THE UNIVERSITY OF CHICAGO

MODULAR DESIGN FEATURES OF A PEPTIDE AMPHIPHILE MICELLE VACCINE PLATFORM AND THEIR IMPACT ON AN IMMUNE RESPONSE

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This dissertation is dedicated to my wife who encouraged me to pursue my dreams and finish my dissertation.

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Abstract

Inducing a strong and specific immune response is the hallmark of a successful vaccine. Nanoparticles have emerged as promising vaccine delivery devices to discover and elicit immune responses. Modular platforms are attractive for their engineerability and broad potential applications. Fine-tuning a nanoparticle vaccine to create an immune response with specific antibody and other cellular responses is influenced by many factors such as shape, size and composition. Peptide amphiphile micelles are a unique biomaterials platform that can function as a modular vaccine delivery system, enabling control over many of these important factors. Peptide amphiphiles (PAs) consist of a hydrophilic peptide antigen conjugated to a hydrophobic lipid tail. The PAs then self-assemble into micelles, with the micelle characteristics determined by the chemical composition of the PA and micelle preparation methods. PA micelles contain a large design space, so it is important to have a basic understanding of how each design feature can affect the platform's interaction with the immune system.

In this dissertation, the structure, composition, and biodistribution properties of PA micelles are evaluated for their ability to impact an immune response against a Group A Streptococcus B cell antigen (J8). Through structural design and physical characterization, micelles are shown to self-assemble into either short rod-like or long cylindrical shapes. Analyzing these shape effects on the immune response showed that cylindrical micelles induced higher antibody titers than rod-like micelles, providing evidence that the cylindrical micelle shape is important to induce immune responses and a possible mechanism of action. Shape was also seen to impact the activation profile of dendritic cells, B cells and T cells. Assembly into cylindrical micelles also stabilizes the secondary structure of peptide antigens, which may impact

the immune response raised. In composition, the hydrophobic/hydrophilic interface of PA micelles enabled the precise entrapment of amphiphilic adjuvants which were found to not alter micelle formation or shape. These heterogeneous micelles significantly enhanced murine antibody responses when compared to animals vaccinated with non-adjuvanted micelles or soluble J8 peptide supplemented with a classical adjuvant. PAs were also shown to traffic more efficiently to the lymph node than free peptide. Characterization of these design features and their impact on an immune response provides a valuable foundation of knowledge to apply when expanding the peptide amphiphile micelle platform to other vaccine applications.

1 Peptides in immunoengineering

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1.1 Introduction

1.1.1 Progress and challenges in engineering immunity

The immune system plays a critical role in our health. When functioning correctly, the immune system continually protects us from the most common to the most deadly and mysterious maladies: common influenza to HIV and cancer. When functioning incorrectly, autoimmune diseases such as diabetes and multiple sclerosis ensue. Understanding how the immune system functions and learning how to rationally control it to protect against or treat disease has been a challenge historically burdened by the immunology and medical fields. Recently, exciting strides in immunological discoveries and investigational tools have allowed outsiders to enter the fold, chief among them being the biomaterials field.[1] With biomaterials as an engineering tool, rational manipulation of the immune system at the cellular and molecular level has expanded, shedding new light on the functioning of the immune system while also showing promising preclinical and clinical data for more effective and safer treatments.[2] As a flourishing subset of the biomaterials field, peptides have been used as a major design component of the immune engineering strategy. Peptides, because of their chemically defined nature, engineerability, and range of complexity, provide useful functionalities when designing strategies for vaccination, cancer immunotherapy and the treatment of autoimmune disorders. This chapter will examine the peptide-based strategies for immune modulation in detail and discuss the promise and outstanding challenges facing the field of immune engineering. A

summary of all the peptides, their sequences, and their applications mentioned in this chapter will appear at the end of this chapter (Table 1.3).

1.1.1.1 Key cellular actors in the immune system

A brief review of the immune system as a whole and the activities of central immune cells during an immune response is important to discuss before delving into the role peptides play in the immunoengineering field today. The immune system is a collection of specialized cells that collaborate to protect against invading pathogens.[3] The cells belong to one of two arms of the immune system, the innate immune system or adaptive immune system. Innate immune cells such as neutrophils and macrophages are the rapid response arm of the immune system. These cells rapidly respond to pathogen invasions through the receptors they express that recognize conserved molecular motifs (i.e., flagella) characteristic of bacteria, viruses, and fungi, to quickly phagocytose (internalize) pathogens and secrete reactive oxygen species or cytokines (soluble cell signaling molecules that aid in cell-cell communication in immune responses).

While the innate immune system is the first, immediate response to a pathogen, the adaptive immune system is a secondary response which follows the processing and recognition of an antigen. The adaptive immune system is comprised of T cells and B cells. More specifically, there are two types of T cells. First, $CD4^+$ helper T cells are cells that secrete cytokines to direct the function of other immune cells, including innate cells, natural killer cells and B cells. The second type of T cell is the $CD8^+$ killer T cell that recognizes and destroys infected or transformed cells. B cells are responsible for producing antibodies that bind to and neutralize the ability of pathogens to invade host cells and/or promote their phagocytosis. The adaptive immune response is a very specific response because of the clonal development of the immune system where each T cell and B cell expresses a unique T cell receptor (TCR) or B cell

receptor (BCR), respectively. These receptors are formed in part by a process of DNA recombination, creating a wide variety of receptors that can recognize any microbial antigen (an antigen is essentially any biological molecule from a pathogen that is recognized by a TCR or BCR).[4] When a T cell or B cell binds an antigen, this specific cell undergoes massive proliferation, generating a pool of effector cells within ~7 days following exposure, all specific to that antigen. During these 7 days, the innate immune system is attempting to clear the pathogen until the adaptive immune system steps up to finish clearing out the invasion. Following pathogen clearance, a majority of these effector B and T cells (~90%) enter a phase of programed cell death, leaving behind a small group of long lived memory cells that provide fast memory protection if the same antigen or pathogen ever invades again.[5]

A final important group of immune cells are the antigen-presenting cells (APCs), which connect the innate arm to the adaptive arm of the immune system. APCs, including the most famous dendritic cell (DC), are responsible for activating naïve T cells, and in some cases B cells.[6, 7] DCs act like innate immune cells because they reside in all peripheral tissues, constantly collecting antigens from the surrounding fluid. Similar to innate cells, DCs keep an eye out for and respond to pathogen invasions or tissue damage through the receptors they express (most studied are Toll-like receptors) that recognize conserved molecular motifs.[8] Unlike innate cells that remain at the site and fight the initial stages of the infection, once activated by a danger signal, DCs migrate from their peripheral tissue through they lymphatic vessels to local draining lymph nodes, where they physically present antigen to T cells and B cells. T cell activation is achieved through the loading of short (8 – 15 amino acids) peptide fragments of antigens into the cleft of major histocompatibility complex (MHC) molecules displayed on the DC surface. DC's and other antigen presenting cells have MHC class II, but all

other nucleated cells in the body can also present peptides with MHC class I. These peptides are surveyed by the TCRs of T cells, and on finding a cognate peptide, T cells become activated by the DC to proliferate and carry out their adaptive immune response. In this way, DCs are the connection between the innate arm and the adaptive arm of the immune system.

The complex activities of individual immune cells and their interactions that make up the immune system summarized above (greatly oversimplified) is viewed as a network of potential for the immunoengineering field. In this chapter, we aim to summarize the numerous ways in which bioengineers, chemical engineers, material scientists, chemists, and physicists (often in collaboration with immunologists) use peptides as tools to probe or manipulate immune responses for therapeutic ends.

1.1.2 Peptides in immunoengineering

The purpose of this chapter is to highlight the current ways which peptides are being used in immunology as well as the future ways which peptides can be used to assist in or directly tackle the challenges of vaccine development, immunotherapy and the broader immune engineering field as a whole. Peptides are used for many purposes, as has been described many times throughout this textbook already. In cell biology, peptides are usually considered intermediate degradation products on their way to full degradation. The resulting free amino acids can then be used to build new proteins. Until 1986, it was never considered that peptides could have an essential role during an immune response: as specific antigens that DCs present to T cells and B cells for activation.[9] Over the past three decades, not only have we uncovered the natural function of peptides in immunology, but we've also come to identify and utilize other functions for peptides in immunology.

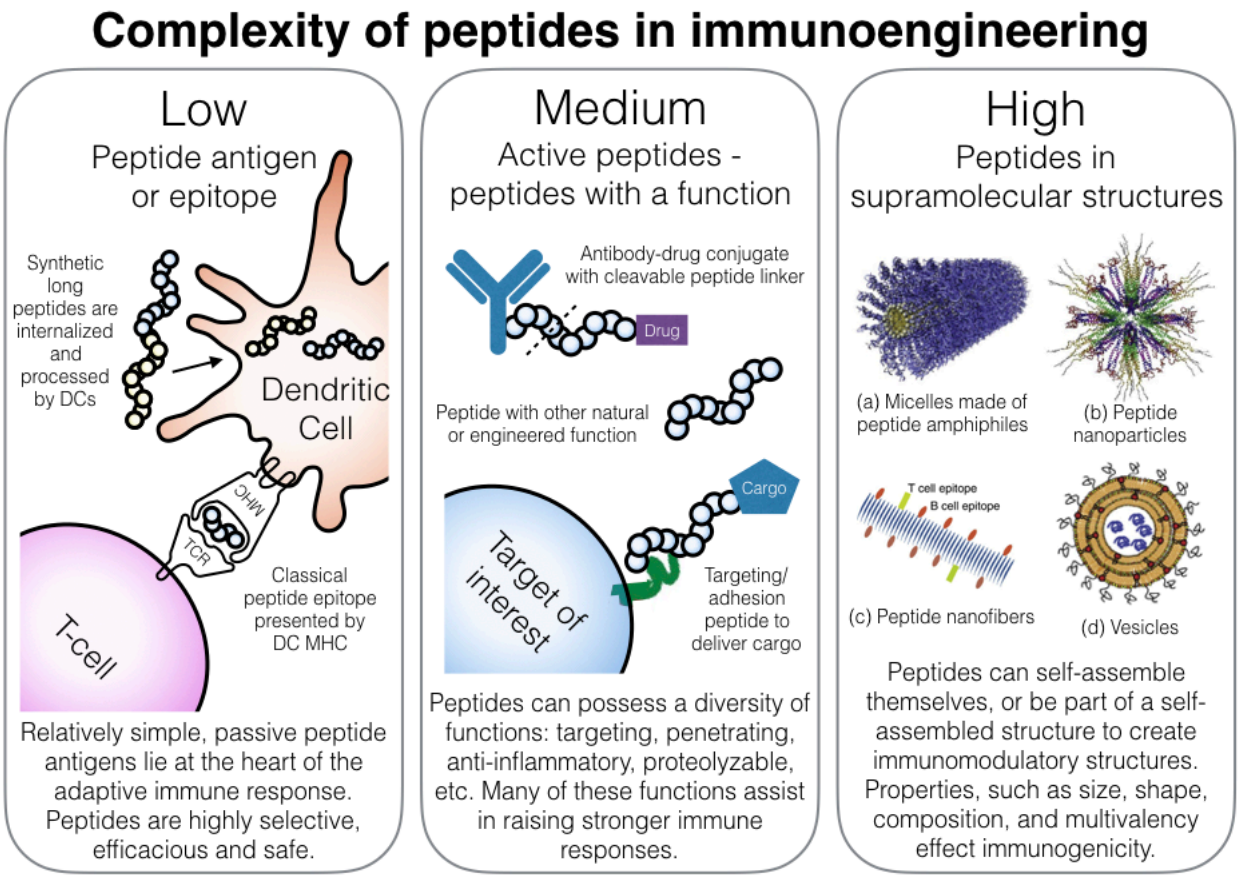

Figure 1.1 - Peptide-based biomaterials for immunoengineering. As complexity increases, both breadth and strength of available functions expand for these systems. The range of engineered complexity allows for the selection of optimal system benefits for each application. (a) Reprinted from Black et al.[10]; Copyright (2012), with permission from Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (b) Reprinted from Kaba et al.[11] (c) Reprinted from Wen et al.[12]; Copyright (2015), with permission from Elsevier. (d) Reprinted from Moon et al.[13]; Copyright (2011), with permission from Nature Publishing Group.

Peptides encompass a wide spectrum of complexity (Figure 1.1). In the first level of complexity, peptides act as relatively simple, passive antigens. Moving up in complexity, peptides can possess functional/active properties. In the highest level of complexity, peptides can form supramolecular/assembling structures. As complexity increases, both breadth and strength of available functions expand for these systems. The range of engineered complexity allows for the selection of optimal system benefits for each application. While a majority of the immune

engineering strategies currently in the clinic today are based on traditional drug development approaches – antibodies, recombinant proteins, small molecule drugs – simple peptides, which act mainly as antigens, also make an appearance in treatments. Many peptide antigens in commercially available drugs come from the first, lower tranche of complexity. These peptides are chosen for exhibiting the general, natural properties of peptides as a category: highly selective and efficacious, as well as, safe and well tolerated. These properties are the primary differentiating factor of peptides compared with small molecules and provide peptides with an excellent starting point for the design of immune therapies.

While opportunities still exist for the design and use of peptide antigens in their simplest form, a range of peptide technologies have been emerging that represent the opportunities and future directions within the peptide immunoengineering field. Beyond a peptide's use as an antigen, immunoengineering opportunities exist with the next level of peptide complexity: functional or 'active' peptides. Peptides can possess a diversity of functions, including targeting (cell binding, integrin binding, surface binding), penetrating, anti-inflammatory, proteolyzable, and combinations thereof.[14] Moreover, the tailorable nature and chemically defined properties of peptides leaves much room for the discovery and development of additional functions in the future. Many of these functional peptides assist in the basic design principle that immunomodulatory compounds much reach their target cell types to exert their effects. Using peptides to target, attach, or enzymatically release drugs in target lymphoid tissues or within specific immune cells can dramatically increase their efficiency and potency. With these enhanced functions and directed tissue and/or cellular targeting, engineered peptides can also lead to safer vaccines and therapies.[15-18] Immunomodulatory drugs (such as small molecules or cytokines) typically act on a broad range of cell types, causing them to have severe toxicity

side effects resulting in their failure as a drug.[19, 20] Functional peptide incorporation to the engineered system has the potential to modulate the right target cells at the right location and avert typical toxicity.

Continuing along the complexity scale, peptides can self-assemble by themselves or be a part of a self-assembled supramolecular structure. These peptide particles can possess immunomodulatory functions, acting as antigens, adjuvants (immune response booster), immune potentiators, or a combination thereof. These engineered platforms acquire their immunological properties from many controllable features, including size, shape, multivalency and tightly controlled molecular content of different functional components.

In the end, a successfully engineered immunotherapy involves orchestrating the activities of a broad range of immune cells to elicit a specific immune response. Peptides, with the range of complexities they possess, are ideal for engineering these immune responses. Promising data discussed in this chapter suggest that peptides will have an important role to play in the future of the burgeoning field of immune engineering.

1.2 Peptides as antigens – immunogenic peptides to engineer immune responses

Peptides fulfill many roles in immunology, yet none are more important than their role as immunogenic epitopes driving the adaptive immune response. Epitopes – T cell epitopes, B cell epitopes, or any other part of an antigen that is recognized by the immune system – lie at the heart of the adaptive immune response. It has been known for over 30 years now that peptides are critical factors for instructing the immune system to mobilize against foreign invaders. With our knowledge of how peptides are used to alarm the immune system (their processing and

presentation on MHC), the question is how to use this information to modulate the immune system to treat or prevent infections, cancer, and autoimmunity.

