

A self-assembling peptide acting as an immune adjuvant

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The development of vaccines and other immunotherapies has been complicated by heterogeneous antigen display and the use of incompletely defined immune adjuvants with complex mechanisms of action. We have observed strong antibody responses in mice without the coadministration of any additional adjuvant by non-covalently assembling a T and B cell epitope peptide into nanofibers using a short C-terminal peptide extension. Self-assembling peptides have been explored recently as scaffolds for tissue engineering and regenerative medicine, but our results indicate that these materials may also be useful as chemically defined adjuvants. In physiological conditions, these peptides self-assembled into long, unbranched fibrils that displayed the epitope on their surfaces. IgG1, IgG2a, and IgG3 were raised against epitope-bearing fibrils in levels similar to the epitope peptide delivered in complete Freund's adjuvant (CFA), and IgM production was even greater for the self-assembled epitope. This response was dependent on self-assembly, and the self-assembling sequence was not immunogenic by itself, even when delivered in CFA. Undetectable levels of interferon-gamma, IL-2, and IL-4 in cultures of peptide-challenged splenocytes from immunized mice suggested that the antibody responses did not involve significant T cell help.

biomaterial | epitope | nanofiber | self-assembly | vaccine

The development of vaccines and other immunotherapies has been challenged by imprecise antigen display and the use of heterogeneous immune adjuvants whose mechanisms of action are complex and incompletely understood. Synthetic peptides are useful as antigens because their precise chemical definition allows one to specify the exact epitopes against which an immune response is to be raised. However, peptides are poorly immunogenic by themselves and require coadministration with strong adjuvants. Although many adjuvants have been investigated for peptide immunotherapies to date, current strategies such as particulates (1, 2), oil emulsions (3), toll-like receptor (TLR) ligands (4), immunostimulating complexes (ISCOMs) (5), and other biologically sourced materials (6, 7) utilize chemically or structurally heterogeneous materials, making their characterization, mechanistic understanding, and regulatory approval challenging (1, 8, 9). This situation has motivated the pursuit of self-adjuvanting or adjuvant-free systems (10–12).

A broad goal in the field of biomaterials is to produce synthetic scaffolds capable of presenting multiple cell-interactive components in spatially resolved networks (13, 14). To accomplish this, supramolecular self-assembly is rapidly becoming a synthetic method of choice (15–17). One strategy that has received attention recently is based on fibrillized peptides, peptidomimetics, and peptide derivatives, which are being explored for a variety of biomedical and biotechnological applications, most notably as scaffolds for regenerative medicine (18, 19) and defined matrices for cell culture (20, 21). In these applications, self-assembled materials provide several advantages, including multifunctionality, multivalency, synthetic definition, molecular specificity, and control over the nanoscale positioning of ligands and other biomolecular features (17). In our laboratory, we have

previously developed self-assembled, multicomponent matrices for cell culture using a short fibrillizing peptide, Q11 (Ac-QQKQFQFEQQ-Am) (16, 17, 22, 23). This peptide, like other previously reported short fibrillizing peptides (21, 24–26), β -hairpins (27), peptide-amphiphiles (19, 28), and peptide derivatives (29), self-assembles in salt-containing aqueous environments to form networks of β -sheet-rich nanofibers. It is also capable of displaying functional amino acid sequences or chemical groups on the surface of its self-assembled fibers. For example, adding cell-binding amino acid sequences to the N terminus of Q11 leads to self-assembled fibrils that functionally present the cell-binding peptides on their surfaces (16). Q11 with an N-terminal cysteine and a C-terminal thioester can undergo native chemical ligation after assembly, which can be used to stiffen the fibrillar network (23). These peptides can also be mixed to display precise combinations of different ligands (16).

During initial development as scaffolds for regenerative medicine, Q11 and other self-assembling peptide-based materials have been found to be minimally immunogenic. In previous work, we found that Q11 and Q11 with N-terminal cell-binding RGDS sequences elicited little to no antibody responses in mice (16). Negligible tissue responses were also observed for β -sheet fibrillizing RAD16-II peptide assemblies injected within rat (30) or mouse (18) myocardium. Low antibody titers have also been reported in rabbits and goats for RAD16 peptides (24). Only one study to our knowledge has observed an inflammatory response to RAD16 peptides in rats, but the causes were not known (31). The minimal immunogenicity of these materials observed to date is clearly advantageous for applications in regenerative medicine, but previous work has focused largely on amino acid sequences that are already found in endogenous proteins, for example the RGDS cell-binding sequence from fibronectin (16, 32). Because of this, strong epitopes have effectively been avoided. In the present work, we sought to determine the extent to which the previously observed low immunogenicity also applied to peptide sequences containing stronger epitopes. Our results indicate a surprisingly robust antibody response generated against a self-assembled peptide containing antigenic determinants from ovalbumin known to be recognized by both T cells and B cells. This indicates that in certain circumstances, self-assembled peptides can serve as powerful chemically defined adjuvants.

