (Figure 4). Similar trends in antigen-specific IFN- γ and IL-4 production were observed from endogenous T cells in mice that did not receive OT-II cells, as assessed on week 9, one week after the second boost (Figure 4b), although the increase relative to pOVA alone was not statistically significant. Consistent with the prevailing theory that inflammation and T cell activation are correlated, T cell responses to OVAQ11 were substantially weaker than the responses raised by immunization with pOVA-alum.

3.5. Rapid development of germinal center B cells and TLR-4-independent antibody production

T_{fh} cells are critical for the germinal center reaction, which generates high-affinity B cells, plasma cells and memory B cells [37]. Thus the modest T_{fh} cell response induced by OVAQ11 was surprising based on previous reports by our group [15–17] and others [38] that high-titer antibody responses are raised by Q11-based materials. We therefore investigated the development of pOVA-specific GC B cells. Mice were immunized s.c. with OVAQ11 or PBS, and endogenous pOVA-binding GC B cells (GL-7⁺ Fas⁺) were quantified on day 14 after primary immunization by flow cytometry (SI Methods). GL-7⁺ Fas⁺ pOVA-binding GC B cells were detectable after immunization with OVAQ11 (2.0 ± 0.4 % of all pOVA-binding B cells) but not after immunization with the same molar quantity of pOVA adjuvanted with alum (Figure 5a). Furthermore, pOVA-specific antibodies were observed after immunization with OVAQ11 but not with pOVA-Alum (Figure 5b and Supporting Results). We note that alum is a potent and reliable adjuvant for whole proteins and for peptide-carrier conjugates, but it is known to be a poor adjuvant for unconjugated peptides [39]. Indeed, we confirmed that OVA protein adjuvanted with alum raised strong IgG responses, as did OVAQ11 formulated with alum (Figure 5b), illustrating, in the latter case, that the absence of carrier function in the unconjugated peptide/alum system could be restored by employing Q11. Antibodies raised by OVAQ11 cross-reacted with whole OVA protein in ELISA assays; the log₁₀ IgG titers at week 5 were 3.2 ± 1.1 and 3.6 ± 1.1 for ELISAs against pOVA and OVA protein, respectively, in this experiment (mean $\pm$ std dev, $n = 5$). Interestingly, both of the alum-adjuvanted formulations raised detectable IgE responses, whereas OVAQ11 alone did not (Figure 5b).

Because lipopolysaccharide (LPS) is a frequent laboratory contaminant, a TLR4 agonist, and a T cell-independent mitogen for B cells [40], we investigated the requirement of TLR-4 in the immunogenicity of OVAQ11. TLR-4 knock-out mice immunized with OVAQ11 raised

strong antibody responses comparable to WT mice, indicating that TLR-4 ligation was not required in the mechanism of action of these peptide self-assemblies to elicit antibodies (SI Figure 4). This observation is consistent with our OVAQ11 formulations having negligible amounts of endotoxin (< 0.01 EU per 100 μ L dose) [41].

3.6. Nanofiber immunization raises high-affinity and neutralizing antibody responses against peptide antigens

The modest Tfh response raised the possibility that the quality of the antibody response would be inferior to immunizations that elicited more robust Tfh responses. We therefore interrogated the quality of the antibody responses to the peptide antigen by surface plasmon resonance (SPR), comparing OVAQ11 to both Alum and CFA (Figure 6a-c and SI Figure 5). At 7 days following the second boost, the quantity of antibodies that bound to the pOVA-coated chip was similar in sera from mice immunized with OVAQ11, OVAQ11 with alum, and pOVA-CFA. Importantly, the dissociation rate, which together with binding is a measure of antibody affinity, was also similar. pOVA-Alum sera did not produce a measurable SPR signal, consistent with the minimal antibody titers detected by ELISA. Thus, Q11 nanofibers differ from alum and other classical adjuvants in that they induce development of peptide-specific germinal center B cells and production of high-affinity antibodies without inflammation at the injection site and with relatively modest T cell responses compared to alum adjuvant.

