Modulating Adaptive Immune Responses to Peptide Self-Assemblies

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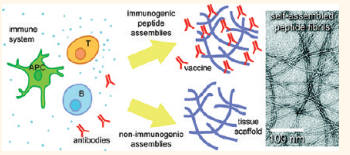
ABSTRACT Self-assembling peptides and peptide derivatives have received significant interest for several biomedical applications, including tissue engineering, wound healing, cell delivery, drug delivery, and vaccines. This class of materials has exhibited significant variability in immunogenicity, with many peptides eliciting no detectable antibody responses but others eliciting very strong responses without any supplemental adjuvants. Presently, strategies for either avoiding strong antibody responses or specifically inducing them are not well-developed, even though they are critical for the use of these materials both within tissue engineering and within immunotherapies. Here, we investigated the molecular determinants and immunological mechanisms leading to the significant immunogenicity of the self-assembling peptide OVA-Q11, which has been shown previously to elicit strong antibody responses in mice. We show that these responses can last for at least a year. Using adoptive transfer experiments and T cell knockout models, we found that these strong antibody responses were T cell-dependent, suggesting a route for avoiding or ensuring immunogenicity. Indeed, by deleting amino acid regions in the peptide recognized by T cells, immunogenicity could be significantly diminished. Immunogenicity could also be attenuated by mutating key residues in the self-assembling domain, thus preventing fibrillization. A second self-assembling peptide, KFE8, was also nonimmunogenic, but nanofibers of OVA-KFE8 elicited strong antibody responses similar to OVA-Q11, indicating that the adjuvant action was not dependent on the specific self-assembling peptide sequence. These findings will facilitate the design of self-assembled peptide biomaterials, both for applications where immunogenicity is undesirable and where it is advantageous.



KEYWORDS: self-assembly · vaccine · biomaterial · tissue engineering · scaffold

Self-assembly of short peptides has become a popular synthetic route for soft materials intended for biomedical applications such as cell delivery,[1−4] drug delivery,[5−8] regenerative medicine,[2,9−13] and vaccines.[14−17] Progress has been made recently toward these applications, including demonstrations of regeneration in infarcted myocardium,[2] evidence of repair in spinal cord injuries,[10] and the elicitation of strong adjuvant-free antibody responses for vaccines and immunotherapies.[15] However, as the development of this class of materials continues, it is becoming clear that they can exhibit a wide range of immunogenicity. For example, peptides that form β-sheet fibrils and hydrogels have generally not raised detectable antibody responses in animal models,[2,9,18,19] but we have previously observed fibrillized peptides eliciting strong, specific antibody responses without the presence of any supplemental immune adjuvants.[15] This wide range of antibody responses, from undetectable to significant, makes this class of materials potentially useful both for tissue engineering and for vaccine development, but only if the molecular determinants of their immunogenicity can be effectively understood and controlled.

One advantage of self-assembled peptide materials is their significant degree of multivalency,[20−23] which is known to contribute to immunogenicity for fibrillized peptides,[15,24] other peptide materials,[16,17,25−27] and polymers.[28] Multivalency can enable the repetitive display of ligands or epitopes for improved affinity and avidity with specific receptors,[22,23,29−31] but it can also lead to enhanced antibody responses. The most basic distinction between the possible mechanisms for eliciting antibody responses is whether they are dependent on the involvement of T cells or not.[31,32] Most proteins elicit T-dependent responses, where antigens are processed and presented in MHC class-II molecules of antigen-presenting cells (APCs) such as dendritic cells. This complex is then recognized by T helper cells, which become activated and differentiate. These cells then provide help to B cells that have encountered the same antigen, causing them to mature into antibody-producing plasma cells. T-dependent antigens tend to elicit recall antibody responses that are class-switched and of high affinity upon secondary immunization, both of which are generally desirable in vaccines.[33,34] Alternatively, in T-independent responses, B cells can be

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stimulated to produce antibodies in the absence of MHC class-II-restricted T cell help. T-independent antigens tend to have repeating subunits in multivalent arrays that are capable of cross-linking B cell antigen receptors, an event that then stimulates the B cell to produce antibodies directly.[31,32,35] Given the proteinaceous yet highly repetitive nature of fibrillar peptide assemblies, it has been conceivable that both mechanisms could be operative for them, but in order to develop strategies for controlling immune responses against them, it is necessary to understand which process is dominant. For example, intervening in T-independent responses might entail the development of strategies to modulate the interactions between fibrils and B cells, whereas modulating a T-dependent response might entail strategies aimed at dendritic cells, T cells, or antigen processing and presentation.