Results

Peptide Design and Supramolecular Assembly. The peptide Q11 was previously designed as a self-assembling transglutaminase substrate (22) and was a variation on the DN1 peptide originally described by Aggeli and coworkers (25, 33). For the work

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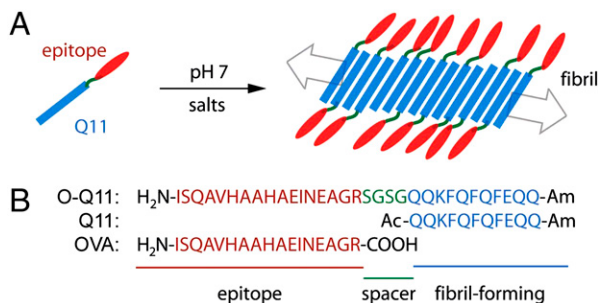


Fig. 1. Schematic (A) and sequences (B) of epitope-bearing self-assembling peptides. The Q11 domain (blue) assembles into fibrillar aggregates, displaying the epitope sequence (red) at the end of a flexible spacer (green).

reported here, we designed a peptide containing a Q11 self-assembling domain in tandem with OVA₃₂₃₋₃₃₉, a 17-amino acid peptide from chicken egg ovalbumin (Fig. 1). OVA₃₂₃₋₃₃₉ (ISQAVHAAHAEINEAGR, hereafter referred to as OVA) is an H-2^b-restricted class II peptide containing multiple antigenic determinants, including known T and B cell epitopes (34). The self-assembling epitope peptide (O-Q11) was produced by solid phase synthesis and included a hydrophilic Ser-Gly-Ser-Gly spacer between the OVA and Q11 domains, with the OVA domain positioned at the N terminus (Fig. 1B).

When dissolved in water at concentrations of 40 mM and lower, O-Q11 formed no visible precipitate. However, similar to Q11 and to other previously reported Q11 derivatives (16, 17, 22, 23), it formed networks of laterally entangled fibrils when it was first dissolved in water and then added to salt-containing buffers such as phosphate-buffered saline (PBS), (Fig. 1A, Fig. 2). By TEM, O-Q11 in PBS appeared as long, unbranched fibrils with widths of about 15 nm (Fig. 2C). Although the length of these fibrils was not directly measured, the relative scarcity of fibril ends in TEM images suggested that the fibril length was on the order of microns. Circular dichroism of O-Q11 showed a single concentration-dependent minimum at 229–232 nm (Fig. 2E). This spectrum is consistent with a high degree of β -sheet or β -turn structure, and it is similar to the spectra of Q11 and mixtures of Q11 with other previously reported ligand-bearing Q11 derivatives (16, 22).

Epitopes Were Functionally Displayed on O-Q11 Fibrils. The availability of the OVA epitope on the surface of O-Q11 nanofibers was confirmed by TEM and by ELISA. To label epitopes in TEM samples, an N-terminally biotinylated O-Q11 was synthesized with a single biotin tag directly adjacent to the OVA sequence. This biotinylated peptide produced fibrils that appeared morphologically similar to O-Q11 (Fig. 2D). Five-nanometer streptavidin-conjugated gold particles were then used to probe epitope availability on the fibril surface, with unmodified Q11 serving as a negative control. Biotin-O-Q11 fibrils stained strongly with streptavidin-gold, whereas Q11 samples bound negligible numbers of particles (Fig. 2B–D), demonstrating that a significant portion of the peptides' N termini were available on the surface of O-Q11 fibrils. To confirm this finding and to quantify the availability of the entire epitope, ELISA was employed. Plates coated with OVA and O-Q11 peptides were probed with antisera from mice immunized with OVA, either with or without complete Freund's adjuvant (CFA). Antisera from CFA-adjuvanted groups showed similar titers of OVA-reactive IgG, whether measured on OVA plates or on O-Q11 plates, and both showed low backgrounds for antisera from nonadjuvanted groups (Fig. 2F). This result indicated that the OVA epitope was functionally presented on the surface of the Q11 fibrils, and that plates coated with O-Q11 fibrils produced similar signal strengths to plates coated with the nonfibrillized OVA peptide, allowing the