1.2.1 Considerations for the design of peptide antigens

1.2.1.1 Choosing the peptide antigen

A variety of considerations need to be made during the design of a peptide antigen, depending on the context with which the peptide will be used. Chief among the considerations is the identification of immuno-dominant domains of epitopes that are capable of inducing an immune response.[21] The immune phenotype required to treat or prevent a disease will determine the epitopes chosen. For example, extracellular pathogens or infections may be effectively neutralized by an antibody response, which could dictate the selection of an immuno-dominant B cell epitope. On the other hand, intracellular pathogens or cancers may require the identification of epitopes that induce cytotoxic T cell responses.[22, 23] In addition to the effector B cell or cytotoxic T cell responses, a helper T cell response may also be crucial, requiring the identification of an activation solution for that cell as well.[23, 24] From among the epitopes that are found to induce the desired immune response, the next challenge is be to identify the epitope(s) or peptide(s) that activate the immune response to the correct magnitude that can provide protective immunity. The processing, presentation, and association of the candidate peptide antigen by antigen presenting cells (DCs) in such a highly MHC heterogeneous human population is another issue that requires consideration.

1.2.1.2 Improving the peptide antigen

A peptide antigen should be chosen first for its immunogenic characteristics, as described above. However, when deploying the peptide in vivo, some peptide characteristics such as stability, affinity and delivery, should be improved upon in order to obtain sustained

immunological effects. As described in earlier chapters, one major drawback of peptides is their short half-life. Peptides have a short half-life in circulation, are rapidly cleared through the kidney by filtration, and are also destroyed by many extracellular peptidases.[25] Over the years, however, many strategies have been devised to mitigate this problem. A number of these strategies are discussed in more detail elsewhere in the text, so we will briefly touch on the strategies most pertinent to immune engineering.

Solving peptide stability through chemical modification employs one of the most advantageous properties of peptides. Prodrugs or propeptides are stable, chemically altered peptides that are processed into the desired antigen on location. Propeptides are designed to have the antigenic sequence flanked by additional amino acid sequences preferred by dominant endopeptidases. Combining this with blocking N- or C-terminal groups may further protect from degradation and allow the antigen sequence to arrive in tact and perform its immunological function.[26] Stability may also be increased by delivering the peptide with an adjuvant.[27] Many adjuvants rely on creating a depot, in which peptides are protected from immediate degradation, and to which immune cells will be attracted and activated by simultaneous stimulation of peptides and adjuvant molecular motifs.[28-30] Peptide antigen stability can also be increased through incorporation into larger structures such as antibody drug conjugates, supramolecular peptide structures, liposomes, or other assemblies, many of which will be discussed later in this chapter and other chapters.

Among all the peptide antigens that produce an immune response, many will bind with relatively high affinity to MHC molecules, biased by the selection criteria. But even if the peptide antigen can induce an immune response and bind to MHC molecules, the binding may still have room for improvement. In other words, a peptide's natural affinity or on/off rates may

not be ideal and this may provide an area of opportunity to improve the immune response. These parameters are important to the development of an immune response because it stands to reason that such peptides that make more stable MHC molecules will have more time to activate the immune system.[31, 32] Improving peptide affinity to MHC molecules can be achieved in a few different ways. One way is with a chemical biology approach to improve the interactions of the MHC with the peptide cargo, primarily through modifying the N- and C termini and anchoring residues of the peptide. For peptide side chains, small modifications may sometimes improve affinity, but care must be taken to not affect the MHC-peptide complex surface for fear of altering the ability of a T cell receptor to recognize the complex. Finally, a more direct way to improve affinity is to just directly conjugate the peptide to the MHC through molecular biology/protein engineering techniques, as some researchers have done.[33]

1.2.2 Peptide antigens modulating immune responses

There are many ways an immune response may need to be modulated, including inducing or suppressing a particular response. Because of a peptide's specificity and chemical definition, it can be engineered to be useful in almost any case required. Autoimmune diseases (such as Type 1 diabetes and multiple sclerosis) arise when T cells that recognize self-proteins are not completely eliminated before leaving the thymus.[34] It is possible to control these autoreactive cells through mechanisms of tolerance in the peripheral tissues, but if these mechanisms fail, disease ensues. In Type 1 diabetes, one reason why tolerance mechanisms fail is because the patient happens to be homozygous for a particular MHC molecule. 45% of type 1 diabetes patients are homozygous for HLA-ER3 or HLA-DR4, although this is present in only 3% of the population.[35] These MHC molecules selectively present a peptide from a tissue specific protein, which are then recognized by self-recognizing T cells that were not deleted in T cell

maturation. If there are a set of dominant peptides in this interaction, modifying these peptides to become less able to associate with T cells[36] or to selectively activate regulatory T cells (T_{reg}s), could stifle the autoimmune response.[37] Researchers have successfully used this approach in mouse models of defined and homogeneous T cell responses. However, the strategy becomes more complicated if multiple populations of T cells or T_{reg}s need to be antagonized or activated, as is the case in human translation. To realize a strategy for human Type 1 diabetes, more studies are currently underway.

Another clever peptide approach to treating type 1 diabetes involves tolerizing polyspecific $CD8^+$ T cells that recognize multiple self-epitopes expressed by pancreatic islet cells.[33] Tolerization in this approach is attempted with iron oxide nanoparticles conjugated with autoantigen peptide – MHC complexes (pMHC-NPs). In this antigen-specific strategy, it was found that pMHC-NPs stimulated self-antigen-specific $CD8^+$ T cells and led to the expansion of autoregulatory memory-like T cells. These autoregulatory T cells kill APCs that present autoantigens, thereby preventing the activation of autoreactive $CD8^+$ T cells. If the activation of polyspecific autoreactive $CD8^+$ T cells are also inhibited, normal glycemic levels in nonobese diabetic mice can be established (Figure 1.2). It is thought that the expansion of the autoregulatory memory-like T cells may be a result of TCR cross-linking by the autoantigen peptide loaded pMHC-NPs.[33]

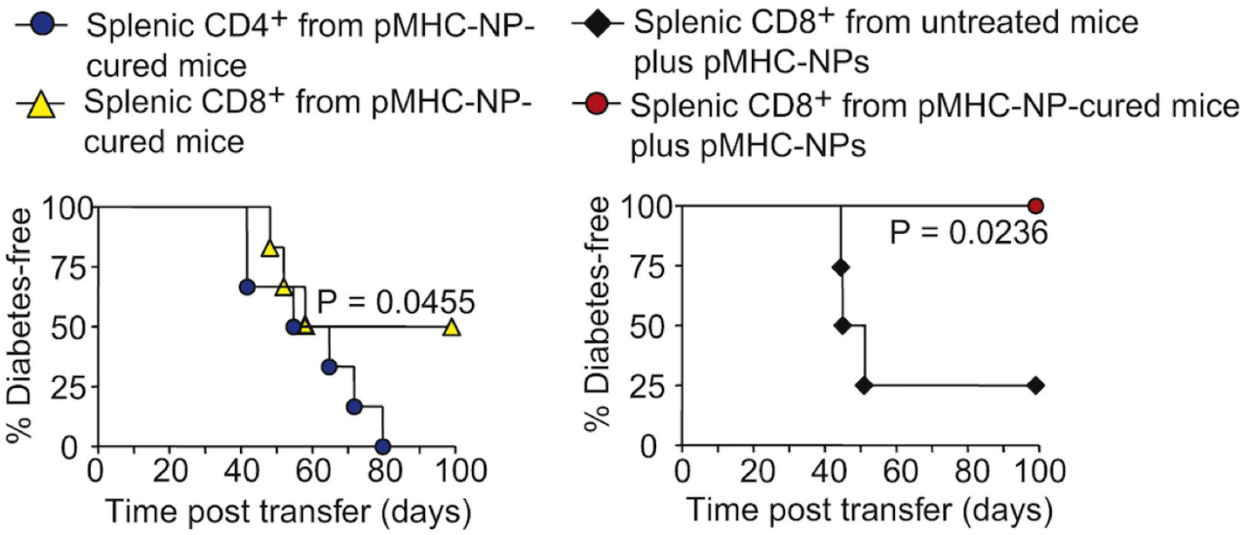

Figure 1.2 - Peptide-MHC-Nanoparticles (pMHC-NPs) restore Normoglycemia in Diabetic NOD Mice. Left: pMHC-NP-expanded $CD8^+$ cells from cured NOD mice (n = 6; 5 x 10^6 $CD8^+$ cells, containing < 5 x 10^4 pMHC-NP cells) suppress the adoptive transfer of diabetes by splenic T cells from prediabetic NOD mice into NOD.*scid* females compared to total $CD4^+$ cells (5 x 10^6) from cured donors (n = 6). Right: pMHC-NP treatment (twice/week) potentiates the transfer of protection by splenic $CD8^+$ cells from pMHC-NP-cured mice into T cell- reconstituted NOD.*scid* hosts (n = 3) but cannot induce protective activity in splenic $CD8^+$ T cells from untreated NOD donors (n = 4). Reprinted from Tsai et al.[33]; Copyright (2010), with permission from Elsevier.

Cancer therapy with peptide antigen is a conceptually simpler task than autoimmune disease. If a tumor peptide antigen is defined, injecting the peptide to activate a T cell response against this antigen could result in an immune response against the tumor. This strategy has been applied in the treatment of premalignant lesions of cervical cancer in human patients, in melanoma tumors, and many more cancer mouse models.[38-42] In general, tumor microenvironments weaken the local immune system's ability to develop a strong immune response against the tumor. By injecting a peptide vaccine and raising an immune response elsewhere in the body, this can help to induce a local immune response to clear the tumor. Of course, a major player in this response is the DC. When presented the correct peptide antigen, DCs can activate a strong and specific immune response against tumors. In mouse models, it is enough to provide the minimal 9-11 amino acid peptide sequence recognized by $CD8^+$ T cells in

order to get an immune response.[38] But, by delivering a longer peptide which included both MHC class I and MHC class II sequences, a stronger immune response can generally be raised.[38] Delivering this longer, combined peptide resulted in a T cell response against human papillomavirus (HPV) proteins and clearance of the premalignant cervix tissue in patients.[38, 43] A longer peptide could perform better in vaccines for multiple reasons; longer peptides are retained longer in circulation, and combining both MHC class I and MHC class II peptides increases the probability of a DC processing and presenting both peptides on its surface to activate a strong $CD8^+$ T cell response.[44-46] Additionally, delivering a longer peptide allows the immune system of each individual to process and trim the peptide in slightly different ways. This is important because human tumor microenvironments are complex and all slightly different, which means the peptide antigens required for an immune response are all slightly different between patients. Therefore, delivering a longer peptide allows the peptides to be trimmed more naturally.

1.3 'Active' peptides – peptides with a function

While a peptide's basic function as an antigen in immunology is exceptionally important, peptides can offer much more functionality beyond that role. Due in large part to a peptide's chemical definition and tailorable nature, peptides can possess a diversity of functions, including targeting (cell binding, integrin binding, surface binding), penetrating, anti-inflammatory, proteolyzable, and combinations thereof. Moreover, the tailorable nature and chemically defined properties of peptides provides opportunities for the discovery and development of additional functions in the future.

1.3.1 Targeting peptides

Peptides that target specific cell types involved in the immune systems have several key uses, including antigen delivery for vaccine development, delivery of immunosuppressant agents for the treatment of autoimmune diseases, and redirection of an immune response to eradicate diseased cells. Unlike antibodies, peptides are easy to synthesize in large quantities, and their smaller size improves tissue penetration.[47] Additionally, peptides can be chemically modified to alter affinity, charge, hydrophobicity, stability and solubility. Because of these tailorable qualities, peptides can display antibody-like affinities for their receptors, making them effective targeting agents.[48] Actively targeting immune cells in order to modulate immune responses is a heavily and actively explored area, and here we will discuss promising examples of this strategy.

1.3.1.1 Targeting the innate immune system

Neutrophils are the "first responders" at sites of inflammation and play an important role in providing the initial defense against invading pathogens through phagocytosis of microbes and secretion of cytokines and reactive oxygen species.[49] Inflammation caused by neutrophils are important to the initiation of a complete immune response, however, prolonged neutrophil-mediated inflammation can lead to tissue damage and the pathogenesis of diseases such as arthritis, cancer, and COPD.[50] Targeting neutrophils to mediate excessive inflammation is an attractive strategy, so long as the beneficial role of neutrophils and inflammation are not completely inhibited. One strategy begins by using collagen IV targeting peptides. Collagen IV is a useful target for tissue injury applications because this type of collagen is abundant on the basal lamina – a layer of extracellular matrix secreted by epithelial cells. These collagen IV targeting peptides were used to deliver polymer nanoparticles carrying the anti-inflammatory

peptide Ac2-26. Isolated from annexin A1 N-terminus, Ac2-26 (Ac-AMVSEFLKQAWFIENEEQEYVQTVK) was discovered for its sequence similarity to uteroglobin, and functions as a mimetic peptide that acts on the G-protein–coupled formyl peptide receptor, ALX/FPR2.[51-54] After intravenous administration, the collagen IV peptide targets and delivers the encapsulated Ac2-26 peptides to sites of tissue injury. In a hind-limb ischemia-reperfusion tissue injury mouse model, this resulted in a 30% reduction in neutrophil recruitment to the site of injury (Figure 1.3).[55] Targeting collagen IV during an injury is one way to deliver cargo to neutrophils, though it may be delivered to other cells as well. To have even more specific delivery to a neutrophil, the peptide WAWVWLTETAV can be used. This peptide was isolated against the FcγRIIA receptor, a receptor only expressed by neutrophils and mononuclear phagocytes, and was shown to mediate cell internalization and degradation of an OVA model antigen after binding.[56]

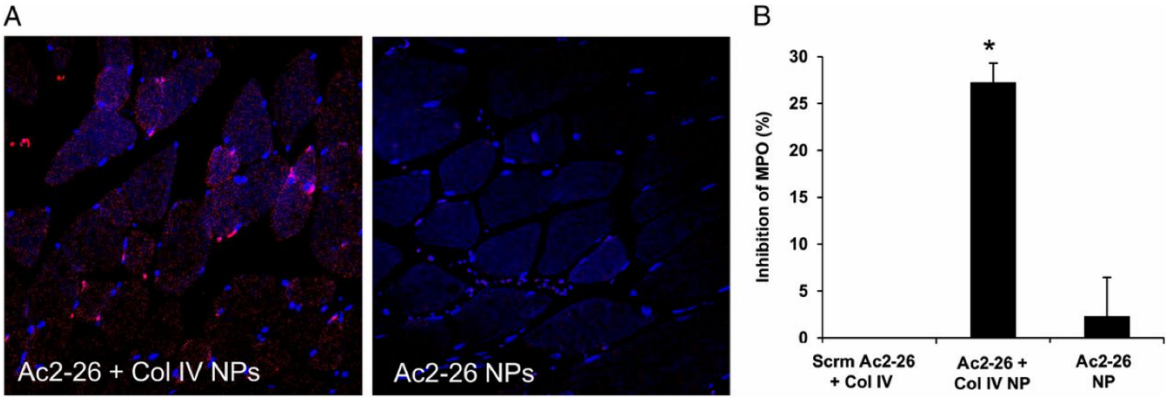

Figure 1.3 - Collagen-IV–targeted Ac2-26 NPs (Col IV NPs) limit Polymorphonuclear Neutrophils (PMN) infiltration into injured tissue. Ischemia was induced by placing a tourniquet around the hind limb for 1 h. After 1 h, the tourniquet was released and vehicle, Scrm Ac2-26 Col-IV NPs, Ac2-26 Col-IV NPs, or Ac2-26 NPs were injected i.v. Reperfusion was carried out for 1 h. The gastrocnemius muscle tissue was harvested and (*A*) sectioned for confocal imaging using a Nikon A1R microscope, 20× magnification. Images are representative of $n = 3$. (*B*) Gastrocnemius tissue was lysed and homogenized to assess PMNs using a myeloperoxidase (MPO) ELISA ($n = 3$; mean ± SEM). The data are plotted as inhibition of tissue MPO. *$P < 0.05$ Col-IV for Ac2-26 NPs vs. Ac2-26 NPs or vs. Scrm-Ac2-26 Col-IV–targeted NPs. Reprinted from Kamaly et al.[55]; with permission from PNAS.