To functionally assess the quality of the immune response to nanofiber vaccination, we investigated the protective potential of the anti-OVA antibody response in a Hemagglutinin Inhibition (HAI) assay for Influenza A. Influenza A viruses begin the infectious cycle by attaching to sialic acid moieties on mammalian cells via the globular head of an envelope protein, hemagglutinin (HA). *In vitro*, this causes the agglutination of mammalian and avian erythrocytes, and serum HAI titers > 32 are associated with clinical protection against influenza infection [42]. Here, we measured the levels of neutralizing antibodies in an HAI assay using a strain of influenza (A/HK/X31 H3N2) that had been engineered to express pOVA within the HA head (pOVA-flu; Figure 6d-e) [29]. Mice were immunized and boosted at week 4, and serum was collected at weeks 5 – 6 (ELISA titers are provided in SI Figure 6). OVAQ11 sera had high ELISA titers of anti-pOVA and had correspondingly high HAI titers against pOVA-flu, considerably greater than 32 ($p < 0.05$). The majority of pOVA-CFA sera also had high ELISA and HAI titers, while pOVA-Alum sera had minimal anti-pOVA antibodies and no activity in this HAI assay. Neutralization was antigen-specific, because immunized sera did not prevent agglutination of a control (wildtype) virus that lacked the pOVA epitope. These results confirm that the high-affinity antibodies induced by Q11 nanofibers are neutralizing towards targeted surface epitopes, suggesting the potential for a protective response *in vivo*.

4. Discussion

New subunit vaccine technologies are primarily focused on protein or peptide antigens that require adjuvants to improve the vaccine's immunogenicity. For example, the newest generations of proposed seasonal and pandemic influenza vaccines, aiming to move away from the standard egg-based production of whole viruses, utilize recombinant proteins such as HA adjuvanted with a stable oil-in-water emulsion (e.g., MF59) [43, 44] or, more recently, a two-part adjuvant system incorporating a TLR-4 agonist within a similar emulsion [45, 46]. Like alum, these adjuvants enhance adaptive immunity by stimulating local inflammatory responses at the injection site, which then lead to dendritic cell recruitment, maturation of essentially all antigen-presenting cells in the vicinity, and antigen uptake and transport to the draining lymph nodes [34, 36, 47]. Many recently developed nano- or micro-particulate subunit vaccines also deliberately incorporate inflammatory

activators of APCs [48, 49], including CpG DNA [50], endotoxin derivatives [51], or dextran sulfate [52]. Yet, achieving regulatory approval of new adjuvants continues to be difficult, largely due to concerns about potential local or systemic reactogenicity and other adverse effects [1, 3].

In contrast to the prevailing inflammation-based strategy for adjuvant and vaccine development, here we utilized an antigen-displaying peptide-nanofiber vaccine to elicit high-titer, high-affinity, and neutralizing IgG responses, without exogenous adjuvants or measureable local inflammation. The antibody response to the OVAQ11 fibers was CD4 T cell dependent [15, 16], associated with germinal center B cell formation, and yielded high affinity antibodies that were functionally neutralizing in a HAI assay to influenza virus. Only the DCs that took up OVAQ11 demonstrated increased expression of CD80 and CD86. Unexpectedly, macrophages that internalized OVAQ11 did not demonstrate increased expression of CD80 and CD86. We speculate that this restricted APC activation may be responsible for the lack of inflammation observed at the injection site. Consistent with this finding, OVAQ11 elicited only modest T cell differentiation into effector subsets, including germinal-center related Tfh cells. This immunological profile contrasts strongly with the classical adjuvant, alum, which elicits strong local inflammation, T cell proliferation, and T cell differentiation, but weak antibody responses to the peptide antigen, pOVA, in the absence of a carrier. In addition to their non-inflammatory character, these self-assembled peptide-based materials also have the advantage of being well suited for the precise display of multiple different peptides simultaneously [53, 54], which should enable the development of multivalent vaccines containing optimized sets of peptide epitopes.

The generality of these findings to other types of self-adjuvanting particles remains to be demonstrated, however we note that some types of self-adjuvanting nanoparticles have been shown to deliver antigen directly to dendritic cells in the lymph node through lymphatic flow [13, 19], while other engineered particles are phagocytosed and processed by antigen presenting cells in vitro without upregulating activation markers [25]. We speculate that these may be two potential mechanisms by which self-adjuvanting particles, including beta-sheet nanofibers, could bypass the need for tissue inflammation. Once in the lymph node, nanoparticles must still be able to activate antigen presenting cells in order to be self-adjuvanting [20]; a possible mechanism was identified for Pluronic-stabilized polypropylene sulfide nanoparticles, which activated the complement cascade to generate in situ the signals for dendritic cell activation [13, 55]. Finally, while we have focused on antibody responses in this work, self-adjuvanting nanoparticles have also been shown to elicit effective cytotoxic T cell responses by displaying an epitope for CD8 T cells [10, 12, 56, 57]. For example, epitope-conjugated peptide amphiphiles that self-assembled into nanometer-sized micelles were shown to elicit protective immunity *in vivo* without appreciable DC maturation or activation *in vitro* [56]. Thus, self-assembled peptide nanofibers and related self-adjuvanting nanoparticles provide an approach to activating both arms of adaptive immunity.