We have previously developed the self-assembling peptide Q11 (QQKFQFQFEQQ), which is amenable to functionalization with a range of small chemical moieties or short peptides. Upon fibrillization of the Q11 domain, these functional components are displayed on the surface of the nanofibers.[15,18,20,36] We have investigated Q11 as an oligomerizing domain for coassembling multiple functional components, including chemical groups for cross-linking[36] and cell-binding ligands.[18,20] We have found Q11 to be nonimmunogenic by itself or even when administered in complete Freund's adjuvant (CFA),[15] and Q11 peptides appended with RGD cell-binding ligands have also failed to raise strong antibody responses.[18] However, Q11 coupled to a model antigen peptide from ovalbumin (residues 323−339 from chicken ovalbumin bearing known T and B cell epitopes) raised high titers of anti-OVA antibodies without the need for supplemental adjuvant.[15] This response depended entirely on the chemical conjugation of the OVA epitope to the self-assembling Q11 domain, as the antibody response was completely abrogated when the peptide linkage between the OVA epitope and the Q11 assembling domain was broken.[15] In this previous work, it has been unclear whether the antibody responses have been dependent on T cells or not. While we did not observe cytokines indicative of a T-dependent response such as IL-2, IL-4, or interferon-γ in peptide-stimulated splenocyte cultures, we did observe broad classes of antibody types in the sera of the immunized mice, usually a hallmark of T-dependent responses.[37]

The goal of the present work was to provide insight into factors that can be used to control the immunogenicity of β-sheet fibrillar materials. We conducted experiments aimed at definitively ascertaining the T cell dependence of antibody responses raised against OVA-Q11, at the same time investigating the durability of the antibody responses, as previous work was limited only to short-term experiments on the order of five weeks.[15] We also investigated mechanisms for ablating their immunogenicity, by mutating both the epitope domain as well as the peptides' ability to self-assemble. Finally, we investigated a second fibrillizing peptide, KFE8 (FKFEFKFE),[38] to determine whether the responses observed for OVA-Q11 may also apply to other fibrillizing peptides.

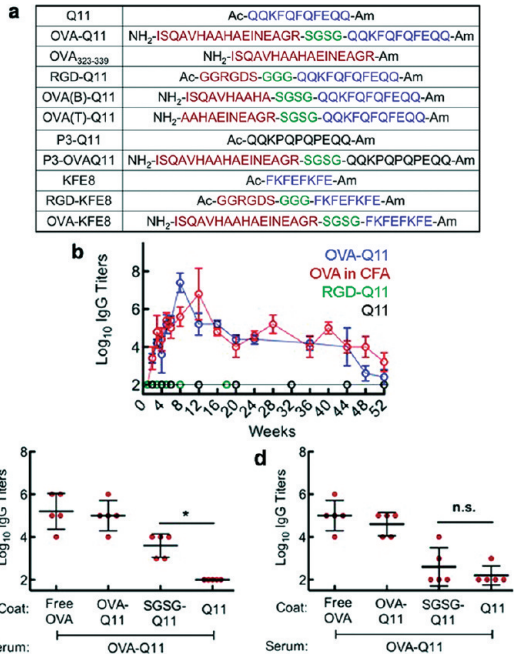

Figure 1. Antibody responses to self-assembled peptide antigens were epitope-dependent, long-lived, and primarily directed against the N-terminal epitope domain. List of peptides investigated in this study (a, fibrillization domain shown in blue, spacer in green, and epitope in red). Durable antibody responses lasting at least one year were raised against OVA-Q11 and OVA peptide in CFA, but not RGD-Q11 or Q11 (b, a single boost containing 1/2 the primary immunization dose was given after 4 weeks for all groups). Using ELISA, antisera raised against OVA-Q11 were probed against various peptide fragments (c,d, sera collected 5 weeks (c) or 24 weeks (d) after primary immunizations). No significant differences in titers were observed between antibodies reactive to the free OVA antigen and the fibrillized OVA-Q11 at either time point. Some antibodies were raised against the SGSG-Q11 peptide at week 5 (c), but these diminished by week 24 (d); *$p < 0.05$ by ANOVA using Tukey posthoc test.

RESULTS

Antibody Responses to Self-Assembled Peptides Were Long-Lived and Epitope-Dependent. Previous work indicated that OVA-Q11 raised strong antibody responses,[15] whereas RGD-Q11 did not,[18] but it was not determined whether these responses were transient or maintained over long periods of time. We therefore investigated antibody responses over one year in mice immunized with OVA-Q11, OVA peptide in CFA, RGD-Q11, and Q11 (sequences in Figure 1a). Immunization with OVA-Q11 produced a surprisingly durable antibody response that was detectable for at least 52 weeks, similar in magnitude and longevity to the free OVA peptide