1.3.1.2 Targeting the adaptive immune system

Human Immunodeficiency Virus (HIV) is a terrible infection for which there is no cure. During infection, $CD4^+$ T cells are a primary target of the virus. This has motivated a number of immunoengineering strategies to target therapeutic agents to $CD4^+$ T cells to block HIV replication, and peptides are frequently used in the targeting strategy. For example, lipid nanoparticles containing an encapsulated antiretroviral drug, idinavir, used specific CD4 co-receptor targeting peptides to target T cells. Pretreatment of $CD4^+$ T cells with the peptide targeted lipid nanoparticles resulted in a reduced number of infected cells compared to non-targeted lipid nanoparticles in vitro (Table 1.1).[57]

Table 1.1 - Intracellular Indinavir Concentrations After Incubation with Soluble or Lipid-Associated Indinavir Nanoparticle. Reprinted from Endsley et al.[57]; Copyright (2012), with permission from Lippincott Williams & Wilkins, Inc.

	Intracellular Indinavir (ng/10^5 CEM×174 Cells)			
	Day 0		Day 4	
Indinavir Formulation	**6.25 μM**	**25 μM**	**6.25 μM**	**25 μM**
Soluble	2774 ± 53	3772 ± 1066	53 ± 1	124 ± 4
DSPC: DSPE-mPEG				
Untargeted	34 ± 10†	102 ± 3†	7 ± 0.1†	5 ± 0.4†
CD4-BP2 LNP	3105 ± 164	3584 ± 62	93 ± 1†	148 ± 1†
CD4-BP4 LNP	3584 ± 62†	6586 ± 328†	162 ± 4†	282 ± 5†

*To quantify Indinavir levels after enhanced delivery to cells with LNPs, triplicate 10^5 CEM×174 cells were incubated with 6.25 or 25 μM indinavir formulations, for 30 minutes at 37°C, washed, pelleted, then lysed in 100 μL of acetonitrile and 100 μL of water was added to each sample. 10 μL of each sample in duplicate was injected onto a Zorbax SB-C18 column with mobile phase consisting of a 0.1% acetic acid solution in methanol. The mass spectrometer was operated in API-ESI^+ mode, and the analytes were detected using selected ion monitoring at m/z 603.7–623.7 to detect indinavir.

†Significant at $P < 0.05$ compared with soluble drug.

API-ESI^+, atmospheric pressure ionization-electrospray ionization; DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine.

1.3.1.3 Targeting antigen presenting cells

Dendritic cells (DCs), as mentioned, are the cells that connect the innate immune system to the adaptive immune system. DCs are constantly on the lookout for foreign antigens, and after capturing an antigen, present peptides to activate naïve T cells, B cells, natural killer cells and natural killer T cells.[58] Due to their ability to activate all of these different immune pathways, DCs are ideal targets for immune engineering. Because of this, several DC specific targeting

peptides have been isolated for the purposes of specifically delivering antigens to dendritic cells. One such peptide, the p30 (CGRWSGWPADLC) peptide, was isolated against the DC-specific marker CD11c/CD18 and was later used for vaccination.[59, 60] Chicken ovalbumin (OVA) is a T cell dependent antigen commonly used as a model antigen for studying antigen-specific immune responses. In this vaccination, peptides on the surface of an OVA antigen loaded liposome targeted DCs with the p30 peptide and produced a much stronger OVA specific antibody response compared to the control OVA liposomes without the targeting peptide. In another example, the p30 DC targeting peptide was grafted onto plasma membrane vehicles derived from B16-OVA cells, a metastatic murine melanoma cell line that secretes OVA. Vaccinating mice with these peptide membrane vehicles before introducing B16-OVA cells into the mice significantly reduced the number of lung metastases. Additionally, mice bearing existing B16-OVA tumors that underwent subsequent vaccination with the peptide membrane vehicles induced antigen specific T cell priming and antibody production, followed by dramatic anti-tumor responses (Figure 1.4).[60]

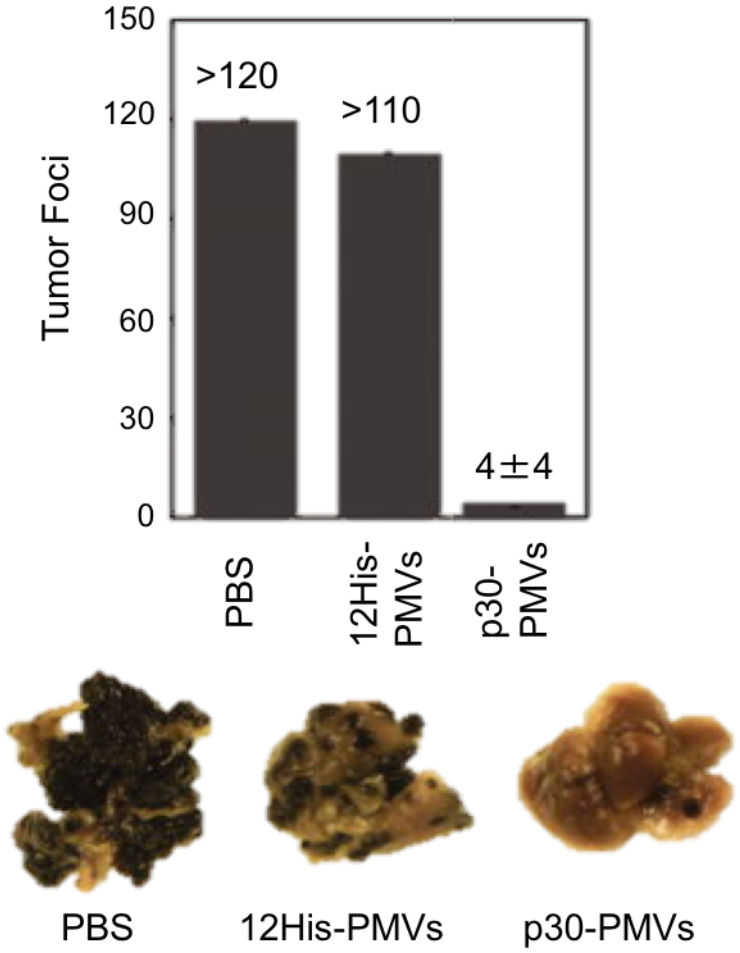

Figure 1.4 - Ag-bearing liposomes engrafted with peptides that interact with CD11c/CD18 induce potent Ag-specific and antitumor immunity. Vaccination with plasma membrane vehicles modified with the dendritic-cell-specific peptide CGRWSGWPADLC (p30) leads to anti-tumor activity. Naïve mice were injected iv with B16-OVA cells on day 0. At days 2, 8, and 14 different groups of mice (five mice per group) were vaccinated with PBS or B16-OVA-derived plasma membrane vehicles modified with the control peptide 12His or the dendritic-cell-specific peptide p30. At day 21, the lungs were removed from the mice, and tumor foci were counted via microscopy. (Top) Bars indicate the mean number of tumor foci for each vaccination group, and this number is indicated above each bar. (Bottom) Representative lung images from each vaccination group. Reprinted from Faham et al.[60]; Copyright (2010), with permission from John Wiley and Sons, Inc.

In recent years, microbiome research has made many promising advances. Because of the microbiome's wide reaching influence throughout the body, it has garnered interest from many different fields, including immunology. Immunoengineering offers the opportunity to expand

applications in this exciting new field of research. The intestines are lined with a single layer of epithelium for protection, making it difficult for most things to pass through, or out, of the gut.[61] Microfold cells (M cells) are immune cells located in Peyer's patches of the intestinal epithelium that sample antigens in the gut and transport them a short distance to a binding pocket in which immune cells such as lymphocytes or macrophages dock.[61] Because M cells can carry antigens out of the gut to other immune cells in order to facilitate an immune response, M cells are very desirable targets for orally available vaccines. Engineering delivery directly to the M cells for initiation of an immune response resulted in the identification of the Co1, P8 and P25 peptides (Co1: SFHQLPARSPLP; P8: LETTCASLCYPS; P25: VPPHPMTYSCQY).[62, 63] Kim et al. elucidated the delivery of Co1 peptide by fusing it to the fluorescent protein EGFP. These peptides, and in particular the Co1 peptide, are able to specifically bind M cells and transport across the intestine in vivo when injected into mouse intestine, as well as bind to tissue sections of human intestine as evidenced by immunohistochemistry. After oral administration, the targeting peptide-fused antigen (Co1-EGFP) enhanced immune responses by raising 2 to 3-fold higher antibodies against the fused antigen compared with those of the control antigen without targeting peptide (Figure 1.5). In addition, the use of the targeting peptide supported a skewed Th2-type immune response against the fused antigen. Th2 immune responses are typically raised against extracellular infections or parasites, which makes sense in this context because the EGFP represents an extracellular infection. Because of Co1's ability to raise strong immune responses and shown binding to human M like cells in culture, Co1 peptide has high potential for clinical translation in the effort to develop an oral vaccine.

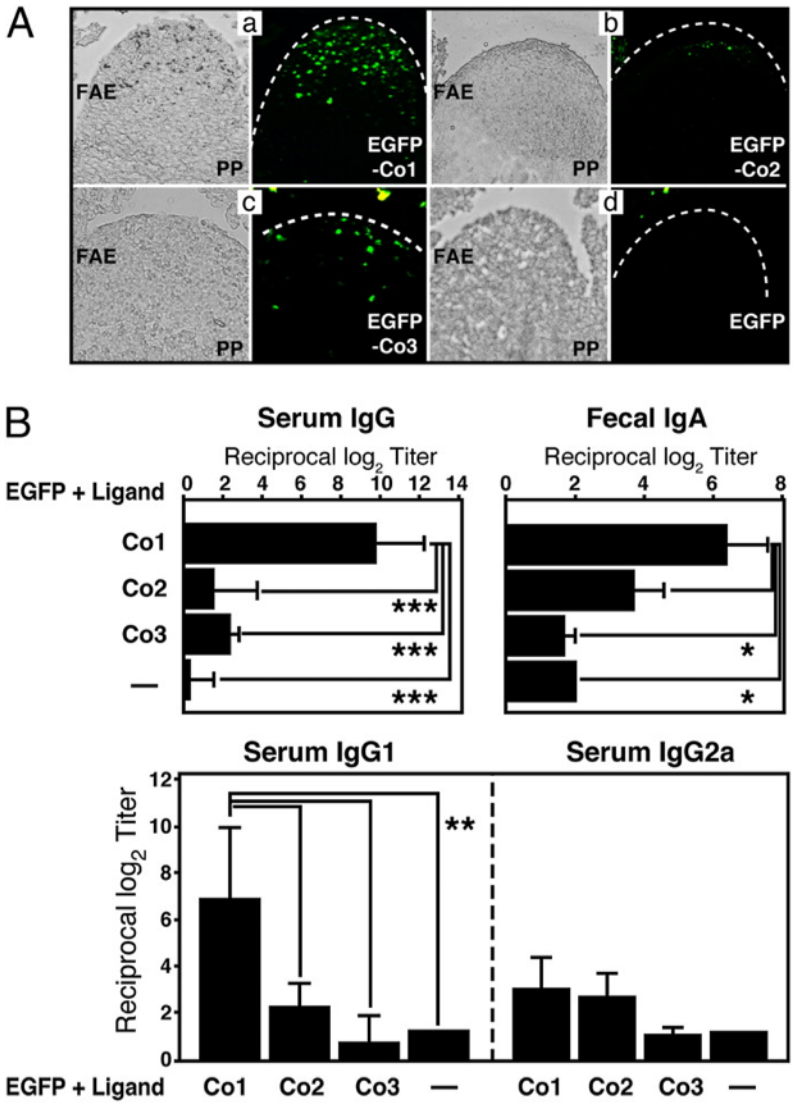

Figure 1.5 - Targeting of ligand-fused EGFP into Peyer's Patches (PPs) and induction of EGFP-specific mucosal and systemic immune responses after oral administration of the ligand-fused antigens. A, Immunohistochemical analysis of mouse gut loops incubated with (a) EGFP-Co1, (b) EGFP-Co2, (c) EGFP- Co3, and (d) EGFP (green) alone using the ex vivo loop assay. Left and right columns of each panel represent the results from light and fluorescence microscopy, respectively (original magnification 310). B, Levels of EGFP-specific serum IgG and fecal IgA (upper panel) and EGFP-specific IgGs of different subclasses (lower panel) in the sera after oral administration of ligand-fused EGFP or EGFP alone. Results were calculated as the reciprocal of the geometric mean $\log_2$ titer. Reprinted from Kim et al.[63]; Copyright (2010), with permission from the American Association of Immunology.

1.3.1.4 Other targeting peptides in immune engineering

One of the most heavily studied peptides in the context of biomaterials and immunoengineering has been RGD (Arg-Gly-Asp). RGD was originally developed as a small molecule capable of functioning analogously to the 10^{th} type-III domain of fibronectin, which is a high molecular weight protein of the extracellular matrix (ECM) that binds to special cell membrane receptors called integrins. RGD has undergone exhaustive exploration and is now known to bind promiscuously to many different integrin subtypes and many different ECM molecules beyond fibronectin.[64, 65] RGD peptide has been studied in many contexts, but one example with single-walled carbon nanotubes (SWNTs) is especially relevant to immunoengineering. In this example, SWNTs were coated with lipid-tailed poly(ethylene glycol) and terminated with RGD. These particles were shown to accumulate at tumor sites in mouse models of cancer.[66] This was first attributed to the preferential accumulation of SWNTs in tumors via the enhanced permeability and retention (EPR) effect and the ability of the RGD peptide to bind to integrins expressed on tumor vasculature on the surface of tumor cells.[67, 68] EPR is the property by which molecules of certain sizes tend to accumulate in tumor tissue much more than they do in normal tissues.[69] Upon further investigation using intravital microscopy, it was shown that in addition to accumulation in the tumor due to EPR, 25% of intravenously injected SWNTs were taken up preferentially by $Ly6C^{hi}$ monocytes in the circulation, which were then recruited to the site of the tumor in response to inflammation. The RGD peptide played a key role in that RGD conjugation to SWNTs increased the recruitment of $Ly6C^{hi}$ monocytes into the tumor interstitium and resulted in increased accumulation of SWNTs at the tumor site (Figure 1.6).[70] Although RGD is powerfully useful in some contexts, caution must be taken because of its broad integrin binding properties. For example, if cell-specific behaviors

are required, or if a defined set of integrins need to be activated and bound, the broad RGD integrin binding may not be desired.[65]