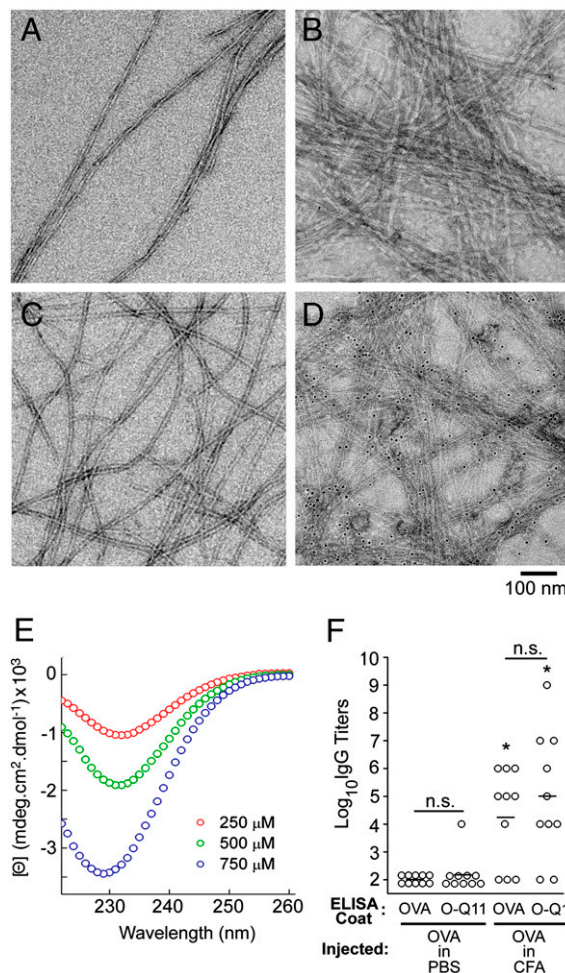


Fig. 2. Q11-based peptides self-assembled into β -sheet fibrils that displayed functional epitopes on their surfaces. Q11 (A) self-assembled into long, unbranched fibrils that did not bind streptavidin-gold (B). O-Q11 (C) also formed long, unbranched fibrils, and biotin-O-Q11 bound streptavidin-gold (D), indicating availability of the N terminus on the fibril surface. O-Q11 possessed a predominant β -sheet structure by CD (E). OVA antisera reacted similarly to ELISA plates coated with OVA or O-Q11 (F). Each point represents one mouse's serum; bars represent the mean. * $p < 0.01$ compared to OVA coat/OVA in PBS injection, by ANOVA with Tukey HSD post hoc testing. n.s., not statistically different.

sera of mice immunized with different peptides to be compared. Slightly higher titers were observed for the O-Q11-coated plates in both cases, but it was not statistically significant.

High IgG Titers Were Elicited by O-Q11 Without Adjuvant in Mice. To investigate how fibrillization affected the immunogenicity of OVA, C57BL/6 mice were immunized subcutaneously with the different peptides and boosted with additional peptide at 28 d (see *Methods*). Serum was collected 7 d after the boost, and levels of various immunoglobulins were measured. It was found that fibrillized Q11 alone did not raise any detectable IgG, whether delivered with or without CFA (Fig. 3A and B, raw data in Fig. S1). This result reiterated our previous findings that Q11 was not immunogenic (16) and further indicated that Q11 continued to be nonimmunogenic even when delivered in CFA. Also, we previously showed that Q11 and functionalized Q11 peptides did not induce cell death in cultures of primary human endothelial cells, indicating that the basic Q11 sequence was noncytotoxic as well as nonimmunogenic (16, 23).

In surprising contrast to the immeasurably low immunogenicity of Q11, O-Q11 elicited high IgG titers without any added