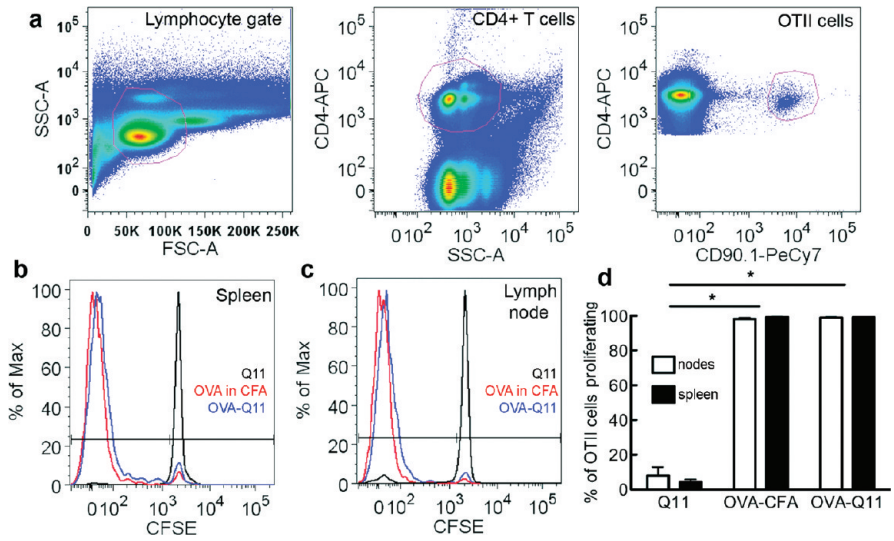

Figure 2. OT-II CD4+ T cells proliferated in response to fibrillized OVA-Q11. The gating process is shown for distinguishing adoptively transferred CFSE-labeled OT-II cells from endogenous CD4+ T cells (a). Proliferation of adoptively transferred OT-II cells in the spleens (b) and lymph nodes (c) of mice immunized with Q11, OVA-CFA, or OVA-Q11. Percentage of proliferating cells in the spleen and lymph nodes (d); $p < 0.01$ by ANOVA using Tukey's posthoc test.

administered in CFA (Figure 1b). The responses increased after the boost at week 4, reached maximum levels at week 6, and declined slowly thereafter but remained significant until the study was terminated at 1 year. Unlike OVA-Q11, neither RGD-Q11 nor Q11 raised any detectable antibody responses, even in the long term (Figure 1b). Moreover, mixed immunizations containing coassembled OVA-Q11 and RGD-Q11 elicited responses only against the OVA-Q11 component (Supporting Information, Figure S1). Both OVA-Q11's durable response and RGD-Q11's long-term lack of response are important for vaccine development and regenerative medicine, respectively. In the former, antibody responses raised against peptide assemblies can be long-lasting, and in the latter, nonimmunogenic peptide assemblies can remain nonimmunogenic, even when combined with other immunogenic Q11 variants, a feature that is encouraging for the development of materials that can avoid sensitization.

In order to gain insight into potential locations where mutations could be made to modulate the response, we next determine which stretches of amino acids in OVA-Q11 the observed antibodies were raised against. We conducted ELISAs probing antisera from mice immunized with OVA-Q11 against plates coated with different fragments of OVA-Q11, including OVA peptide (antigen), OVA-Q11 (antigen-linker-Q11), SGSG-Q11 (linker-Q11), or the Q11 domain by itself. At five weeks post first immunization, antibodies raised against OVA-Q11 bound to the free OVA peptide, full-length OVA-Q11, and the linker-Q11 sequence (Figure 1c). Over time, however, antibodies against the linker-Q11 fragment (SGSG-Q11) diminished, with only a small response observed by week 24 (Figure 1d). At these later time points, the antibody response appeared to be directed primarily toward the OVA epitope portion of the peptide, suggesting that antibodies became increasingly antigen-specific over time. No significant differences were observed between antibody titers measured on plates coated with the free OVA peptide or OVA-Q11 at either time point.

Antibody Responses Against OVA-Q11 Were T Cell-Dependent. Given the inconclusiveness of our previous findings regarding T cell involvement[15] and the importance for material design to definitively assign T-independent or T-dependent status to fibrillized peptide materials, we undertook to resolve this issue. Using adoptive transfer assays and genetically modified mice, we found that the response to OVA-Q11 was indeed T cell-dependent. In the adoptive transfer experiments, mice were injected with CFSE-labeled OT-II CD4+ T cells, which specifically recognize the $OVA_{323-339}$ epitope.[39] These mice were then immunized with peptides, and proliferation of the OT-II CD4+ T cells was measured *via* the dilution of the CFSE label using flow cytometry. Robust proliferation of OT-II CD4+ T cells was observed in the lymph nodes and spleens of mice immunized with both OVA-Q11 and OVA delivered in CFA, as indicated by the dilution of the CFSE label (Figure 2). Quantification of the shift in the CFSE intensity indicated that more than 95% of the transferred cells in both OVA-Q11 and OVA-CFA immunized mice had proliferated. In contrast, only a small shift in CFSE intensity was observed for Q11-immunized mice, comparable to naïve mice receiving similar numbers of OT-II cells but no immunizations (Figure S2). Taken together, these data clearly indicated that antigen-bearing fibrils were capable of stimulating the proliferation of OT-II CD4+ T cells, whereas the Q11 domain of the peptides did not by itself stimulate these T cells.