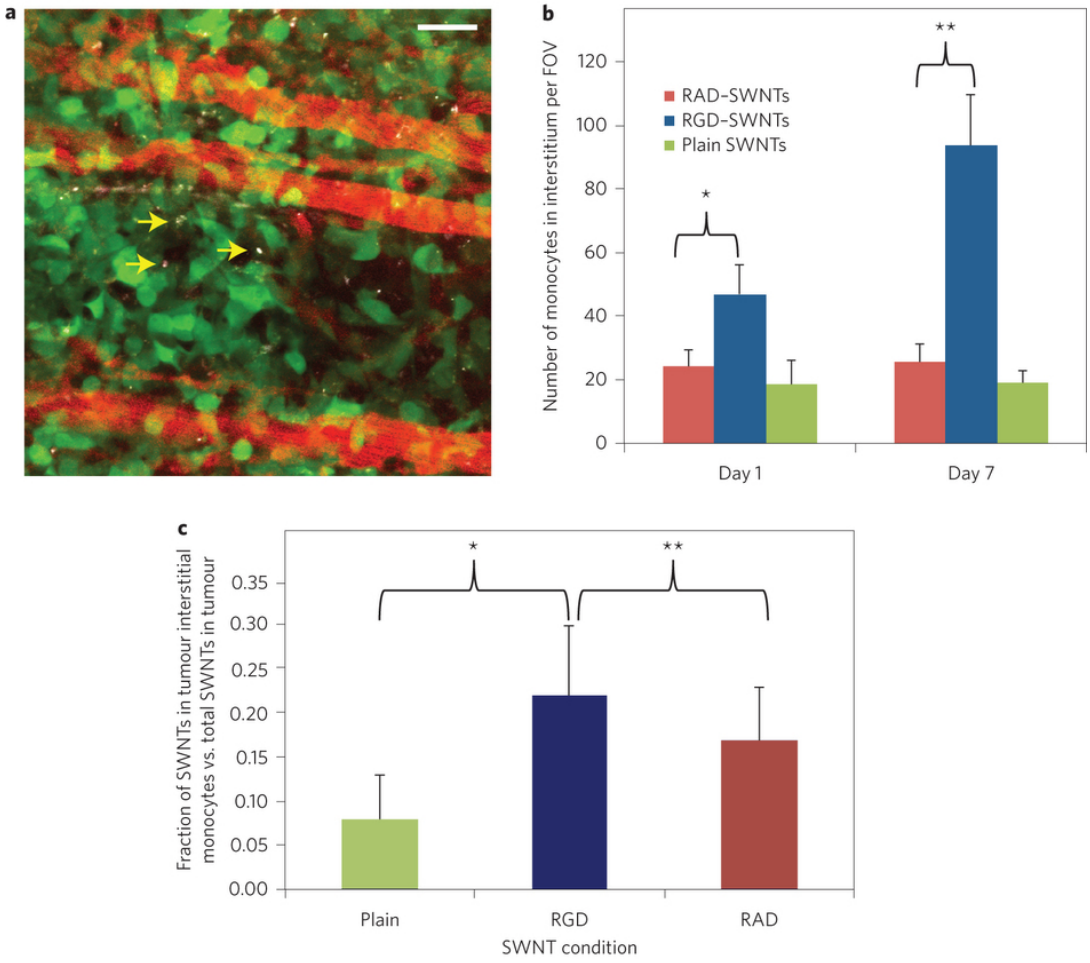

Figure 1.6 - SWNT-laden monocytes enter the tumor interstitium in a peptide-dependent manner. a, Representative intravital micrograph of a tumor region (tumor cells, green; blood vessels, red; SWNTs, greyscale). Yellow arrows point to several SWNT-laden monocytes within the tumor interstitium. Scale bar, 50 μm. b, Bar graph showing that significantly more monocytes carrying RGD–SWNTs accumulate in tumor interstitium than monocytes carrying RAD–SWNTs and plain SWNTs on days 1 and 7 p.i. of SWNTs. Moreover, significantly more RGD–SWNT-laden monocytes are in the interstitium per field of view (FOV) on day 7 than on day 1 ($P < 0.001$). *$P < 0.05$; **$P < 0.0005$. Error bars represent s.e.m. c, SWNTs can enter the tumor via a variety of mechanisms, such as leakage through blood vessel pores. This graph shows the relative amounts of SWNTs in the tumor interstitium that were ferried in via the Trojan horse monocytes compared with all SWNTs within the tumor interstitium as a function of peptide on day 1 p.i. More than 20% of SWNTs in the tumor interstitium in the RGD–SWNT condition are carried in via monocytes. *$P < 0.0001$; **$P < 0.05$. Error bars represent s.d. Reprinted by permission from Macmillan Publishers Ltd: Nature Nanotechnology[70], copyright (2017).

Another promising, more recently developed adhesion peptide is the erythrocyte-binding peptide ERY1 (H_2N-WMVLPWLPGTLDGGSGCRG-$CONH_2$).[71] As mentioned earlier, researchers are exploring new approaches based on induction of antigen-specific immunological tolerance for the treatment of auto-immune diseases. In this case, the antigen-specific tolerization approach involves using erythrocyte (red blood cell)-binding peptides, with the premise that as erythrocytes circulate, age, and are cleared, the erythrocyte surface-bound antigen payload will be cleared tolerogenically along with the eryptotic debris. ERY1 targets and attaches to the erythrocyte-specific cell surface marker glycophorin A after i.v. injection (Figure 1.7). In an ovalbumin (OVA) model antigen proof of concept experiment, it was shown that erythrocyte binding antigen is collected much more efficiently than free antigen by splenic and hepatic immune cell populations and hepatocytes, and that it induces antigen-specific deletional responses in $CD4^+$ and $CD8^+$ T cells.[72, 73] In a separate application for the same ERY1 system, a tolerance induction strategy was applied to prevent antigen specific humoral (antibody) immune responses to therapeutic proteins. In this tolerance induction strategy, they demonstrate that binding the clinical therapeutic enzyme *Escherichia coli* L-asparaginase to erythrocytes in situ antigen-specifically abrogates development of antibody titers by >1000-fold and extends the pharmacodynamics effect of the drug 10-fold in mice, tolerizing mice to multiple subsequent doses of the wild-type enzyme.[74] This tolerance strategy, led by the erythrocyte binding peptide ERY1, shows strong translational promise to enable more effective and safer treatment with therapeutic proteins and drug candidates that are hampered by immunogenicity, as well as in the treatment of autoimmune diseases such as type 1 diabetes and multiple sclerosis.

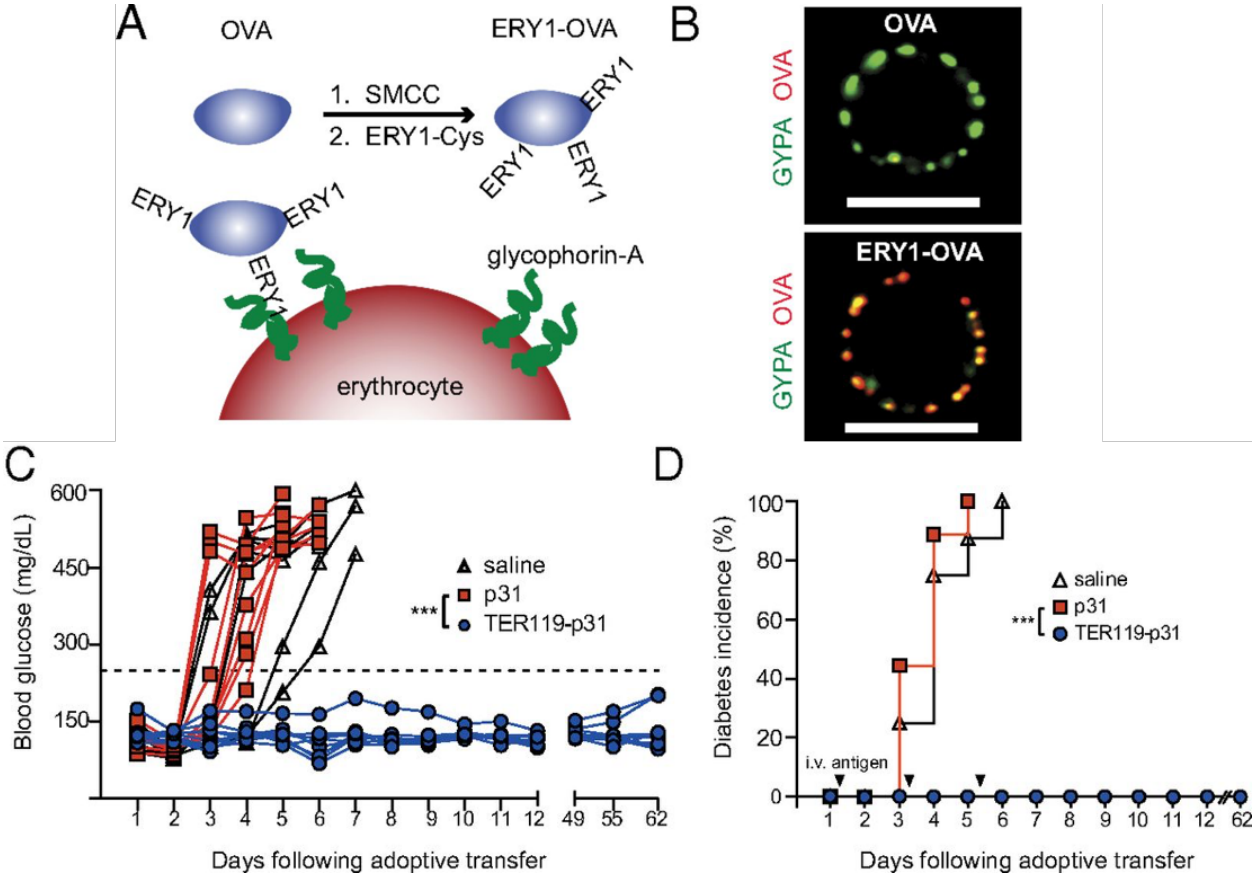

Figure 1.7 - ERY1-OVA binds the equatorial periphery of mouse erythrocytes with high affinity and protects mice from T cell-induced autoimmune type 1 diabetes. (*A*) Schematic of conjugation of ERY1 peptide to OVA, resulting in binding to erythrocyte surface glycophorin-A. (*B*) High-resolution confocal microscopy images of mouse erythrocytes labelled ex vivo with (green) anti-mouse glycophorin-A (GYPA) and (red) either OVA (*Upper*) or ERY1-OVA (*Lower*). (Scale bar = 5 μm.) (*C*) Glycemia monitoring as measured by daily blood glucose measurements following adoptive transfer of diabetogenic BDC2.5 $CD4^+$ T cells and a tolerogenic treatment regimen of either saline, p31, or TER119-p31 ($n = 8$, $n = 9$, and $n = 9$, respectively). ***$P < 0.0001$. (*D*) Diabetes incidence rate quantified by measurements in *C*; arrows indicate antigen administration time points. ***$P < 0.0001$. Reprinted from Kontos et al.[72]; with permission from PNAS.

1.3.2 Enzyme cleavable peptides for immunoengineering

As described above, peptides in immunoengineering have been used to bind very specific cells and cell surface receptors. Another fantastic feature of a peptide is that its specificity extends beyond just the cell surface. Peptides can also interact with specific enzymes and subcellular compartments. Immunoengineering strategies can use peptide sequences with

distinctive binding constants and degradation kinetics to various proteases in order to direct the most desired immune response or other outcome for a particular application.

As a comparison to targeting peptides, monoclonal antibodies (mAb) are also an attractive immunoengineering tool, thanks to their extracellular target specificity.[75-78] However, the effectiveness of directly triggering cell death through extracellular activation of intracellular protein pathways can be complicated and highly cancer cell/tissue type dependent. Instead of relying on these complicated pathways to death, mAbs can be used to kill cells more directly by simply carrying and delivering a toxic payload to the target. These combined particles, termed antibody-drug conjugates (ADCs), have undergone considerable optimization since they were first used more than three decades ago, and have recently shown major success.[79-82]

The delayed success of antibody-drug conjugates was mainly due to the complex, detailed design of their conjugation chemistry; linking antibody to cargo (the linker). Design parameters for this critical segment are that; (i) the conjugation of antibody to cargo should occur in mild medium as to not do damage to the antibody; (ii) the linker must be resistant to proteases in the serum during circulation in order to deliver the cargo to the target intact; and (iii) once the cargo is delivered to the target, it is commonly desired that the cargo be internalized by the cell and released from the antibody so that the cargo can perform its function. Luckily, when a mAb binds to its target, endocytosis is frequently triggered and the ADC is taken into an acidic environment where the cargo is released from the antibody through the acid labile linker.

The development of acid labile linkers significantly advanced ADC technologies, and improved their efficacy and tolerability. Some examples of the first acid labile linker chemistries to be used include hydrazones and disulfide bonds. The advantage of hydrazone is that this linker

is highly unstable at pH 5, largely allowing for the complete intracellular release of active drug. Unfortunately, hydrazones are only moderately stable at neutral pH in circulation (Figure 1.8).[83] Disulfide bonded linkers on the other hand, such as glutathione and cysteine, show higher stability than hydrazones in circulation because of steric hindrance and the simple fact that thiols are generally not present outside the cell (Figure 1.8).[84] However, this strong stability can persist even within the lysosome, leaving disulfides incompletely degraded, thus preventing the cargo from being released.[85] One added limitation to the disulfide linker technology is the extensive and complicated purification procedures required to produce homogeneous product. In order to produce the desired molecule, solvent chemicals to disrupt excess disulfide bonds may damage the stability of mAbs.

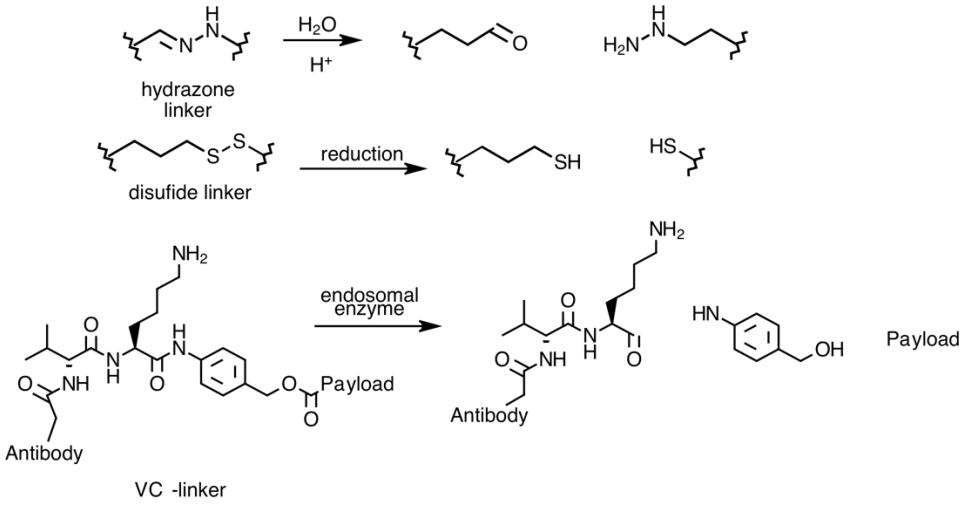

Figure 1.8 - Antibody drug conjugate linkers and their cleavage mechanisms.

Since each of these linkers had limitations, the search for an improvement continued. Ultimately, cleavable peptides emerged as the lead candidate to advance ADCs. Peptide linker technologies presented significant advantages over those non-peptide linkers.[86] Chief among the advantages is that the hydrolysis of peptide-based linkers is enzymatic. This allows the cleavage sequence within the peptide linker to be selected based upon which enzymes are

preferentially expressed by the target tumor cells or solid tumor masses. Therefore, the cargo will only be cleaved and released from the mAb within a target cell, and be shuttled out of non-target cells by the continued progression of lysosomes to exosomes. In addition, the peptides will not be cleaved in the systemic circulation because the peptides can only be cleaved by the intracellular tumor-associated proteases. Peptide linkers are also biocompatible, producing safe byproducts after the cargo is delivered.