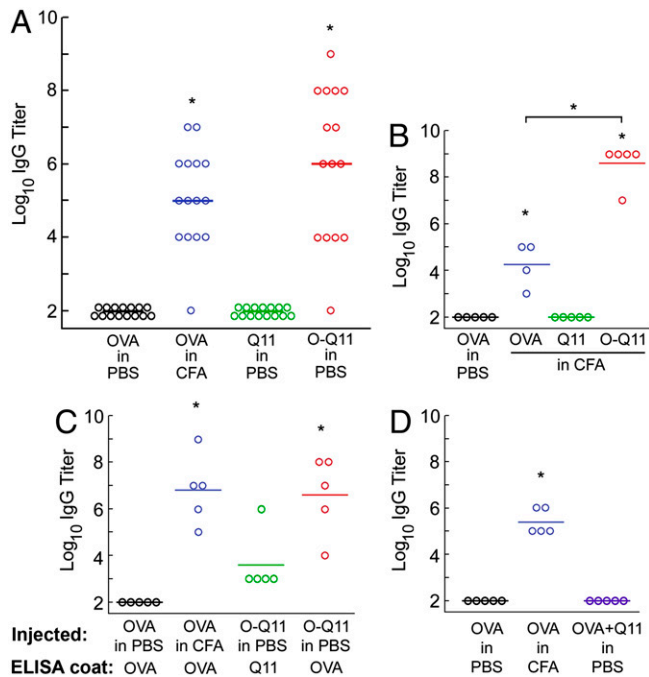


Fig. 3. Fibrillization by the Q11 domain strongly adjuvanted IgG responses to OVA. Similar titers of total IgG were raised against O-Q11 delivered in PBS and OVA delivered in CFA, whereas Q11 or OVA delivered in PBS did not elicit a response (A). Q11 was nonimmunogenic even in CFA, whereas CFA increased IgG titers for O-Q11 (B). O-Q11 antisera were strongly cross-reactive to OVA-coated ELISA plates and showed a small amount of reactivity to Q11-coated plates that was not statistically significant (C). The adjuvant activity of Q11 was entirely dependent on covalent conjugation between the fibrillizing domain and epitope domain, as mixtures of Q11 and OVA did not raise any OVA-specific IgG (D). Each point represents one mouse; bars represent the mean. * $p < 0.01$ by ANOVA with Tukey HSD post hoc testing, compared with OVA in PBS, or between groups as indicated.

adjuvant (Fig. 3A, raw data in Fig. S1). Even higher titers were produced when it was delivered in CFA (Fig. 3B). Anti-peptide IgG titers were similar between mice injected with O-Q11 in PBS and OVA peptide in CFA, indicating that the Q11 sequence itself functioned as a strong adjuvant, presumably by assembling the OVA peptide into nanofibers. O-Q11 antisera also bound to OVA-coated plates, demonstrating that the epitope was conserved (Fig. 3C). O-Q11 antiserum reactivity to OVA-coated plates also excluded the possibility that the high antibody titers found in O-Q11 antisera were a measurement artifact arising from increased antigen density on fibril-coated ELISA plates. In addition, endotoxin levels were less than 0.3 EU/mL for all samples (Table S1), eliminating the possibility that inadvertent contamination caused the observed adjuvant effect. O-Q11 antisera also reacted with Q11, though at smaller, statistically insignificant levels (Fig. 3C), possibly indicating a small degree of epitope spreading to the Q11 domain.

We hypothesized that the multivalent surface display of the epitope on the fibrils was the source of Q11's strong adjuvant properties, but alternate explanations were possible. For example, if Q11 functioned as an adjuvant by activating TLRs, similarly to LPS or unmethylated CpG motifs, or if it simply slowed the diffusion of the epitope from the injection site by surrounding it with fibrils (the so-called "depot effect"), then outright conjugation of the epitope and the fibril would not be required. Additionally, if Q11 fibrils functioned in a manner similar to particulate adjuvants such as aluminum salts, whereby the adsorption and entrapment of the antigen onto and within the particle is sufficient for adjuvancy, then conjugation would also not be required. We did not investigate TLR activation in this study,

but to determine whether covalent conjugation between the epitope sequence and the self-assembling sequence was required for the strong antibody responses observed for O-Q11, we measured responses to unconjugated mixtures of Q11 peptide and OVA peptide. Notably, the strong antibody response generated by O-Q11 was completely abolished in the absence of covalent coupling between the Q11 fibrillizing domain and the epitope domain (Fig. 3D). No detectable IgG was observed for mixtures of OVA and Q11. This result indicated that Q11's adjuvant properties were dependent on its covalent attachment to the epitope peptide.

In order to determine the nature of the immune response to Q11-adjuvanted peptides, the isotypes of the responding antibodies were evaluated. For both OVA in CFA and fibrillized O-Q11, the dominant antibody isotype was IgG1, with smaller amounts of IgG2a, IgG2b, IgG3, and IgM being produced for both (Fig. 4). Comparing CFA-adjuvanted responses with Q11-adjuvanted responses, IgG1, IgG2a, and IgG3 were produced in statistically similar quantities, but IgG2b production was greater in the CFA-adjuvanted group, and IgM production was greater in the Q11-adjuvanted group.