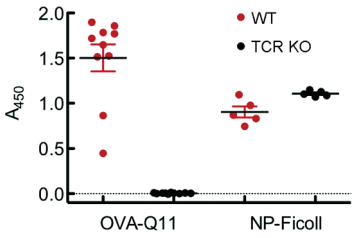

Figure 3. Antibody responses to OVA-Q11 were T cell-dependent. T cell receptor knockout mice (TCR KO, *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$) did not produce antibodies detectable by ELISA against OVA-Q11, but they did respond to a known T-independent antigen, nitrophenyl-Ficoll (NP-Ficoll). WT mice responded to both OVA-Q11 and NP-Ficoll.

We next determined whether T cell help was in fact required for an antibody response against OVA-Q11. Knockout mice lacking functional $\alpha\beta$ and $\gamma\delta$ T cell receptors (*Tcrb*$^{-/-}$*Tcrd*$^{-/-}$) were immunized with OVA-Q11 and compared with wild-type controls. Strikingly, antibody responses against OVA-Q11 were completely abolished in mice lacking functional T cell receptors (Figure 3). In contrast, both knockout and wild-type mice responded to nitrophenyl-Ficoll, a well-known T-independent antigen. Taken together, the results of the OT-II adoptive transfer experiments and the lack of an antipeptide response against OVA-Q11 in T cell receptor knockout mice confirmed that CD4+ T cells are indispensable for eliciting antibody responses against OVA-Q11.

Having determined that antibody responses to assemblies of OVA-Q11 were strongly T cell-dependent, we sought to attenuate them by mutating epitope regions in the $OVA_{323-339}$ antigen portion of the peptide. We constructed two peptides having deletions in either the putative B cell epitope or T cell epitope of $OVA_{323-339}$, based on a previous report that mapped the epitope locations within this peptide for C57BL/6 mice.[39] The peptide OVA(B)-Q11 contained only the putative B cell epitope, whereas OVA(T)-Q11 contained only the putative T cell epitope (sequences in Figure 1a). By TEM, it was observed that both OVA(B)-Q11 and OVA(T)-Q11 self-assembled into fibers in PBS, as did 1:1 mixtures of the two (Figure 4a–c). In mice, neither peptide raised strong antibody responses (Figure 4d) nor did mixtures of the two peptides. Thus it is clear that a contiguous $OVA_{323-339}$ antigen is required for the immunogenicity exhibited by full-length OVA-Q11. However, it cannot be ruled out that the deletions affected the activity of the remaining epitopes, which could explain why the response was not reconstituted when the peptides were mixed.

Collectively, these results offer some guidance for the design of clinically useful self-assembling peptide biomaterials. Given the dependence of a strong antibody response on both T cell involvement and the presence of a T cell epitope, one could seek to ensure that no functional T cell epitopes are present in a

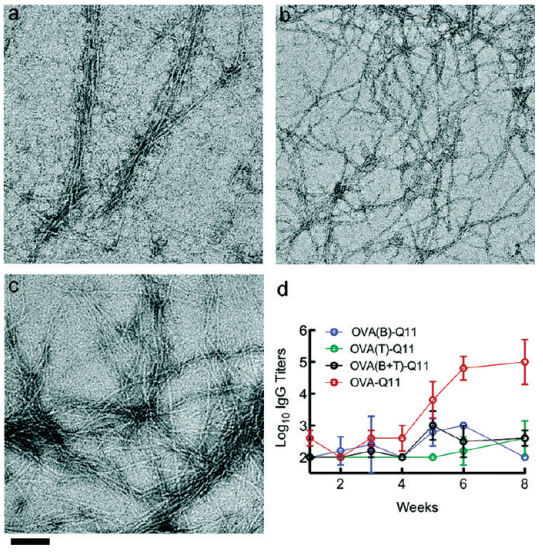

Figure 4. Nanofibers were formed by OVA(T)-Q11, which lacked the putative B cell epitope of $OVA_{323-339}$, (a); by OVA(B)-Q11, which lacked the putative T cell epitope (b), and by cofibrillized 1:1 mixtures of the two peptides (c). Scale bar is 100 nm. Mice immunized with OVA(B)-Q11, OVA(T)-Q11, or cofibrillized mixtures (OVA(B+T)-Q11) did not raise strong antibody responses comparable to OVA-Q11 (d).

self-assembled peptide biomaterial intended for clinical applications such as regenerative medicine, where an adverse immunogenicity would be undesirable. Conversely, in applications such as vaccination, where a strong immune response is desirable, the opposite strategy of ensuring the inclusion of a properly restricted T cell epitope would appear to be a good one. Our results indicate that these strategies are effective at rendering Q11-based self-assemblies either strongly immunogenic or minimally so.