Cathepsin B enzyme was found overexpressed in wide variety of human cancers, often associated with the tumor cell membrane.[87] Thus, cathepsin B enzyme cleavable linkers are among the most advanced linkers used with ADCs for the treatment of cancer. As mentioned before, the internalization of mAbs occurs through endocytosis. Endo-lysosomes degrade their contents using proteases, such as cathepsins, in acidic pH. Cathepsins recognize and cleave specific amino-acid sequences. These sequences have been extensively studied with cathepsin-B being the most popular endocytotic cysteine-based protease.[88, 89] Cathepsin-B is rarely found in the extracellular matrix or elsewhere outside the cell, and therefore conjugates produced with cathepsin-B cleavable linkers are likely to be stable in circulation.[90]

Initially, Dubowchik et al, studied a library of enzyme cleavable dipeptide linkers and measured the rate of drug (doxorubicin) release by enzyme activity.[90, 91] According to Dubowchik's studies, in the presence of cathepsin enzyme, Phenylalanine-lysine (FK), Valine-Lysine (VK) and Valine-Citrulline (VC) showed the fastest cleavage kinetics. With the addition of a spacer such as para-(amino benzoyl) (PAB), the cleavage reaction rates were improved even further, to half-life times of 8, 9 and 240 min, respectively. While in vitro studies were a great place to start, in vivo experiments revealed that cleavage kinetics were vastly different between the three sequences. The cleavage kinetics of VC with PAB spacer stood out amongst the rest,

showing impressively high cleavage as identified through drug efficacy. The cleavage mechanism of VC linkers is shown in Figure 1.8. In this reaction, enzyme interacts with VC dipeptide and cleaves through the N-terminus of the peptide, which triggers the 1,6-elimination of carbon dioxide on PAB and contaminant release of the free drug in parent amine form.[92] According to extensive studies, the VC peptide linker shows superior stability in human plasma, robust cleavage after endocytosis, and potent, antigen specific cytotoxicity when compared to non-peptide linkers.[90]

Following the initial studies on enzyme cleavable peptide linkers, many fields saw their potential and quickly adopted the linkers. The immunoengineering field applied the linkers to ADCs because of their superior properties compared to previously used acid-liable linkers. One of the first ADC studies to use these peptide linkers was performed by Doronina et al., who wanted to deliver monomethyl auristatin E (MMAE).[93] MMAE cannot be delivered systemically because of its severe toxicity and adverse effects. Therefore, Doronina wanted to test whether delivering MMAE as part of an ADC could circumvent the toxicity and potentially improve MMAE safety and efficacy. To test this, three linkers (FK, VC, and hydrazone) in an otherwise unchanged ADC system, were compared against one another for their ability to kill cancer cells and prevent toxic effects by MMAE. The results showed that hydrazone was far less stable in plasma compared to the peptide linkers. Moreover, comparison of the dipeptide linkers showed that FK and VC both activated the enzyme trigger robustly; however, FK was found to be less stable in plasma. In vitro experiments of the same study demonstrated that peptide-linked ADC were highly potent; inducing 10-100 fold greater immunologically dependent cell death compared to the corresponding hydrazone linker ADC.[93]

Of course, with such dramatic success brought on by essentially only a change to a peptide linker, enzymatically cleavable peptide applications have been greatly repeated and extended. Burris et al switched the antibody to a fully-human mAb (CR011) to target breast cancer.[94] More specifically, the antibody targets anti-glycoprotein NMB (GPNMB), also known as osteoactivin, that is expressed in many breast cancer types and associated with an increased risk of cancer recurrence. The ADC was prepared with MMAE and a VC peptide linker, and phase II studies have met the criteria for advancement (Table 1.2). Other examples of ADCs under clinical trials that use the VC peptide linker are shown in Table 1.2.

Table 1.2 - Examples of Antibody Drug Conjugates prepared with cleavable VC dipeptide

Antibody Drug Conjugate	Antibody	Cancer type	Status	Reference
Brentuximab vedotin (SGN-35)	Anti-CD30	Lymphomas	Approved	[95]
Glembatummumab vedotin (CDX-011)	Anti-GPNMB	Breast Cancer	Phase II	[94]
SGN-75	Anti-CD70	Renal Cell Carcinoma	Phase I	[96]
AGS-22M6E	Anti-Nectin fully human IgG	Solid tumors	Phase I	[97]
PSMA ADC	Anti-PSMA fully human IgG1	Prostate cancer	Phase II	[98]

ADCs have also been prepared against the CD30 antigen on Hodgkin's disease and the LeY antigen on carcinomas. In vivo tests of anti-CD-30-MMAE (SGN-35), prepared with the VC peptide linker, showed no sign of toxicity in healthy mice up to a 30 mg/kg concentration even though a 1 mg/kg dose is known to be therapeutic to a xenografted mice model.[99] The human trials of SGN-35 produced high response rates; 75% for Hodgkin's lymphoma and 87% for anaplastic large cell lymphoma, and was approved by the Food and Drug Association (FDA) (Table 1.2).[95] Another interesting example of an ADC using the VC peptide linker for

Hodgkin's lymphoma is SGN-75. SGN-75 is a drug conjugated version of naked antibody SGN-70; a humanized anti-CD70 IgG1 mAb. CD70 is a member of a TNF superfamily that is transiently expressed on activated T- and B-lymphocytes, natural killer cells, and mature dendritic cells.[100-103]. In normal tissues, CD70 has very limited expression. However, in several malignancies, such as renal cell carcinoma and non-Hodgkin lymphoma cells, CD70 overexpression is observed.[104, 105] SGN-70 demonstrated antitumor activity, which was associated with dose-dependent antibody-dependent cell-mediated cytotoxicity.[106] Addition of a drug to this mAb, like MMAE through an enzyme-cleavable VC dipeptide linker, SGN-75, enhanced the anti-tumor activity in phase I studies.[96] (Table 1.2)

Biologic conjugate drug design, simplified by the knowledge and use of such effective peptide linkers, has seen an explosion of success. With such robust and specific enzymatically cleavable peptide linkers, the bottleneck for development of new ADCs has shifted away from linker design. Instead, discovery of new biomarkers specific to various cancer cells is now backlogging the future pipeline. Examples of ADCs on deck for success include ADCs against nectin-4 and PMSA. Nectin-4 is a transmembrane type I protein that has been discovered on epithelial cancer cells. The ADC prepared with VC linker of enfortumab vedotin binding to nectin-4 showed high anti-tumor efficacy in xenografted mice models of several solid tumors.[97]

The use of cancer associated enzyme-responsive peptide linkers for the delivery of anti-cancer agents significantly improved over the course of the last decade. ADCs, many of which are under clinical trials or already FDA approved, showed impressive success in cancer treatment. The specificity of peptides only to the cancer and prompt response potential, make these linkers superior linkers for the delivery of the drugs to the carcinogenic cells, while not

damaging the healthy cells. The studies on exploiting specific biomarkers and antigens for different types of cancer can improve the fight of humanity against cancer, with great help provided by enzyme-specific cleavable peptide linkers.

1.4 Peptides in supramolecular structures

A peptide's role in immunology and immunoengineering is not only critical, but also practical. Peptide antigens are an essential part of the immunologic response, and functional 'active' peptides make new technologies such as ADCs possible, as described above. Nonetheless, even with the previously described examples of successfully engineered systems, many complex diseases still remain, for which engineered immunotherapies do not exist. These diseases will require the activation of a complex and specific immune response not easily induced. Complementary to their antigenic and functional roles, peptides can also be a part of larger structures designed to present antigen or provide immunity-promoting secondary signals. Peptides themselves can self-assemble or be part of a self-assembled supramolecular particle through specific and engineered supramolecular interactions. These peptides in higher complexity structures can be used to instruct the immune system via a different approach than normal peptide antigen. These structures can provide signals to the immune system by (i) the compounds they present/encapsulate or (ii) by virtue of the structure or composition of the structure itself. While a more general overview of the uses of self-assembling peptides is discussed elsewhere in this textbook, here we would like to highlight in particular the role that peptides in supramolecular structures play in immunological applications.

1.4.1 Advantages of supramolecular assembly for immunological applications

In the field of immunoengineering, the antigenic function of peptides has inspired their incorporation into supramolecular assemblies. The resulting assemblies have been incredibly

diverse and elegant in design, employing peptides for a wide array of functions ranging from coatings to encapsulation to driving the assembly itself, among others. Because peptide supramolecular assemblies in particular can simultaneously incorporate immunologically active peptides while inducing an immunological response by virtue of the structure or composition of the assembly itself, these systems have commanded great interest from the research community.

The immune system is highly sensitive to inputs, requiring very specific signals to raise the correct immune response. The way an antigen is presented matters just as much as, if not more than, the antigen itself. Supramolecular assemblies offer the ability to provide this fine-tuned immunological response by rational modification of the moieties responsible for driving assembly in tandem with modification of the immunologically active peptides themselves. Through rational design, it is possible to modify the size, shape, and composition of the supramolecular assembly, as well as any cargos carried in the core or on the surface of the assembly. The fine control of the assembly's structure allows for the precise functioning of the assembly required to achieve the desired immune response. For example, because assemblies are composed of many monomers, supramolecular assemblies can be designed to act as potent adjuvants for their cargo or even exhibit self-adjuvanting properties to promote cellular or humoral response due to the local density of immunogenic moieties present on the surface of the assembly.[10, 107, 108] Furthermore, these multifunctional assemblies can be designed to precisely control the relative amount of each functional component of the assembly to fine-tune the resulting immune response.[109]

Through rational design, supramolecular assemblies are also capable of controlled, targeted localization, increasing the bioavailability of both the enclosed cargo and the assembly itself.[110] Localization is particularly crucial for immunological applications, as "on-target"

effects exhibited at undesired locations have been associated with severe toxicity.[19, 20] The use of targeting moieties in combination with nanoparticles can therefore mitigate the toxicity of immunomodulatory compounds while improving their ability to elicit desirable immune responses.

1.4.2 Peptide assemblies

Peptides themselves are capable of self-assembly into supramolecular structures. In the field of immunoengineering, self-assembling peptides have been used to create platforms capable of highly controlled presentation of antigen. Due to the variety of amino acid building blocks available, peptide assemblies in particular are highly modular and have extensive design capabilities. The assembling peptides can be designed to contain specific secondary structure to control the interactions between peptides, allowing for tuning of the shape and size of the assembly as well as the density of antigen presented at the surface. As a result, it is possible to achieve a more specific immune response, making peptide-peptide assemblies potentially useful as vaccines.

1.4.2.1 Assembly of peptides into fibers

One clever vaccine system relies on coiled-coil self-assembly of peptides. In this case, self-assembly is achieved through the detailed design of a linear peptide monomer. This monomer has three domains: a central "self-assembly" domain with two coiled-coil oligomerization sequences, flanked on the N and C terminus by the antigenic domains (antigen domain 1 – self-assembly domain – antigen 2 domain). The self-assembly sequence contains a pentameric and a trimeric coiled-coil sequence so that the monomer will not coil on itself, but coil only in the presence of other monomers under the correct conditions. The antigen domains are highly modular and can each accommodate multiple epitopes, allowing for great control of

overall epitope presentation. In a study utilizing this system as a malaria vaccine, by attaching a B cell epitope, a T helper epitope and a cytotoxic T cell epitope to this system, the assembly was able to generate a persistent humoral and cellular response to provide protection from malaria parasites for up to one year.[11]

Vaccines utilizing the self-assembly of β-sheet forming peptides into fibers have also been shown to provoke strong immune response.[15] The strong Hydrogen-bonding residues responsible for β-sheet formation permit the attachment of a wide variety of antigens to the assembling domains without disrupting supramolecular assembly of the β-sheets into fibers.[11] Fibrillar structures formed by self-assembling peptides seem to be potent adjuvants without exhibiting inflammatory responses on their own, making fiber assemblies a particularly useful delivery mechanism of vaccines.[107]

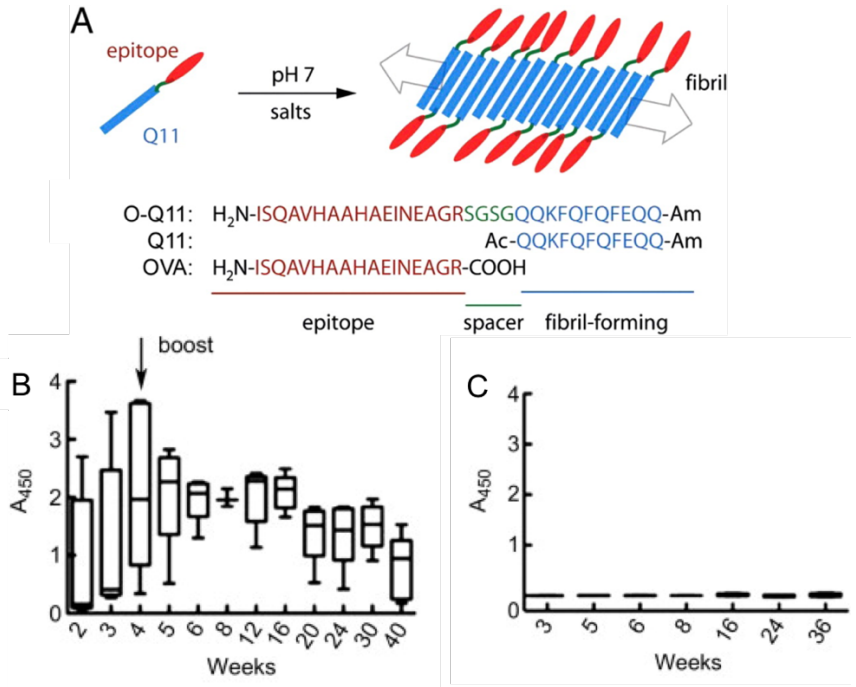

Figure 1.9 - β-sheet fiber-forming peptide vaccine platform. (a) Schematic representation of formation of Q11 fiber assembly and sequence of Q11-OVA, Q11, and ovalbumin epitope (OVA323-339). (b, c) Antibody response to $(NANP)_3$-Q11 (b) and soluble Q11 (c) in B6 mice. Reprinted from Rudra, J. S., et al.[108]; with permission from PNAS.

A prominent example of a β-sheet fiber-forming peptide tested as a potential vaccine platform is the "Q11" peptide reported by Rudra et al. The peptide (Ac-QQKFQFQFEQQ-am, Figure 1.9A) forms fibers 5-10 nm wide and up to one micron in length.[108] The fibers were nonimmunogenic, even when coadministered with mycobacterial Complete Freund's Adjuvant. However, when ovalbumin B- and T-cell epitope ($OVA_{323-339}$) were attached to Q11 fibers and administered to mice, high levels of IgG1, IgG2a and IgG3 antibodies against OVA developed without the use of adjuvant. Interestingly, when the hydrogen bonds driving Q11 self-assembly were disrupted, no immune response was induced. Without the self-assembly, the system becomes merely an injection of soluble Q11 peptide, which is known to have weak immunogenic properties. This suggests that the assembly itself was essential to the function of the vaccine.[107] The vaccine was further shown to induce strong humoral response against malaria parasite when the malaria peptide $(NANP)_3$ was coupled to Q11 (Figure 1.9B,C). [111] More recently, Rudra has shown that $OVA_{257-264}$-Q11 vaccine was capable of eliciting cytotoxic T-cell response in mice, raising the possibility of using the Q11 system in response to viral infections or tumors.[112] The modularity and tunability of Q11 peptide was demonstrated in an experiment in which fibers were formed via coassembly of the B-cell epitope E214-Q11 and the T-cell epitope PADRE-Q11.[109] Modulating the concentrations of each epitope changed the antibody and helper T-cell immune response to the vaccine in a manner dependent on the respective concentrations of each epitope. Furthermore, Chen et al. have demonstrated the safety of the OVA-Q11 vaccine, by showing that $OVA_{323-339}$-Q11 fibers did not induce inflammation at the site of injection nor cell death.[113]

One potential challenge facing the use of Q11 is that its fast fiber formation kinetics can lead to aggregation, which can reduce the potency of the Q11 constructs. To address this,

Hudalla et al. report the use of a peptide (MALKVELEKLKSELVVLHSELHKLKSEL) whose kinetics permit slow formation of a β-sheet, which they called a "β-tail" (Figure 1.10A-C). The slow assembly kinetics of the β-tail peptides was shown to improve their incorporation into Q11 fibers and reduce aggregate formation, providing a platform by which to incorporate antigens into the system. Hudalla proceeded to demonstrate that β-tails attached to fungal cutinase, when co-assembled with Q11, could raise antibody titers against cutinase. Furthermore, assemblies incorporating β-tail-cutinase and β-tail-GFP were able to raise titers against both proteins (Figure 1.10D). These observations suggest that the β-tail system could be used to incorporate multiple types of antigen, modifying the relative ratios of β-tail-antigen constructs to modulate the resulting immune response.[114]

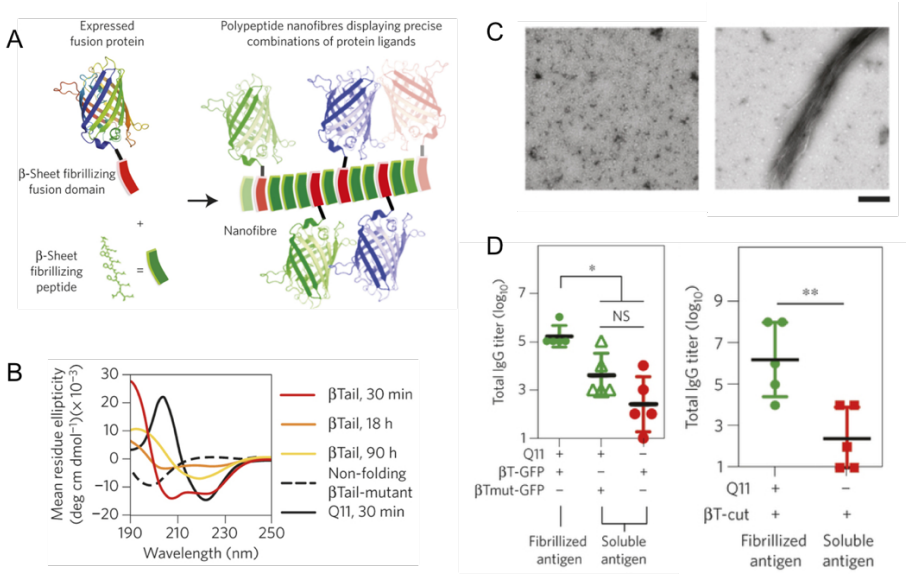

Figure 1.10 – Antigen delivery via β-tail peptides nanofibers. (a) β-tail formation from a fusion of a β-sheet fibrillizing peptide and an antigenic peptide. (b) Circular dichroism shows that β-tails undergo a slow transition to β-sheet structure, while Q11 transitions quickly and a non-functional β-tail mutant retains a random coil structure. (c) TEM images of β-tail peptide at 30 min (left) and 100 h (right). Scale bar = 200 nm. (d) Antibody titers of C57BL/6 mice injected with β-tail-GFP (left) or β-tail-cutinase (right) incorporated into Q11 fibers are greatly increased compared to freely soluble β-tail-antigen (red) or mutated β-tail/Q11 assemblies (left, empty triangles). *p<0.05, **p<0.01. Reprinted by permission from Macmillan Publishers Ltd: Nature Materials[114], copyright (2014).