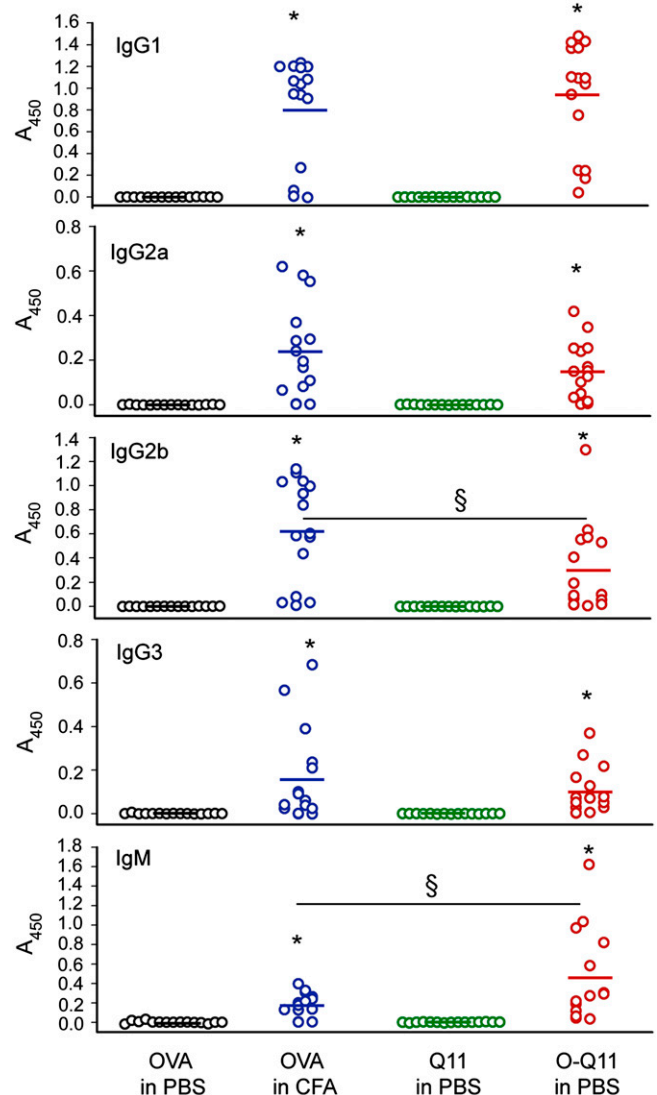


Fig. 4. Antibody isotypes in sera from mice immunized with peptides. Each point represents one mouse; bars represent the mean. * $p < 0.01$ compared with OVA delivered in PBS by ANOVA with Tukey HSD post hoc test. § $p < 0.05$ as indicated.

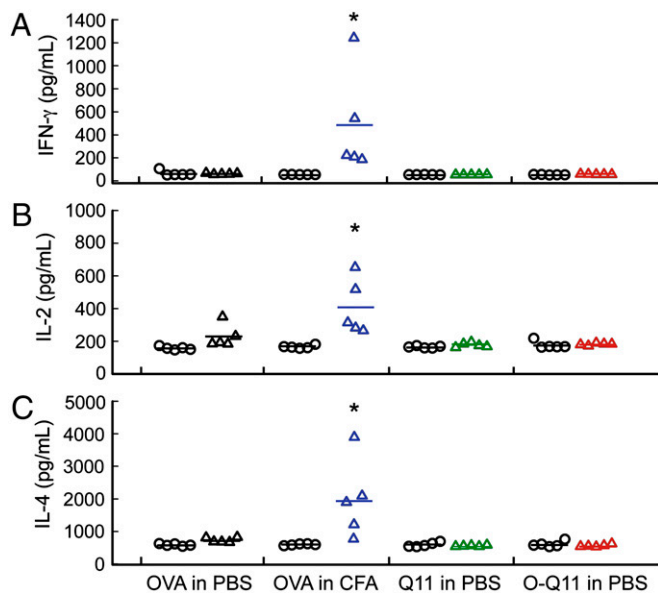


Fig. 5. IFN- γ , IL-2, and IL-4 production in peptide-challenged (triangles) and unchallenged (circles) splenocyte cultures. Each point represents one mouse; bars represent the mean. * $p < 0.01$ compared with corresponding unchallenged control, by ANOVA with Tukey HSD post hoc test.

To investigate the involvement of T cell help in the immune responses to O-Q11, splenocytes from immunized mice were challenged *in vitro* with the peptides, and the production of interferon- γ (IFN- γ), interleukin-2 (IL-2), and interleukin-4 (IL-4) was measured. These three cytokines were selected to provide measures of either a Th1 response (IFN- γ and IL-2) or a Th2 response (IL-4). However, somewhat surprisingly, when splenocytes from immunized mice were challenged *in vitro* with the immunizing peptide, they did not produce significant levels of any of these three cytokines compared with positive controls (Fig. 5). This lack of a strong cytokine response, robust antibody production, and an elevated IgM response in the groups adjuvanted with Q11 is suggestive of a mechanism that does not depend strongly on T cell help.