Antibody Responses to Self-Assembling Peptides Were Dependent on Fibrillization. The multimerization of antigens has been a longstanding strategy for enhancing their immunogenicity.[31,32,35] Previously, this phenomenon has been exploited for generating antibodies against weak immunogens like polysaccharides,[27] antigenic peptides,[14–16] or even self-epitopes.[25,26] It has been our speculation that the noncovalent self-assembly of Q11-based materials has been a key factor underlying their observed immunogenicity,[15] but to conclusively determine this, we designed and investigated the immunogenicity of nonfibrillizing variants of OVA-Q11. The phenylalanine residues in Q11 are thought to play a key role in the peptide's self-assembly through hydrophobic interactions and $\pi-\pi$ stacking, so we mutated Q11's phenylalanines to the β-breaking residue proline. Because a single Phe→Pro mutation in Q11's central phenylalanine position did not fully prevent self-assembly, we mutated all three of Q11's phenylalanines to proline. The resultant peptide, P3-Q11, did not form fibrillar structures by TEM nor did the antigen-bearing version of it, P3-OVAQ11

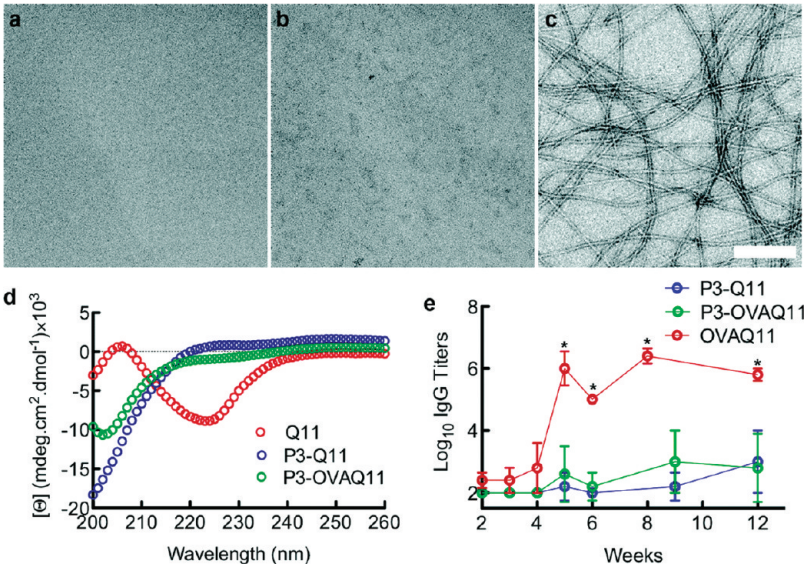

Figure 5. Peptides P3-Q11 (a) and P3-OVAQ11 (b) did not form fibrillar structures by TEM. OVA-Q11 is shown for comparison (c), scale bar is 100 nm for (a−c). P3-Q11 and P3-OVAQ11 adopted a random coil secondary structure, whereas Q11 adopted a β-rich secondary structure by CD (d, 100 μM peptide in 10 mM phosphate buffer/140 mM KF/pH 7.4). Immunizing mice with P3-Q11 or P3-OVAQ11 led to significantly diminished antibody responses compared to OVA-Q11 (e, *$p < 0.01$ by ANOVA with Tukey posthoc; all mice received a booster immunization after four weeks containing 1/2 the dose of the primary immunization).

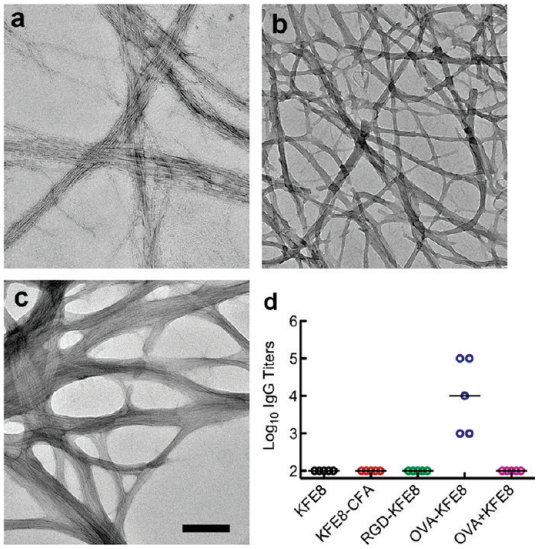

Figure 6. Peptides KFE8 (a), RGD-KFE8 (b), and OVA-KFE8 (c) all formed fibrillar assemblies by TEM (scale bar 100 nm for all images). In mice, only OVA-KFE8 was immunogenic, consistent with the behavior of Q11 previously (d). KFE8 was not immunogenic by itself, in CFA, as RGD-KFE8, or when mixed with OVA$_{323-339}$.