5. Conclusion

In this study, we demonstrate that a biomaterials-based self-adjuvanting vaccine does not elicit the cytotoxicity and inflammation that characterize traditional alum-based adjuvants. Yet, the material nevertheless elicited a modest T cell response and significant germinal center formation in the lymph nodes, and it produced high-affinity and neutralizing antibody responses in an *in vitro* flu model. These data indicate that it is possible to design materials that decouple inflammation from the strength of the immune response, and suggest that this and other nanoparticulate self-adjuvanting materials may comprise a broad platform for the development of potent and non-reactogenic vaccines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank N. Joseph for interpreting the histology slides and J. Williams for providing the OTII mouse strain. This publication was made possible by grant numbers AI094444 (NIH/NIAID) and EB009701 (NIH/NIBIB) to the University of Chicago and grant number HHSN266200700008C (NIH/NIAID) to the University of Rochester, and by the Chicago Biomedical Consortium with support from the Searle Funds at The Chicago Community Trust. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH or the Chicago Biomedical Consortium.

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Figure 1. Structure and cytotoxicity of Q11-based materials compared to Inject Alum
 (a) OVA-Q11 fibers and (b) Inject Alum observed by TEM. (c) J774.1 macrophages exposed to Alum (top) or Q11 (bottom) at 10 % of their standard adjuvant concentrations for 4 hr, with viable (green) and non-viable (red) cell staining. (d) Percentages of non-viable J774.1 macrophages after 4 hr exposure to Alum or Q11. Concentration is given as the fraction relative to the standard adjuvant concentrations used for mouse immunizations (40 mg/mL total Al/Mg salt for Inject Alum; 2 mM, or 7 mg/mL, for Q11 peptide).

Figure 2. OVAQ11 immunization does not generate detectable local inflammation

(a) Injection of mouse footpads with OVAQ11 did not cause inflammation, whereas pOVA-Alum caused significant swelling and acute inflammation and necrosis (white arrows in H & E-stained tissue sections collected at day 8). Scale bars are 200 μ m. Schematics at the right illustrate the immunization conditions for the pOVA antigen assembled into nanofibers via the Q11 assembly domain or adsorbed on alum. N = 3 mice per group. (b) Cellular and cytokine responses were analyzed in lavage fluid 20 hr after intraperitoneal injection. (top) Phenotyping by flow cytometry indicated that OVAQ11 did not recruit inflammatory cells, similar to unadjuvanted pOVA or PBS, whereas pOVA-Alum recruited significantly higher numbers of inflammatory cells. N = 3 mice per group, one of two independent experiments is shown. (bottom) Bead-based immunoassay showed no increase in inflammatory cytokines after OVAQ11 immunization, whereas alum immunization induced 5 out of 6 cytokines in the panel. Abbreviations: Macrophages (mac), conventional dendritic cells (cDC), inflammatory DCs (iDC), plasmacytoid DC (pDC), neutrophils (neut), inflammatory monocytes (iMono), eosinophils (eosi); monocyte chemotactic protein-1 (MCP-1), keratinocyte-derived chemokine (KC), granulocyte colony-stimulating factor (G-CSF), interleukin (IL). All error bars show mean $\pm$ 1 std. dev. *, $p < 0.05$ compared to OVAQ11 group (1-way ANOVA with Tukey post-hoc tests).

Figure 3. OVAQ11 is internalized by antigen presenting cells and induces targeted dendritic cell activation *in vivo*