Discussion

Simply attaching a short self-assembling amino acid sequence to a peptide epitope's C terminus can dramatically enhance the peptide's immunogenicity. Our results indicate that fibrillizing peptide domains may be useful as simple adjuvant systems for peptide-based immunotherapies, and these results also provide guidance for the development of nonimmunogenic peptide biomaterials within applications such as regenerative medicine. As a means to specifically enhance peptide immunogenicity, the unique Q11-based approach reported here has a number of advantages. First, a self-assembling peptide domain can be easily added to any known epitope using conventional solid phase peptide synthesis. Also, by utilizing self-assembly, highly multivalent nanoscale objects can be directly produced from only one molecule, which enables precision in the production, purity, and study of the material. In contrast, many adjuvants currently employed or under development are composed of multiple molecular constituents or heterogeneous mixtures, making their definition, formulation, purification, and characterization challenging. For example, immunotherapies based on attenuated live viruses or heat-killed organisms contain intrinsic adjuvants such as lipopolysaccharide or unmethylated CpG motifs that are critical to their efficacy (35). Adjuvants based on natural products such as saponins or squalene (7) are by nature associated with some degree of molecular heterogeneity, and particulate adjuvants

such as aluminum salts depend on antigen adsorption or entrapment, processes that are complexly dependent on multiple chemical and physical factors during formulation (1, 35). Accordingly, specific molecular features of current adjuvants are not easily adjusted independently within a given vaccine formulation, making it difficult both to optimize an immunotherapy as well as understand its mechanism of action. Such efforts will be greatly enhanced with the availability of chemically defined adjuvants.

Molecular definition is becoming more favored in the development of immunotherapies, as reflected by the recent interest in microparticle- and nanoparticle-based approaches (2, 36, 37), and on multivalent polymeric ligands (38). Other multivalent synthetic epitopes, such as the polymers generated by ring-opening metathesis polymerization recently investigated by Kiessling and coworkers, also elicit significant antibody production (38). These polymers were investigated *in vivo* using CFA, however, so it is not clear whether they also induce strong responses without adjuvant. Another recent approach employing noncovalent aggregation was based on DNA vaccination and used 103 tandem glutamine repeats to oligomerize protein antigens (39); however, with DNA vaccination it may be challenging to tightly control the ultimate protein dose or the stoichiometries of multiple epitopes. In contrast, the molecular definition inherent in a synthetic self-assembling peptide enables precise dosing and should enable the predictable cofibrillation of multiple different epitope-displaying Q11 derivatives, as has been previously accomplished with cell-binding ligands (16). This may be a useful way to clarify the mechanism of Q11's adjuvant properties and to optimize its use toward a range of therapeutic targets. Although definitive long-term safety studies have yet to be performed, it is encouraging that Q11 and other fibrillizing peptide biomaterials have been found to be noncytotoxic in cell culture (16, 23). At this time, however, it is not entirely clear what the molecular determinants of β -sheet fibril toxicity are, or why some β -sheet fibrils appear to be nontoxic, while others are toxic (40). This aspect requires clarification as fibrillar biomaterials are developed (41). In addition, the lack of a robust cytokine response in the present system may indicate that this approach may not provide for lasting B cell or T cell memory, necessary for vaccinations that would provide long-term immunity. Immunological memory was not investigated in this study, but if it is found in the future that peptide fibrils are unable to provide memory, it may be possible to combine the fibril-forming peptides with other chemically defined adjuvants or other epitopes via coassembly. Regardless of whether the present system is able to produce lasting memory, its ability to produce high antibody titers would be immediately useful in other applications such as bioreagent antibody production against specific peptide epitopes in animals.