(Figure 5a−c). In addition, CD indicated a loss of β-sheet secondary structure for both P3-Q11 and P3-OVAQ11, which instead assumed random coil structures (Figure 5d). Antibody responses in mice against P3-Q11 or P3-OVAQ11 were significantly diminished compared to those raised by OVA-Q11 (Figure 5e), indicating that the loss of fibrillization led to the loss of immunogenicity. While it is possible that the restriction and MHC presentation of the epitopes in P3-OVAQ11 could have changed owing to the three Phe→Pro mutations, it is not likely that this was causative of the diminished immune response owing to the negligible immunogenicity of nonmutated Q11 to begin with. This issue was further addressed with another self-assembling peptide, KFE8, discussed below.

Another Self-Assembling Peptide, KFE8, Exhibits Adjuvanting Properties Similar to Q11. To date, investigations of the adjuvanting properties of short fibrillizing peptides have focused on Q11, so it has not been clear whether this phenomenon is a general feature of peptide self-assemblies or if it is dependent on the specific amino acid sequence of Q11. To determine whether other self-assembling peptides also strongly adjuvant particular epitopes, we investigated the self-assembling peptide domain KFE8 (Ac-FKFEFKFE-Am).[38,40] We synthesized three peptides, KFE8, RGD-KFE8, and OVA-KFE8 (sequences in Figure 1a), determined their capacity to self-assemble, and measured their immunogenicity in mice. All three KFE8-containing peptides self-assembled into fibrillar structures, similar to Q11 and its derivatives (Figure 6a−c). In mice, the immunogenic properties of KFE8 were strikingly similar to those of Q11 (Figure 6d). In particular, KFE8 and RGD-KFE8 by themselves were not immunogenic, nor was KFE8 immunogenic when it was delivered in CFA. However, OVA-KFE8 raised strong antibody responses in the same manner as OVA-Q11. Also like Q11, this response was abolished when the covalent linkage between the OVA epitope and the KFE8 assembly domain was broken, that is, when OVA$_{323-339}$ and KFE8 were mixed and delivered together. These findings are precisely consistent with the behavior of Q11, confirming that immune responses to both self-assembling peptides

are dictated by the nature of the conjugated epitope and the ability to form fibrils, but not on the primary sequence of the self-assembling domain. In this way, the present and previous observations with Q11 are likely to be consistent for the variety of other fibrillizing peptide materials currently under investigation.

DISCUSSION

The work presented here reinforces the concept that the immunogenicity of self-assembling fibrillar peptides can span a wide range, from being essentially nonexistent to being strong and protracted. Both nonimmunogenic peptides (such as Q11, KFE8, and the RGD-containing versions of them) and highly immunogenic peptides (the OVA-containing peptides) were observed here. When strong antibody responses were raised, they were entirely T cell-dependent, and disrupting the T cell epitope of OVA-Q11 was an effective means for diminishing this immunogenicity. The finding of T cell dependence is helpful for the clinical translation of these materials because it allows one to rule out T-independent mechanisms. That is, if a T cell epitope can be avoided, the materials should be minimally immunogenic. In an inbred mouse strain such as C57BL/6, this is relatively simple, but it can be much more challenging for an outbred human population. For clinical use, epitope prediction algorithms under current development may aid in peptide selection or design.[41] Such a strategy would represent a passive diminishment of peptide immunogenicity or the avoidance of structures and sequences that could provoke a strong antibody response. On the other hand, more active approaches may also be possible, for example, co-delivering protolerance cytokines or other immunosuppressive cues, employing co-stimulation blockade agents, or rapidly clearing the peptide from the tissue site. At the same time, it is not clear yet what consequence a strong anti-biomaterial antibody response may have on the clinical performance of self-assembled peptide biomaterials. Indeed, biologically sourced materials in current clinical use such as porcine small intestinal submucosa (SIS) elicit a significant antibody response, but do so without clinical rejection of the xenogeneic material.[42,43] Some degree of immunogenicity may likewise be acceptable in many biomaterials applications for self-assembling peptides.