1.4.2.2 Conjugated peptide assemblies

While hydrogen bonding is an important force that can drive self-assembly, it is not the only force at our disposal. The hydrophobic force is an excellent means of driving self-assembly of hydrophilic peptides. To promote self-assembly via the hydrophobic force, the peptide should contain a hydrophobic component, which could be a peptide sequence composed of hydrophobic amino acids, a hydrophobic polymer, or a lipid or acyl chain. The resulting molecule is capable of self-assembly into a monolayered spherical or cylindrical micelle, or a bilayered vesicle or liposome, depending on the respective properties of the hydrophilic peptide "head" and hydrophobic carbon "tail." For example, the peptide conjugate amphiphile W3K (C_{16}-WAAAAKAAAAKAAAAKA), designed by Shimada et al., self-assembled into spherical micelles in aqueous solution, but as the peptide's secondary structure transitioned from α-helix to β-sheet morphology over the course of days, the micelles gradually elongated to worm-like micelles.[115]

One advantage of the peptide-conjugate assembly strategy is the stabilizing effect it can have on the secondary structure of the peptide epitope. A peptide's secondary structure is a vital parameter of the specific binding of the peptide to its target, so this stabilizing effect generally strengthens the immunogenic function of the peptide. A platform reported by Tirrell et al. utilizes a peptide-dialkyl lipid (diC_{16}) conjugate with self-adjuvanting properties (Figure 1.11A). Using an elongated form of the OVA cytotoxic T-cell epitope ($OVA_{253-266}$, EQLESIINFEKLTE-diC16), the assembly induced a cytotoxic T-cell response that inhibited the growth of E.G7-OVA tumors in mice (Figure 1.11B).[10, 116] Another platform utilizing the J8 B-cell epitope against Group A Streptococcus (QAEDKVKQSREAKKQVEKALKQLEDKVQK-diC16) exhibited strong IgG and IgM antibody response (Figure 1.11C).[117, 118] Notably, the J8-diC16

construct did not require a helper T-cell epitope, whereas the Q11 systems did, though the cause of this difference is unknown.[107]

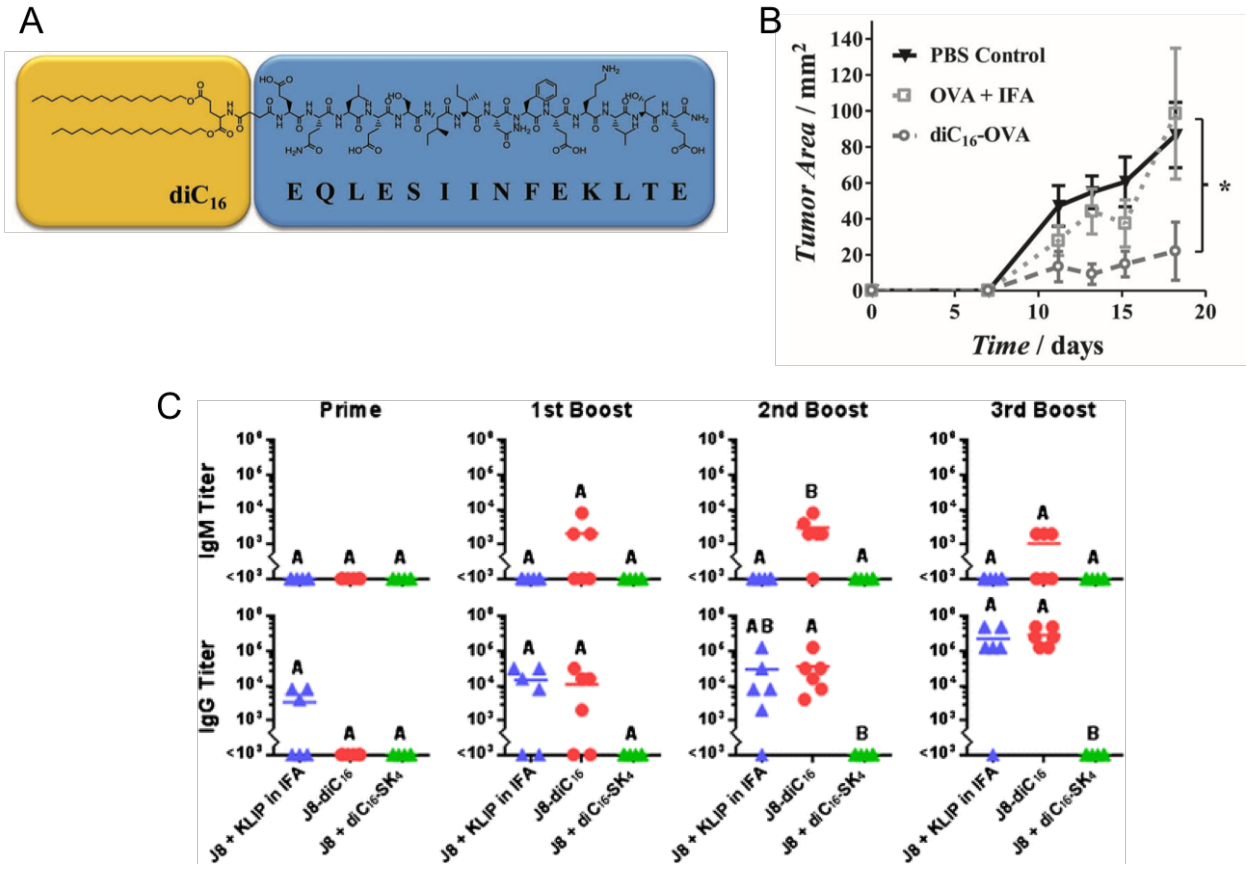

Figure 1.11 – Peptide amphiphile micelle vaccine and immunotherapy. (a) Structure of $OVA_{253-266}$-diC_{16}. (b) $OVA_{253-266}$-diC_{16} inhibits tumor growth in mice. IFA, Incomplete Freund's Adjuvant. *$p<0.05$. (c) J8-diC_{16} raises IgG and IgM antibody titers against Group A Streptococcus. Within each graph, different letters indicate statistically significant differences in mean ($p<0.05$), whereas a shared letter indicates no significance ($p>0.05$). (a,b) Reprinted from Black et al.[10]; Copyright (2012), with permission from Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (c) Reprinted from Trent et al.[117]; Copyright (2014), with permission from Springer.

Peptide amphiphiles are also highly modular. Because an antigenic peptide needs only to be hydrophilic and attachable to the hydrophobic component, a wide variety of antigens may be used. Furthermore, it is possible to synthesize a branched molecule capable of binding multiple antigens, allowing for mixed-antigen systems capable of a more comprehensive response. A

related platform utilizes self-assembling lipoamino acids, comprised of a fatty acid linked to two peptide sequences through the amine moieties of a lysine linker unit. Azmi et al. report the use of a lipoamino acid system utilizing the J14 peptide epitope (KQAEDKVKASREAKKQVEKAL-EQLEDKVK).[119] Building on this platform, Zaman et al. designed a lipopeptide vaccine against *streptococcus* utilizing a C_{16} lipid linked to P25 epitope and J14.[120]

1.4.3 Further delivery strategies

In addition to the use of engineered peptides to drive assembly, other supramolecular systems exist to stimulate immune response. These systems use structures such as viral proteins (as in virus-like particles) or synthetic polymers to form particles. The immunogenic cargo is then delivered attached to the surface or encapsulated within the particles. This section will briefly discuss the strategies utilizing virus-like particles and peptide encapsulation.

1.4.3.1 Virus-like particles (VLPs)

VLPs are a class of supramolecular assemblies composed of an underlying self-assembling structure coated with an antigen of interest. The goal of a VLP is to raise an immune response against the coated antigen, which can be almost anything (peptides, whole proteins, etc.). The self-assembly drivers of a VLP are viral envelope proteins known as capsids. Capsids do not contain any viral genetic material and are therefore noninfectious. Even though the capsid shell is noninfectious, it resembles a real pathogen and still contains conserved pattern associated molecular patterns (PAMPs) recognized by the innate immune system. This allows the VLP to induce strong innate and adaptive immune responses against the conjugated antigen without need for additional adjuvants.[121] In a sense, the VLP itself acts as the adjuvant. However, an unintended drawback of a VLPs innate immunogenicity is that an immune response will be raised against the VLP capsid proteins in addition to the coated antigen that is delivered with it.

Attaching antigens to VLPs therefore enhances the strength of the immune response, but the immunogenicity of the capsids themselves reduces the capacity for fine control of the resulting response. This contrasts with the supramolecular systems mentioned previously where an immune response is raised against the antigen only, not the self-assembling platform it employs (though as mentioned the self-assembled structure is required to raise an immune response). Currently, two VLP vaccines are used in the market: hepatitis B and human papillomavirus.

By modifying non-assembling viral proteins with functionalities that drive self-assembly into a virus-sized particle, one can construct synthetic virus-like-particles (SVLPs). Lipids are a common functionality to add to drive hydrophobic assembly, but the final structural shape often varies. Boato et al. synthesized SVLPs utilizing a sequence derived from respiratory syncytial virus, $HR1_{153-202}$, that organized into trimeric coiled-coils which in turn formed micelles (Figure 1.12).[122] These SVLPs were fused to HIV-1 peptide antigen, and subsequently induced antibody production without adjuvants in rabbits. Another recent study, by Robinson et al. used a similar SVLP to deliver B-cell and helper T-cell epitopes against malaria parasite subcutaneously to mice and rabbits, which were met with strong antibody responses in the animals.[123]

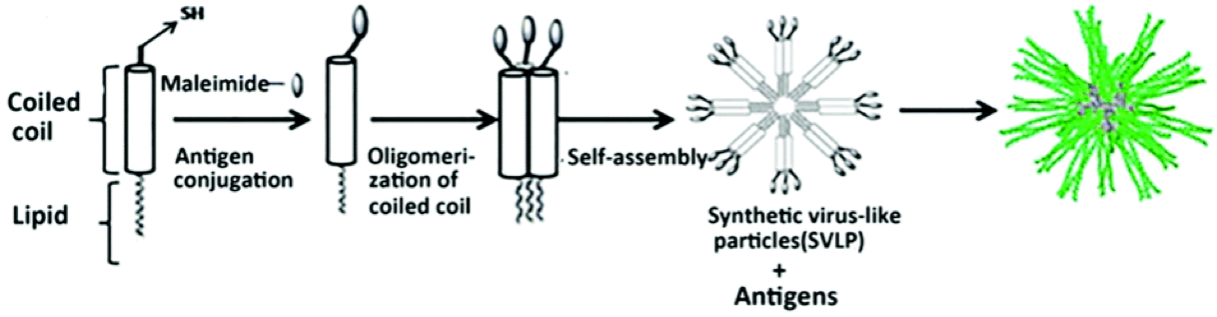

Figure 1.12 - Formation of synthetic VLPs by attaching antigens to self-assembling peptide-lipid conjugates. The resulting structure resembles a virus in size and shape. Reprinted from Yang, et al.[124]; Copyright (2016), with permission from The Royal Society of Chemistry.

1.4.3.2 Encapsulation of peptides in nanoparticle assemblies

An alternative strategy that uses self-assembled particles to deliver immunomodulatory agents is encapsulation. Encapsulation is a thoroughly developed field that utilizes many strategies to deliver antigens of interest (peptides, protein, cytokine, etc.), and has been thoroughly reviewed elsewhere.[125, 126] One primary advantage to encapsulation of antigens, including peptides, is their extended release profile, as freely soluble antigens generally have shorter lifetimes. For example, nanoparticles composed of the biodegradable synthetic diblock copolymer poly(lactic-co-glycolic acid)-b-poly(ethylene glycol) (PLGA-PEG) targeted to tissue injury sites via collagen IV targeting peptide and loaded with the anti-inflammatory peptide Ac2-26 (AMVSEFLKQAWFIENEEQEYVQTVK) preferentially targeted sites of tissue injury and reduced neutrophil recruitment at those sites to promote healing via sustained release on long timescales (Figure 1.13).[55]

A particular class of drug-encapsulating self-assembling particle is the interbilayer-crosslinked multilamellar vesicle (ICMV). ICMVs are multilamellar lipid vesicles that have been stabilized by covalently crosslinking the headgroups of the lipid assembly to the peptide adjuvant loaded within. The covalent bonds increase the density of loaded peptides while also prolonging the release time of encapsulated cargo. In a system reported by Moon et al., the malaria antigen VMP001 was cross-linked with phospholipid vesicles to form ICMVs. The increased density of VMP001 in the ICMVs, in combination with a long release time of two weeks, produced robust immune responses against VMP001 in mice that lasted for over one year.[127]

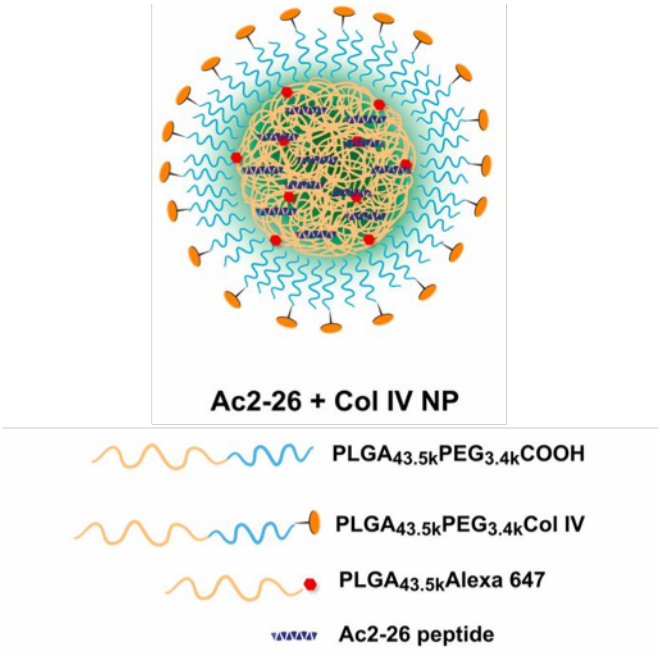

Figure 1.13 - Targeted (Col IV) NPs encapsulating the Ac2-26 peptide were developed using biodegradable polymers via a single-step nanoprecipitation method. The synthesized polymers and Ac2-26 peptide were dissolved in acetonitrile (total polymer 3 mg/mL), and 2% (wt/wt) of the fluorescent PLGA-Alexa 647 was added to all formulations. NPs contained 4% (wt/wt) peptide, and 5% (wt/wt) of the Col IV peptide-conjugated targeting polymer. The organic mixture containing the polymers and peptide was then added dropwise to nuclease-free water (10 mL). The solution was stirred for 2–4 h, and the particles were filtered, washed, and resuspended in water or PBS. Reprinted from Kamaly et al.[55]; with permission from PNAS.