(a) Gating strategy for CD11c⁺ F4/80⁻ MHCII⁺ dendritic cells (DC) and CD11c⁺ F4/80⁺ macrophages (M) after i.p. immunization. (b) Uptake of pOVA-fluorescein or OVAQ11-fluorescein 20 hr after i.p. immunization. Both DCs and Macrophages acquired OVAQ11 in greater quantities (brighter fluorescein staining) and in a larger fraction of the cells than pOVA (pooled cells from n = 2 per group; representative of two independent experiments). (c) Internalization of OVAQ11 by DCs. Cells from the lavage fluid were stained by allophycocyanin-conjugated anti-fluorescein antibody (surface staining) or were pre-blocked with anti-fluorescein antibody, fixed, permeabilized, and finally stained by allophycocyanin-conjugated anti-fluorescein antibody (intracellular staining). Cells were double-positive for allophycocyanin and fluorescein only after permeabilization, indicating that all of the OVAQ11 was intracellular (pooled cells from n = 3 per group). (d) Activation status after OVAQ11 uptake. Only DCs that were positive for fluorescent-OVAQ11 had an increase in CD80 and CD86 expression; neither DCs from the same sample that were negative for OVAQ11 nor macrophages had increased expression of these markers (n = 3 per group).

Figure 4. OVAQ11 induces CD4⁺ T-cell differentiation into T follicular helper and cytokine-secreting cells

Using an OTII adoptive transfer model, (a) pOVA-specific Tfh development was measured by expression of the markers PD-1 and CXCR5 3.5 d after immunization with the indicated condition ($n = 3$; representative of two independent experiments). (b) IL-4 and IFN- γ production were quantified by ELISPOT assays in response to stimulation with pOVA. Cells were collected from draining lymph nodes 3.5 and 7 d after immunization in the OTII cell transfer system (*left*; $n = 3$ mice per time point, pooled to $n = 6$ for analysis; representative of two independent experiments), or at week 9 in the endogenous system (*right*; $n = 3$). Data represent mean $\pm$ 1 SD. *, $p < 0.05$ compared to OVAQ11 group (Student's T test or 2-way ANOVA with Tukey post-hoc tests).

Figure 5. OVAQ11 but not alum induces a germinal center B cell response that correlates with high-titer IgG

(a) Endogenous OVA-specific GC-B cells were detectable by day 14 post-immunization with OVAQ11, but not with pOVA-Alum. B cells labeled with biotin-pOVA were detected by double-positive staining with streptavidin-PE and streptavidin-APC. GC B cells within this population were identified by double-positive staining with GL-7 and FAS, quantified at the right as a percentage of the total pOVA-binding B cells. *, $p < 0.05$ (1-way ANOVA with Tukey post-hoc tests). Pooled data from two independent experiments. (b) IgG and IgM signals against pOVA were strongest after immunization with OVAQ11 and OVAQ11-Alum (left); OVA protein-Alum raised a strong response against the whole protein (upper right). Only alum-adjuvanted vaccines elicited a detectable IgE response at week 9 (lower right; *, $p < 0.05$ versus OVAQ11, 1-way ANOVA with Tukey post-hoc tests). Mice were immunized subcutaneously (s.c.) at week 0 and boosted with half-doses at weeks 4 and 8 (gray arrows). $N = 5$ mice per group. Error bars show mean + 1 SD. Representative of at least two independent experiments.

Figure 6. Antibodies induced by OVAQ11 are high affinity and neutralizing, similar to those raised by pOVA with CFA

(a) Schematic of the SPR experiment, measuring the mass bound to a pOVA-conjugated surface as first serum solution and then buffer were flowed over it. (b) Antibodies induced by OVAQ11 or OVAQ11-Alum bound in similar quantities as those induced by pOVA-CFA; R_0 is the quantity of bound antibody (measured in response units, RU) after 3 min of serum flow, and this value scaled linearly with the concentration of pOVA used to coat the surface. (c) Dissociation rates (k_{off} , s^{-1}) were not significantly different between OVAQ11, OVAQ11-Alum, and pOVA-CFA sera. Measurements of k_{off} were not possible for the pOVA-Alum group because of its low binding. $N = 5$ mice per group; representative of two independent experiments. Error bars show mean $\pm$ 1 SD. Significance tested by 1-way ANOVA with Tukey post-hoc tests. (d) To test the functionality of the antibodies, mice were primed and boosted according to the schedule shown, and haemagglutination inhibition (HAI) assays were performed by incubating the resultant sera with a strain of influenza A engineered to express pOVA in head of the hemagglutinin protein (pOVA-HA; [29] or a control (wildtype) strain. (e) HAI titer was defined as the highest dilution at which agglutination of chicken red blood cells by the influenza virus was inhibited by the sera. OVAQ11 and pOVA-CFA both induced neutralization of pOVA-flu in a majority of immunized mice; no response was observed to the control flu. *, $p < 0.05$ compared to a minimal protective titer of 32 (dashed line), by single sample t-test. Data is from one of two similar independent experiments.