On the other end of the application spectrum, the use of these materials as immune adjuvants is still an emerging concept.[15] Although we show here that antibody responses are T cell-dependent, the full mechanism of adjuvanticity for self-assembling peptides remains to be clarified. For example, in the present study, we did not specifically investigate CD8+ T cell involvement owing to our focus on antibody responses. However, CD8+ T cell responses to these materials could be possible and would be of interest in a variety of applications including vaccines for cancer. It is likely that proper engagement of innate immune cells such as dendritic cells is also key to the long-lived antibody responses observed for OVA-Q11, but these mechanisms have yet to be elucidated. Owing to the particulate nature of self-assembled peptides, their mechanism of adjuvanticity may be similar to other particulate adjuvants such as alum, whose mechanism of action is only now beginning to be understood after over 80 years of use. Alum's adjuvanticity has been explained in part as the combination of many parallel processes, including the "depot effect", allowing slow release of antigens, an improvement of antigen uptake by antigen-presenting cells, inflammation *via* NLRP3 signaling, and the induction of Th2-type immune responses *via* NLRP3 independent signaling.[44] Also, although $OVA_{323-339}$ was used as a model antigen in the present work in order to employ tools such as OT-II cells to clarify T cell dependence, OVA is well-known to be a strong antigen. It will be interesting in the future to determine if specific disease-related peptide epitopes can likewise elicit strong antibody responses, and it will also be interesting to determine if protein antigens attached to the fibrils can be adjuvanted.

CONCLUSION

In the present work, we have begun to develop strategies that can be used to modulate the ability of a self-assembling peptide to either raise a strong antibody response or avoid such a response. By themselves or when conjugated to non-antigenic peptides such as RGD, self-assembling peptides have not elicited significant antibody responses. In contrast, antibody responses raised against assemblies containing the $OVA_{323-339}$ antigen were strong, durable, and primarily focused on the antigenic portion of the peptides. These responses were completely dependent on T cell help and could be significantly diminished by interfering with either the T cell epitope portion of the peptides or their ability to fibrillize. Owing to the strong T cell dependence of these responses, a key strategy for modulating their immunogenicity appears to center on the inclusion or exclusion of an effective CD4+ T cell epitope. These findings are consistent between both Q11 and KFE8, an unrelated self-assembling peptide, and so they appear to be broadly applicable to fibrillar peptide assemblies in general.

METHODS

Peptide Synthesis and Purification. Peptides Q11 (Ac-QQKFQ-FQFEQQ-Am), RGD-Q11 (Ac-GGRGDSGGG-Q11), $OVA_{323-339}$ (ISQAVHAAHAEINEAGR), OVA-Q11 ($OVA_{323-339}$-SGSG-Q11-Am), OVA(B)-Q11 (ISQAVHAAHA-SGSG-Q11-Am), OVA(T)-Q11 (AAH-AEINEAGR-SGSG-Q11-Am), P3Q11 (Ac-QQKPQPQPEQQ-Am), P3-OVAQ11 ($OVA_{323-339}$-SGSG-P3Q11-Am), KFE8 (Ac-FKFEFKFE-Am), RGD-KFE8 (Ac-GGRGDSGGG-KFE8-Am), and OVA-KFE8

($OVA_{323-339}$-SGSG-KFE8-Am) were synthesized using standard Fmoc chemistry on a CSBio136-XT peptide synthesizer and cleaved using standard 95% TFA/2.5% water/2.5% TIS cocktails. Peptides were precipitated in diethyl ether, washed, and lyophilized. Peptides were purified using a Varian ProStar HPLC system, a Grace-Vydac C18 reverse phase column, and water/acetonitrile gradients. All peptides eluted between 25 and 35% acetonitrile, which was removed by centrifugal evaporation. Peptides were lyophilized and stored at −20 °C until further use. Peptide identity and purity (>90% for all peptides used in the study) were confirmed by MALDI-MS and HPLC, respectively. Endotoxin levels of all formulations were within acceptable limits, as measured by the Limulus Amebocyte Lystae (LAL) chromogenic end point assay (Lonza, USA) at the same volume and peptide concentration used for immunizations. The purity and endotoxin levels for all peptides used in the study are provided in the Supporting Information (Table S1).

Transmission Electron Microscopy. Stock solutions of 8 mM peptides were prepared in DI water and incubated overnight at 4 °C. The stocks were diluted to 2 mM in PBS and further incubated for 4 h at room temperature. Peptide solutions (final concentration 0.25 mM) were adsorbed onto carbon-coated 200 mesh lacey grids (Electron Microscopy Sciences). For the coassembled fibrils, OVA(B)-Q11 and OVA(T)-Q11 peptides were mixed as dry powders, dissolved in deionized water, and diluted in PBS. The grids were stained with 1% uranyl acetate for 2 min, washed, dried, and imaged with a FEI Tecnai F30 transmission electron microscope.