1.4.4 Future directions

One major challenge remaining for fiber and cylindrical assemblies is control of their length. While spherical assemblies tend to have well-defined monomer numbers as a result of the properties of the monomer subunits, cylindrical assemblies have a defined cross-radius but poorly defined length. However, overall, supramolecular assemblies present great opportunities in immunomodulation due to their ability to aggregate epitopes, antigens and other immunomodulatory agents into one system in modular fashion. This modularity lends precision to the future engineering of supramolecular assemblies to modulate immune response.

Table 1.3 – Summary of peptide sequences and applications mentioned in Chapter 1

Peptide application	Name of peptide: sequence	Reference
Anti-inflammatory peptide	Ac2-26: Ac-AMVSEFLKQAWFIENEEQEYVQTVK	[51-54]
Neutrophil receptor (FcγRIIA) binding peptide	FcγRIIA binding peptide: WAWVWLTETAV	[56]
Dendritic cell (CD11c/CD18) binding peptide	p30: CGRWSGWPADLC	[59, 60]
M cell binding peptides	Co1: SFHQLPARSPLP; P8: LETTCASLCYPS; P25: VPPHPMTYSCQY	[62, 63]
Erythrocyte binding peptide	ERY1: H_2N-WMVLPWLPGTLDGGSGCRG-$CONH_2$	[71]
Beta sheet fiber forming peptide	Q11: Ac-QQKFQFQFEQQ-am	[108]
Tail added to Q11 in order to incorporate antigens and slow assembly kinetics	β-tail: MALKVELEKLKSELVVLHSELHKLKSEL	[114]
Secondary structure transitioning peptide	W3K: C16-WAAAAKAAAAKAAAAKA	[115]
Chicken egg model antigen	OVA(253-266): EQLESIINFEKLTE	[10]
Group A Streptococcus epitope	J8: QAEDKVKQSREAKKQVEKALKQLEDKVQK	[117]
Group A Streptococcus epitope	J14: KQAEDKVKASREAKKQVEKALEQLEDKVK	[119]
Trimeric coiled-coil peptide derived from respiratory syncytial virus	HR1(153-202): AVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSV LTSKVLDLKNYIDKQ	[122]

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2 Peptide amphiphile micelles self-adjuvant Group A Streptococcal vaccination

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2.1 Summary

Delivery system design and adjuvant development are crucially important areas of research for improving vaccines. Peptide amphiphile micelles are a class of biomaterials that have the unique potential to function as both vaccine delivery vehicles and self-adjuvants. In this study, peptide amphiphiles comprised of a Group A Streptococcus B cell antigen (J8) and a dialkyl hydrophobic moiety (diC_{16}) were synthesized and organized into self-assembled micelles, driven by hydrophobic interactions among the alkyl tails. J8-diC_{16} formed cylindrical micelles with highly α-helical peptide presented on their surfaces. Both the micelle length and secondary structure were shown to be enhanced by annealing. When injected into mice, J8-diC_{16} micelles induced a strong IgG1 antibody response that was comparable to soluble J8 peptide supplemented with two classical adjuvants. It was discovered that micelle adjuvanticity requires the antigen be a part of the micelle since separation of J8 and the micelle was insufficient to induce an immune response. Additionally, the diC_{16} tail appears to be non-immunogenic since it does not stimulate a pathogen recognition receptor whose agonist (Pam_3Cys) possesses a very similar chemical structure. The research presented in this paper demonstrates the promise peptide amphiphile micelles have in improving the field of vaccine engineering.

2.2 Introduction

Streptococcus pyogenes (Group A Streptococcus, GAS) is a Gram-positive bacterium restricted to natural growth in humans where it frequently elicits diseases that range in severity

from mild infections of the pharyngeal mucosa and dermis to life-threatening invasive infections of connective and muscle tissues leading to necrotizing fasciitis, myonecrosis, and toxic shock [1]. Additionally, post-infection sequelae diseases like acute rheumatic fever and glomerulonephritis can arise following localized infections of the nasopharynx and skin. Epidemiological studies estimate that each year greater than 500,000 worldwide deaths are attributable to GAS infections, placing it among the top ten leading causes of death from infectious pathogens [2, 3]. In the United States alone, more than $600 million is spent annually treating diseases caused by this organism [4] with no effective preventative method established short of prophylactic antibiotic usage.

Despite decades worth of research, vaccines against GAS remain commercially unavailable [5]. The primary barriers preventing the successful development of a vaccine include variability of surface proteins [6, 7] and the autoreactivity of antibodies raised against GAS proteins like the highly immunogenic M protein [8]. Safety concerns over protein-based GAS vaccine candidates have been addressed by utilizing peptide antigens from conserved protein domains that do not generate cross-reactive antibodies to host tissues. Specifically, the J8 peptide is a 29 amino acid sequence (QAEDKVKQSREAKKQVEKALKQLEDKVQK) from the C-terminal domain of the GAS M1 protein (M-$5_{336\text{-}364}$) which possesses a conformationally-dependent B cell epitope (SREAKKQVEKAL) and has been found to induce an opsonophagocytic, high-titer antibody response in mice [9, 10] that does not react with human cardiac tissues [11, 12].

Peptides are attractive vaccine candidates since they are typically safer than whole pathogen vaccines, but they are often weak immunogens [13]. To enhance the corresponding host immune response, peptide vaccines are often delivered by aid of a delivery vehicle or with

immune boosting substances termed adjuvants. While promising experiments have been published that employ J8 peptide delivery vehicles [14, 15] or adjuvanted J8 peptide [10, 16], this research has yet to lead to a commercially viable GAS vaccine, so novel systems need to be explored. An effective construct should concentrate the peptide antigen, protect it from degradation, enhance its cellular uptake, and adjuvant its immunogenicity in order to induce a robust immune response [17, 18].

Peptide amphiphiles are a class of biomaterials comprised of peptide-lipid conjugates that undergo self-assembly into micelles in water. They have been shown capable of delivering biologically active peptides for a variety of applications including angiogenesis [19, 20], osteogenesis [21, 22], neurogenesis [23, 24], artherosclerosis treatment [25], cancer therapy [26, 27] and Islet transplantation [28, 29]. Also, peptide amphiphile micelles are comprised of a high concentration of peptide [30], inhibit peptide degradation [31], and can greatly increase peptide intracellular delivery [32]. Recent research by the Tirrell Group has shown that peptide amphiphile micelles that display a tumor-specific cytotoxic T cell epitope can function as a self-adjuvanting vaccine delivery system capable of inducing a tumor suppressing immune response when given prophylactically [33]. In this report, the potential for peptide amphiphile micelles to act as a self-adjuvanting platform for the delivery of a GAS peptide vaccine was investigated.

2.3 Materials and Methods

Peptide and Peptide Amphiphile Synthesis: J8 peptide (QAEDKVKQSREAKKQVEKAL-KQLEDKVQK) was synthesized on Rink amide MBHA resin (Novabiochem) utilizing standard Fmoc solid phase synthesis with the aid of a PS3 Peptide Synthesizer (Protein Technologies, Inc.). The resulting J8 peptide was treated using a concentrated trifluoroacetic acid solution to deprotect side groups and cleave the peptide from resin. High pressure liquid chromatography

with mass spectrometry controlled fraction collection (LCMS; Shimadzu Corp.) utilizing a reversed-phase C8 column (Waters) with a gradient of acetonitrile in Milli-Q water containing 0.1% formic acid was employed to purify J8 peptide. For J8 peptide amphiphiles, the hydrophobic moiety dipalmitoylglutamic acid (diC_{16}) was synthesized by a previously established method [34]. J8 peptide was synthesized similarly to above except the C-terminal lysine was protected with DDE instead of Boc which was used for the other lysines. The peptide was treated with 2% hydrazine in DMF to orthogonally deprotect the C-terminal lysine amine group which was covalently coupled to diC_{16} by an amidation reaction yielding J8-diC_{16} peptide amphiphiles. J8-diC_{16} was further processed and purified by the same methods as the J8 peptide. All samples were lyophilized and stored at -20°C until used. It should be noted that all peptide and peptide amphiphiles were created in a chemical synthesis laboratory using appropriate personal protective equipment to eliminate exposure to biological contaminants.

Micelle Formation and Characterization: To fabricate micelles, J8-diC_{16} peptide amphiphiles were film cast by dissolving them in methanol and evaporating the solvent using nitrogen as a drying gas. Hydration of the films with water or phosphate-buffered saline (PBS) followed by thorough vortexing induced spontaneous micelle formation. The micelles were then allowed to equilibrate overnight. J8-diC_{16} micelles were characterized by previously defined methodologies [33, 35, 36] including critical micelle concentration (CMC) analysis, transmission electron microscopy (TEM), and circular dichroism (CD). CMC was measured by fluorescent sequestration where varying concentrations of J8-diC_{16} were exposed to 1 mM 1,6-diphenyl-1,3,5-hexatriene (DPH) which greatly increases in fluorescence intensity when trapped within the micelle core. Solutions were prepared and allowed to equilibrate for 1 h prior to fluorescent measurement utilizing a Tecan Infinite 200 plate reader (ex. 350 nm, em. 428 nm). The data were

fit with two trendlines which were set equal to one another to determine the fluorescence inflection point (*i.e.* CMC). Micelle morphology was investigated using negative stain TEM. J8-diC_{16} solution (1 uL of 200 μM) was allowed to incubate on Formvar-coated copper grids (Ted Pella, Inc.) for 1 min after which excess liquid was wicked away with filter paper. Grids were then washed with Milli-Q water and then incubated with aqueous phosphotungstic acid (1 wt %) for 1 min before the solution was wicked away. Samples were allowed to air dry and then imaged on a FEI Tecnai 12 TEM using an accelerating voltage of 120 kV. The secondary structure of J8 peptide in solution and confined within the corona of J8-diC_{16} micelles was assessed using CD. CD spectra of 30 μM solutions of J8 and J8-diC_{16} were measured in water at 25°C a total of 5 times and the data was averaged. The data presented represents CD analysis performed at least 3 times per sample. Water was used alternatively to PBS since chloride ions from the PBS can interfere with CD measurements. The data were averaged and a curve from 190 nm to 250 nm was fit using a linear combination of polylysine basis spectra [37] to determine approximate α-helix, β-sheet and random coil peptide secondary structure.

Micelle Annealing and Characterization: To investigate the effect of annealing on micelles, J8 peptide and J8-diC_{16} micelle solutions were prepared as described above. Preliminary experiments employing differential scanning calorimetry showed that J8-diC16 micelles anneal at 40ºC and undergo an irreversible transition at 70ºC as evidenced by the fact that further heat-cool annealing cycles showed no such transition behavior. To ensure annealing has gone to completion, J8 peptide and J8-diC_{16} micelle solutions were heating to 70°C for 1 h and allowed to cool back to room temperature and equilibrate overnight. Micelles were characterized for morphometric changes by TEM using the methodology described previously. Secondary structure of the peptide in solution and on the micelle surface was assessed by CD.

Murine Vaccination: Female BALB/c mice 6-8 weeks old were purchased from Charles River and immunized to investigate the capacity for micelles to induce an antibody-mediated immune response. Previous research has shown that the J8 peptide is a weak immunogen which requires adjuvants to be effective [38]. The strong physical adjuvant Imcomplete Freund's Adjuvant (IFA) was purchased from Sigma-Aldrich. For control groups, a universal T helper peptide antigen termed KLIP (KLIPNASLIENCTKAEL) [39] and a mock peptide amphiphile (diC_{16}-SK_4) were synthesized and purified in the lab similarly to methods detailed above. To confirm that diC_{16}-SK_4 formed micelles, micelle characterization was conducted and a CMC of 4.53 μM was determined (data not shown). J8-diC_{16} and diC_{16}-SK_4 micelles used for vaccination were fabricated by the film deposition, rehydration, and annealing method outlined above. Mice were vaccinated in the nape of the neck at days 0 (prime), 21 (boost 1), 28 (boost 2), and 35 (boost 3) with one of three vaccine formulations:

1) J8 + KLIP in IFA (Positive Control Vaccine) – 12 nmol J8 peptide + 1.33 nmol KLIP in 50 μL IFA and 50 μL PBS
2) J8-diC_{16} (Micelle Vaccine) – 12 nmol J8-diC_{16} in 100 μL PBS
3) J8 + diC_{16}-SK_4 (Mock Micelle Vaccine) – 12 nmol J8 peptide + 12 nmol diC_{16}-SK_4 in 100 μL PBS

Whole blood was collected from mice saphenous veins pre-vaccination on days 21, 28, and 35 as well as on day 42 to analyze for circulating J8-specific antibody response induced by the previous round of immunization. The blood was centrifuged at 10,000 RPM for 10 min to

separate out red blood cells and the supernatant serum was harvested and stored at -20°C until analysis.

Antibody Response Characterization: An enzyme-linked immunosorbent assay (ELISA) was utilized to determine J8-specific antibody titers. Flat-bottom 96-well EIA microtiter plates (Costar) were coated overnight with 100 µL of 10 µg/mL J8 peptide in sodium bicarbonate coating buffer in each well at 4°C. The wells were washed with 200 µL of 0.05% Tween 20 in PBS (PBS-T) three times and then blocked with 200 µL of assay diluent (10% FBS in PBS) for 1 h. The blocking solution was removed and 100 µL of 1:1000 diluted sera samples were added to the top row and then serially diluted two-fold with assay diluent down the plate. After 2 h incubation, wells were washed with PBS-T three times and incubated with 100 µL of 1:3000 diluted detection antibody (IgM, IgG, IgA, IgG1, IgG2a, IgG3, or IgG4; Invitrogen) for 1 h. PBS-T was used to wash wells three times after which 100 uL of Ultra TMB-ELISA substrate solution (Pierce) was added to the wells. Plates were allowed to incubate for 15 minutes in darkness and then optical density (OD) was measured for each well at 650 nm using a Tecan Infinite M200 plate reader. Endpoint antibody titers were defined as the greatest serum dilution where OD was at least twice that of normal mouse serum at the same dilution.

TLR-2 Stimulation: TLR-2 stimulation activity was characterized similarly to a previously established technique [33]. HEK-293 cells transfected to express the TLR-2 receptor on their surface which when stimulated causes a luciferase reporter gene to fluoresce were generously provided as a gift by Professor Greg Barton. The cells were seeded at 10^4 cells per well in a 96-well plate in complete culture media (Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 4.5 g/L glucose, 4 mM L-glutamine, 1 mM sodium pyruvate, 100 µg/mL streptomycin, 100 units/mL penicillin, and 10 µg/mL geneticin) and

allowed to incubate stimulus free for 16 h. Cells were exposed to PBS, 10 μM J8 peptide, 10 μM J8-diC16, or 1 μM synthetic triacetylated lipoprotein (Pam_3Cys-SK_4, Invitrogen), a known TLR-2 agonist and incubated for 6 h. The Promega luciferase assay system was used according to manufacturer instructions and luminescence was measured at 560 nm using a Tecan Infinite M200 plate reader.

Statistical Analysis: JMP software (SAS Institute) was used to make comparisons between groups using an ANOVA followed by Tukey's HSD test to determine pairwise statistically significant differences ($p < 0.05$). Within the figure graphs, groups that possess different letters have statistically significant differences in mean whereas those that possess the same letter are similar.