Owing to the significant production of IgG1, IgG2a, IgG2b, IgG3, and IgM and the relative lack of IFN- γ , IL-2, and IL-4 in cultures of challenged splenocytes, it appears that this fibrillar adjuvant system induces an immune response that does not significantly involve T cell help. Although the molecular definition of the fibrils may allow a clearer understanding of this mechanism of action in the future, at present several explanations are possible, as is the case with other current adjuvants as well (1, 35). It is known that fibrillized peptides tend to be significantly stabilized against proteolytic attack, which would extend the residence time of the peptide at the injection site, the so-called "depot effect". At the same time, it is possible that epitope fibrillization facilitates phagocytosis and activation of antigen-presenting cells. Also, while unlikely, it cannot be ruled out that some degree of TLR activation occurs with fibrillized antigens, because this aspect was not investigated directly. However, the nonimmunogenicity of Q11 codelivered with soluble OVA suggests against this possible mechanism. Although interaction between antigen-specific B cells and T helper cells is usually required for high-affinity antibody production and class switching

from IgM to IgG, T cell-independent (TI) mechanisms of B cell activation and Ig class switching have been known for some time (42–44), and are the most likely explanation for the response observed with Q11. Previous studies with haptenated polymers have shown that highly multivalent synthetic antigens can activate B cells in the absence of T cell help, and O-Q11's mechanism of action may be similar to these previously investigated TI antigens (45, 46). Studies in T cell-deficient mice could be used to clarify this mechanism.

Do the adjuvant properties of β -sheet fibrillized peptides mean that such biomaterials are globally at risk of being inappropriately immunogenic in biomedical applications such as regenerative medicine? We do not believe so, and it is more likely that a confluence of attributes is necessary for producing the strong immune responses we observed. First, there is mounting evidence that most self-assembled peptide biomaterials are well tolerated in vivo (16, 18, 19, 24, 30). In addition, antibodies could not be detected against the unmodified Q11 peptide, even when it was delivered in CFA or when soluble OVA was codelivered with it. Only when OVA was directly conjugated to the Q11 peptide were measurable antibody titers generated. In light of these results, it appears that the immunogenicity of fibrillized peptide biomaterials is dominated by the epitope present on the nanofibers to a much greater extent than the self-assembling nature of the material itself, and immunogenicity can be largely avoided simply by avoiding strong epitopes. Taken further, much of current biomaterials research deals with the biofunctionalization of solid materials, be they self-assembled or covalently constructed, particulate or macroscale, synthetic or biological. In all of these cases, underlying adjuvant activity of the biomaterials themselves is increasingly being shown to modulate the tissue response (47, 48), so the field as a whole will need to establish which modes of surface presentation of biomolecular features lead to nonimmunogenicity, tolerance, or immunogenicity. It is expected that molecularly defined systems such as the one we describe here will directly enable such future studies.

Conclusions

Peptide epitopes that were assembled into nanofibers by a short synthetic fibrillization domain elicited high antibody titers in the absence of any adjuvant. This response was dependent on covalent conjugation between the epitope and fibrillizing domain, and Q11 by itself was nonimmunogenic, even in CFA. Titers of IgG1, IgG2a, and IgG3 were similar between CFA-adjuvanted and Q11-adjuvanted groups, but IgG2b production was greater for CFA-adjuvanted responses, and IgM was greater for Q11-adjuvanted responses. Interferon- γ , IL-2, and IL-4 were not produced in measurable quantities for Q11-adjuvanted responses, which along with the enhanced IgM response suggests that this response is not strongly dependent on T cell help. This strategy represents a simple, chemically defined way of dramatically enhancing antibody responses to peptide epitopes.

Materials and Methods

Peptide Synthesis and Purification. Peptides were synthesized using standard Fmoc chemistry as previously reported (16, 23). For TEM studies, O-Q11 was N-terminally biotinylated on-resin using biotin *o*-nitrophenyl ester. Peptides were purified using a Varian ProStar HPLC system, Grace-Vydac C18 reverse phase columns, and water-acetonitrile gradients. Peptide identity and purity were confirmed by MALDI-MS and HPLC, respectively (Table S1). Endotoxin levels of all immunizations were <0.3 EU/mL by LAL chromogenic endpoint assay (Lonza), well within acceptable limits (Table S1). Endotoxin measurements were conducted on samples containing exactly the same or similar concentrations of peptides as the immunizations (see *SI Text* for detailed methods).

Circular Dichroism. An AVIV 202 CD spectrometer (Aviv Biomedical, NJ), 0.1 cm path length quartz cells, and initial disaggregation in TFA were employed as previously reported (16). Working concentrations of 250 μ M, 500 μ M, and 750 μ M were prepared in degassed water using the absorbance at

257 nm. Owing to peptide fibrillization and the resultant low CD signal strength, spectra below 220 nm could not be measured accurately and so are not reported.