Circular Dichroism Spectroscopy. An AVIV 215 circular dichroism spectropolarimeter was used with 0.1 cm path length quartz cells. Stock solutions were prepared by dissolving the peptides in ultrapure water (Millipore Milli-Q system) and diluted to a working concentration of 100 μM in 10 mM phosphate buffer containing 140 mM potassium fluoride. The pH was adjusted to 7.4. The wavelength range was 190−260 nm, the scanning speed was 0.5 nm/s, and the bandwidth was 0.5 nm. Each spectrum shown is the average of three scans. Under the solution conditions described, adequate signal strength was observed at wavelengths down to 200 nm. Solvent background signals were subtracted.

Animals and Immunizations. Immunizations were performed for C57BL/6 mice or T cell receptor knockout mice (B6.129P2-$Tcrb^{tm1Mom}Tcrd^{tm1Mom}$/J, on a C57BL/6 background, referred to as $Tcrb^{-/-}Tcrd^{-/-}$), purchased from The Jackson Laboratory (Bar Harbor, ME). 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. Peptides were dissolved in sterile water (8 mM stock), incubated overnight at 4 °C, and diluted in sterile PBS (2 mM working concentration) prior to immunizations. To prepare coassembled fibers of more than one peptide, the peptides were combined as dry powders, mixed thoroughly, and dissolved in sterile water. The peptides were allowed to coassemble overnight at 4 °C and fibrillize in sterile PBS. Female mice (6−8 weeks old) were immunized subcutaneously with two 50 μL injections near the shoulder blades, each containing 100 nmol of total peptide. Mice were boosted at 28 days with two additional 25 μL injections, each containing 50 nmol of peptide. Blood was drawn *via* the submandibular maxillary vein, and serum was stored at −80 °C until use.

Determination of Antibody Titers. ELISA plates (eBioscience) were coated with either 20 μg/mL of peptide in PBS (sample wells) or only PBS (control wells) overnight at 4 °C. The plates were blocked with 200 μL of 1% BSA in PBST (0.5% Tween-20 in PBS) for 1 h. Serum was serially diluted in PBS between $1:10^2$ and $1:10^9$ and applied to wells for 1 h at room temperature. Peroxidase-conjugated goat anti-mouse IgG (H+L) (Jackson Immuno Research) (1:5000 in 1% BSA-PBST, 100 μL/well) was then applied for 30 min, and the plates were developed using TMB substrate (100 μL/well, eBioscience). The reaction was stopped using 50 μL of 1 M phosphoric acid, 100 μL of the solution was transferred to fresh plates, and absorbance values were read at 450 nm. The plates were washed between each step with PBST. To determine antibody titers, the mean and standard deviation of the control wells (no coating antigen) was calculated for all serum dilutions. Sample wells whose signal was greater than the mean plus three standard deviations over the control wells were considered as positive titers.

Adoptive Transfer and T Cell Proliferation Assays. OT-II transgenic mice on C57BL/6 background, whose T cells recognize the $OVA_{323-339}$ peptide, carry a marker to distinguish them from naïve T cells from C57BL/6 mice (CD90.1 congenic). *In vivo* CD4+ T cell proliferation was analyzed after adoptive transfer into mice and immunization with the peptides. Briefly, splenocytes and lymph nodes of OT-II+ C57BL/6 mice were processed into single-cell suspensions and enriched for CD4+ T cells by negative magnetic separation (Miltenyi Biotec). The cells were then labeled with 10 μM carboxyfluorescein succinimidyl ester (CFSE) and adoptively transferred into mice (5×10^5 OT-II cells/mouse, retro-orbital injection) 24 h before peptide immunizations. The next day, mice were immunized with 100 nmol of OVA-Q11 in PBS, OVA in CFA, or Q11 in PBS at two different sites subcutaneously. Five days after immunizations, spleens and lymph nodes were harvested, processed into single-cell suspensions, and stained for flow cytometry in 1% BSA in PBS containing APC-labeled anti-CD4 (RM 4−5, BD Biosciences, NJ), PECy7-labeled anti-CD90.1 (HIS51, eBioscience, CA), and 0.02% sodium azide, for 1 h at 4 °C. Anti-CD16/32 (2.4G2.1, University of Chicago Immunology Core) was used to prevent nonspecific antibody binding. After staining, cells were washed twice with FACS buffer and samples were run on a LSR-II flow cytometer (BD Biosciences, NJ) and analyzed for evidence of proliferation as indicated by dilution of CFSE intensity, using FlowJo cytometry analysis software (Tree Star, OR).

Statistical Analysis. Statistical analysis was performed with a Student's *t*-test (two-tailed) or one way ANOVA with Tukey posthoc test, as indicated. Statistical significance was assigned at p values <0.05 (95% confidence interval).

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Supporting Information Available: A table of peptide purity, endotoxin levels, and MALDI mass spectrometry measurements, plus control experiments for Figure 1b and Figure 2. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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