Transmission Electron Microscopy. Peptides were dissolved in deionized water and mixed 6:1 with PBS to produce working peptide concentrations of 330 μ M. After fibrillizing for 4 h, peptides were applied to 400 mesh gold grids with carbon support films, negative-stained with 1% uranyl acetate, and imaged on a Tecnai F30 TEM. For gold staining, prior to negative-staining grids were placed upside-down on a droplet of blocking solution (0.2% acetylated BSA, 0.1% gelatin from cold water fish skin in PBS) for 5 min, then for 2 h on a droplet of 5 nm colloidal gold conjugated to streptavidin (Sigma). Grids were washed once with blocking solution, twice with PBS, and stained with 1% uranyl acetate.

Immunizations. Peptides were dissolved in sterile water (8 mM) and allowed to fibrillize overnight at 4 °C. Stock solutions were then diluted in sterile, endotoxin-free PBS to working concentrations. Female C57BL/6 mice (6–8 weeks old, Taconic Farms, IN) were each given two 50 μ L subcutaneous injections near the shoulder blades, each injection containing 100 nmol of peptide. CFA-adjuvanted groups received the same volume and total peptide dose, prepared by emulsifying peptide/PBS solutions 1:1 in CFA. Mice were boosted at 28 d with two additional 25 μ L injections, each containing 50 nmol of peptide. CFA groups were boosted in incomplete Freund's adjuvant (IFA). Mice receiving mixtures of Q11 and OVA received 100 nmol of each peptide in the first injection and 50 nmol of each in the second. Seven days after the boost, the mice were sacrificed, and sera and spleens were harvested. In all animal work, institutional guidelines for the care and use of laboratory animals were strictly followed under a protocol approved by the University of Chicago's Institutional Animal Care and Use Committee.

Determination of Antibody Titers. High-binding ELISA plates (eBioscience) were coated with either 20 μ g/mL peptide in PBS or PBS alone (for uncoated background subtraction) overnight at 4 °C. Wells were blocked with 1% BSA/0.5% Tween 20 in PBS, and serial dilutions of serum between 1:10² and 1:10⁹ were applied, followed by peroxidase-conjugated goat antimouse IgG (H+L) (Jackson Immuno Research). Washing steps were performed with 0.5% Tween 20 in PBS, plates were developed using TMB substrate (eBioscience), and absorbance values were read at 450 nm (absorbance values shown in Fig. S1). To determine titers for each antiserum, background absorbance values from uncoated wells were subtracted from coated wells, and net absorbances were compared to cutoff values. The cutoff consisted of the mean plus three times the standard deviation of the negative control group (mice receiving OVA without adjuvant) for each corresponding dilution. Any sample dilutions having absorbances above this cutoff value were considered positive readings. The titer was considered as the highest dilution for which it and all lower dilutions had positive readings. If no positive dilutions were present the titer was considered to be 10². Negative control mice (OVA without adjuvant) did not raise detectable IgG, and no single mouse in the negative control groups had absorbance values greater than three standard deviations above the group's mean for a given dilution; therefore all negative control mice are reported as having titers of 10², which is the baseline level of detection for this study. Antibody isotypes were analyzed similarly using a mouse monoclonal antibody kit containing goat antimouse IgG1, IgG2a, IgG2b, IgG3, and IgM (Sigma). See *SI Text* for detailed methods.

Splenocyte Isolation and Challenge. Spleens of the immunized mice were pressed through 70 μ m cell strainers, and isolated splenocytes were washed in RPMI medium containing 10% FBS. Red blood cells were lysed using ACK buffer (150 mM NH₄Cl, 10 mM KHCO₃/0.1 mM EDTA) and washed twice. 1 \times 10⁶ cells/well (96 well plate) were plated in complete T cell medium (S-MEM supplemented with 3.75 mM dextrose, 0.9% L-glutamine, 0.6% essential amino acids, 1.26% nonessential amino acids, 0.9% sodium pyruvate, 9 mM sodium bicarbonate, 95 μ M gentamycin, 140 μ M penicillin-G, 60 μ M streptomycin sulfate, 44 μ M 2-mercaptoethanol, and 10% fetal bovine serum) containing 5 μ g/ml challenging peptide or no peptide. After 24 h, interferon- γ (IFN- γ), IL-2, and IL-4 concentrations were measured in the culture medium using a sandwich ELISA format, capture antibodies for IFN- γ , IL-2, and IL-4, biotin-conjugated detection antibodies, and avidin-horseradish peroxidase (see *SI Text* for detailed methods). Antibodies and reagents were purchased from eBioscience, and cytokine concentrations were calculated from standard curves.

Statistical Analysis. Statistical analysis was performed by ANOVA with Tukey's HSD post hoc comparisons. The data reported in Figs. 3A and 4 represent

three separate experiments, each containing five mice per group. Positive and negative controls were not statistically different between the three trials, and so the data were pooled into the groups of 15 shown.

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