2.4 Results

2.4.1 Synthesis and Characterization of GAS Vaccine Peptide Amphiphile Micelles

With the J8 peptide sequence possessing a C-terminal lysine, the hydrophobic dialkyl tail moiety (diC_{16}) was able to be covalently tethered to the peptide via an amide bond yielding J8-diC_{16} peptide amphiphile (Figure 2.1**a**). J8-diC_{16} amphiphiles were film cast by methanol evaporation and rehydrated to determine if they could self-assemble into micelles. DPH sequestration evident by an exponential increase in fluorescence as a function of increasing peptide amphiphile concentration indicated that J8-diC_{16} forms micelles and does so at a critical micelle concentration (CMC) of 1.71 μM (Figure 2.1**b**). Negative stain transmission electron microscopy (TEM) revealed that J8-diC_{16} formed short cylindrical micelles approximately 5 – 15 nm in diameter and 25 – 125 nm in length (Figure 2.1**c**). In order to determine if micellization affected the J8 peptide, circular dichroism (CD) was employed to determine secondary structure for J8 peptide in solution and in micelles (Figure 2.1**d**). Interestingly, tethering of J8 peptide

within the micelle corona significantly decreased peptide random coil structure while greatly increasing its α-helicity from 0% to 42.5%.

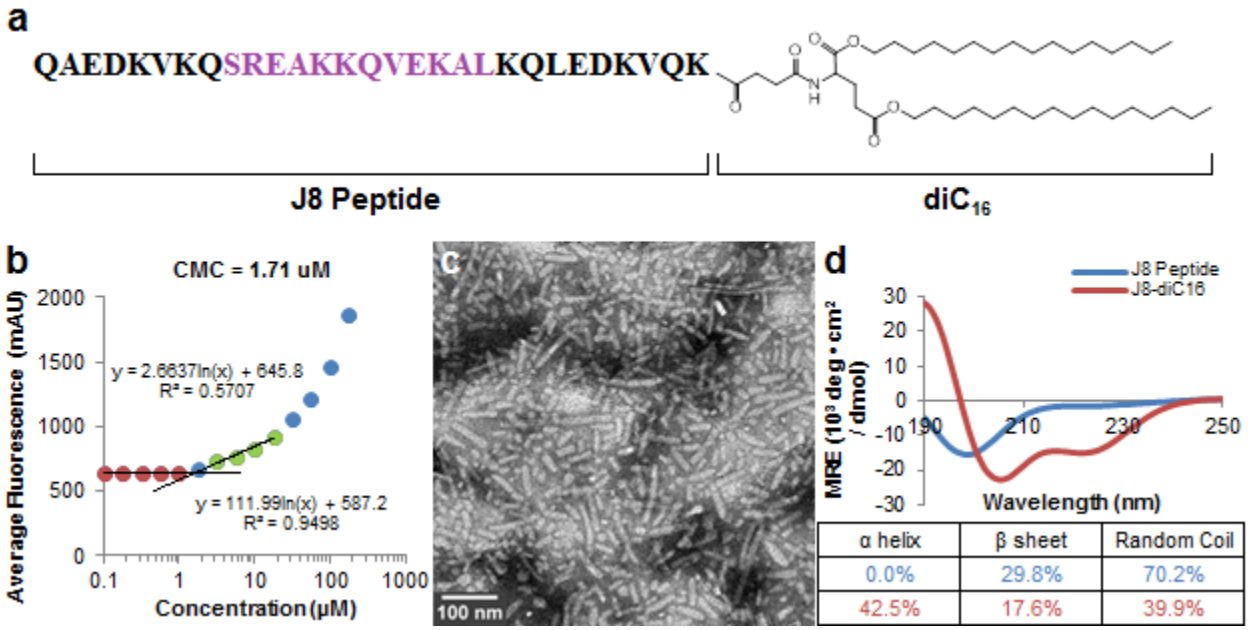

Figure 2.1 – Physical characterization of self-assembled GAS vaccine peptide amphiphile micelles. (**a**) J8-diC_{16} peptide amphiphile structure with the B cell antigen shown in purple. (**b**) The capacity for J8-diC_{16} to form micelles was evaluated by a critical micelle concentration (CMC) assay and a CMC of 1.71 μM was calculated. (**c**) TEM of J8-diC_{16} revealed that PAs form short, rigid, cylindrical micelles. (**d**) CD showed that J8 peptide confined within the corona of micelles possessed altered secondary structure. Curve fitting revealed J8 peptide in solution had no detectable α-helicity whereas micelle-based J8 peptide exhibited significant α-helicity.

2.4.2 Annealing Modulates GAS Vaccine Peptide Amphiphile Micelle Structure

Previous research has shown that micelle morphology and structure can change over time [40] which can be expedited by annealing [41, 42]. After heating J8-diC_{16} to 70°C and allowing it to cool to room temperature, TEM revealed that long, flexible, cylindrical micelles had been formed approximately 5 – 15 nm in diameter and 200 nm – 2 um in length (Figure 2.2**a**). CD revealed that annealing J8 peptide in solution and confined within micelles yielded 0% and 50.5% α-helicity, respectively (Figure 2.2**b**).

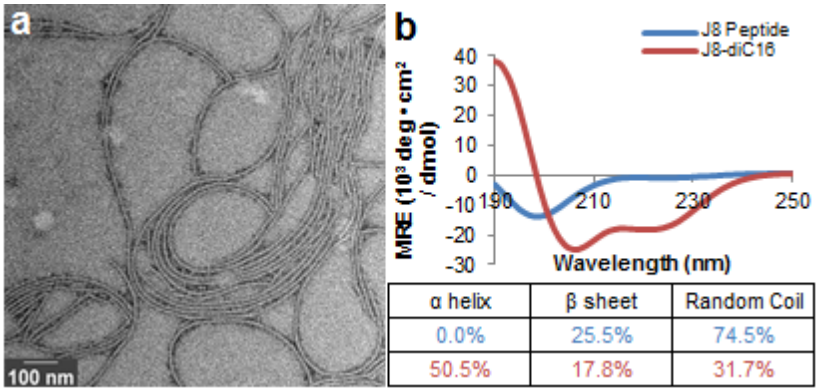

Figure 2.2 - Annealing GAS vaccine peptide amphiphile micelles altered their physical structure. (**a**) TEM of J8-diC_{16} heated and then cooled to room temperature showed micelles transitioned from short, rigid, cylindrical micelles to long, flexible, cylindrical micelles. (**b**) CD revealed that annealing did not alter the α-helicity of J8 peptide in solution, but did appreciably increase the α-helicity of micelle-based J8 peptide.

2.4.3 GAS Vaccine Peptide Amphiphile Micelles Induce Strong Immune Responses

Mice were subcutaneously vaccinated with either GAS vaccine peptide supplemented with conventional adjuvants (J8 + KLIP in IFA), GAS vaccine peptide amphiphile micelles (J8-diC_{16}), or GAS vaccine supplemented with mock micelles (J8 + diC_{16}-SK_4). Harvested serum samples were analyzed by ELISA to determine J8-specific antibody isotype titers (Figure 2.3). Only the J8-diC_{16} vaccine was able to induce appreciable IgM titers which were found to be significantly higher than both J8 + KLIP in IFA and J8 + diC_{16}-SK_4 vaccines after the 2nd boost vaccination. Both J8 + KLIP in IFA and J8-diC_{16} vaccines were able to cause antibody isotype switching as shown by their induction of appreciable IgG titers by the 3rd boost and 2nd boost vaccinations, respectively. The J8 + diC_{16}-SK_4 vaccine did not induce any IgM or IgG antibodies. No vaccine treatment induced an IgA response (data not shown).

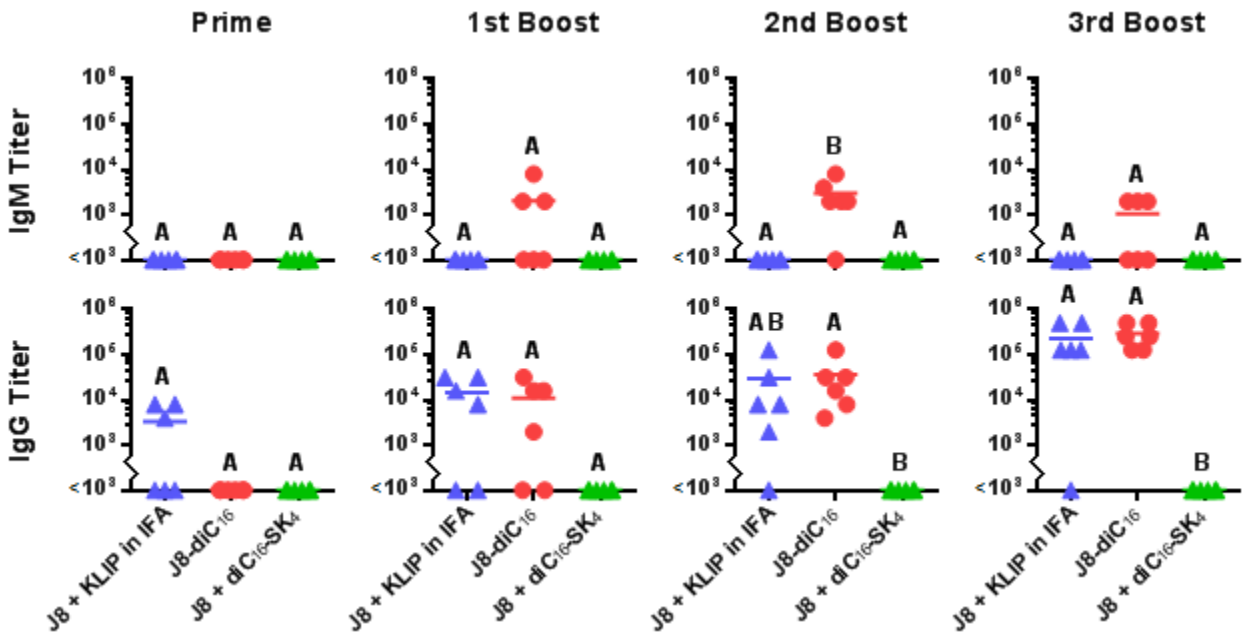

Figure 2.3 - GAS vaccine peptide amphiphile micelles induced strong antibody isotype responses *in vivo*. Only mice given the J8-diC_{16} vaccine produced appreciable IgM titers. Mice given J8 + KLIP in IFA or J8-diC_{16} vaccines produced similar quantities of IgG antibody that directly correlated to the number of vaccinations. In contrast, no antibody titers were observed for J8 + diC_{16}-SK_4 vaccinated mice. While IgA titers were assessed, no mouse produced above background levels. Each point represents one mouse (N = 6); bars represent the mean. Within a graph, groups that possess different letters have statistically significant differences in mean ($p \leq 0.05$) whereas those that possess the same letter are similar ($p > 0.05$).

To further investigate the IgG response induced, ELISAs were conducted on the serum samples to determine IgG subtype titers (Figure 2.4). The data show that IgG1 is the dominant antibody with either J8 + KLIP in IFA or J8-diC_{16} vaccine. A few mice vaccinated with J8 + KLIP in IFA showed appreciable IgG2a titers and a couple of mice given J8-diC_{16} had above background IgG3 titers, but neither of these results was significant. IgG4 was also assayed for but no titers were observed (data not shown).

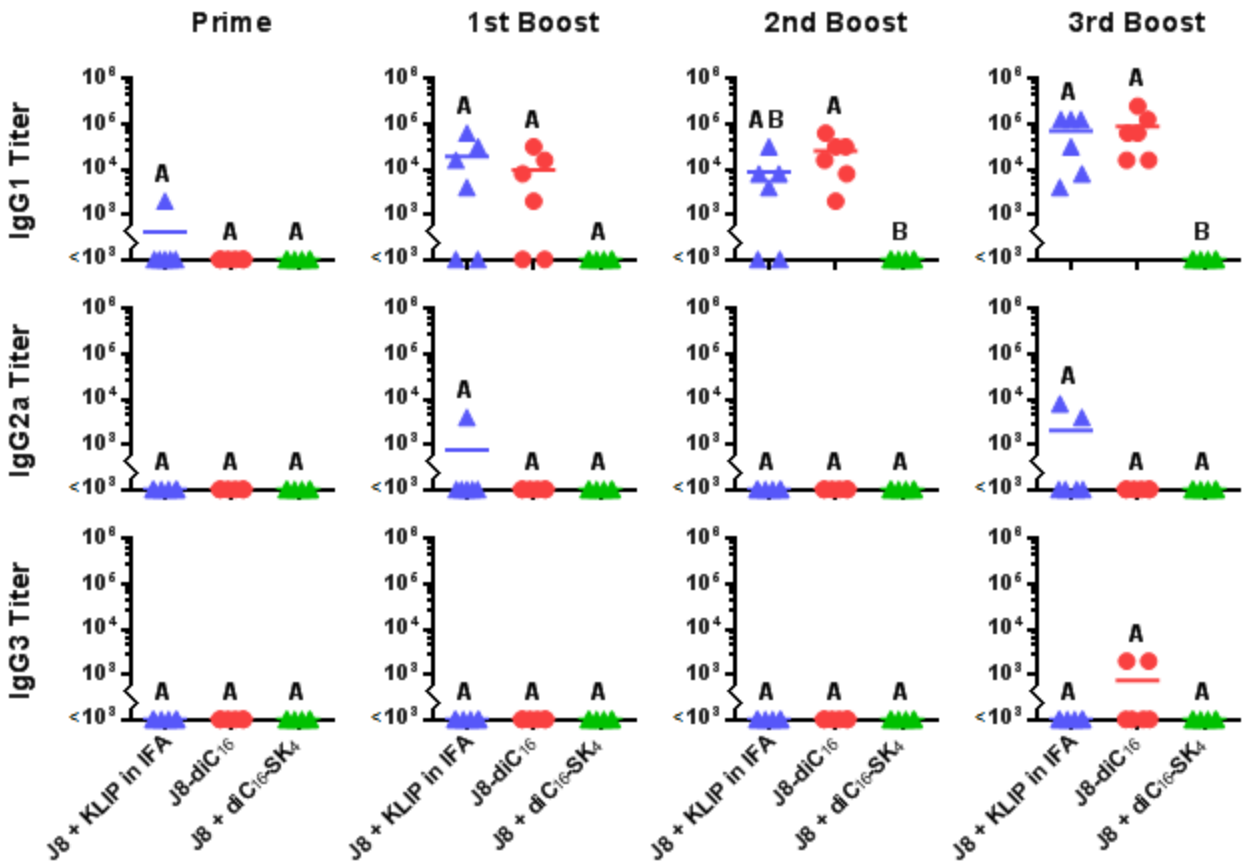

Figure 2.4 - GAS vaccine peptide amphiphile micelles induced strong IgG1-dominant antibody subtype responses *in vivo*. The strong IgG response induced by the J8 + KLIP in IFA or J8-diC_{16} vaccines was found to be predominantly comprised of the IgG1 subtype. While a few mice possessed above background titers for IgG2a and IgG3 dependent on the vaccine formulation they were given, these responses were not statistically significant. Also, no mouse produced above background levels of IgG4 titers. Each point represents one mouse (N = 6); bars represent the mean. Within a graph, groups that possess different letters have statistically significant differences in mean ($p \leq 0.05$) whereas those that possess the same letter are similar ($p > 0.05$).

2.4.4 GAS Vaccine Peptide Amphiphile Micelle Adjuvanticity is Not TLR-2 Mediated

The hydrophobic moiety diC_{16} possesses a similar chemical structure to the known adjuvant Pam_3Cys (Figure 2.5**a**) which is a TLR-2 agonist. To determine if TLR-2 stimulation was responsible for J8-diC_{16} micelle self-adjuvanticity, TLR-2 expressing, luciferase reporter HEK-293 cells were treated *in vitro* with PBS, J8 peptide, J8-diC_{16} micelles, or Pam_3Cys-SK_4 (Figure 2.5**b**). Incubation of cells with PBS or J8 peptide induced no fluorescence indicating an

absence of TLR-2 stimulation compared to the positive Pam_3Cys-SK_4 control. Cells incubated with J8-diC_{16} micelles had similar fluorescence levels to cells exposed to PBS and J8 peptide implying the micelles did not stimulate TLR-2.

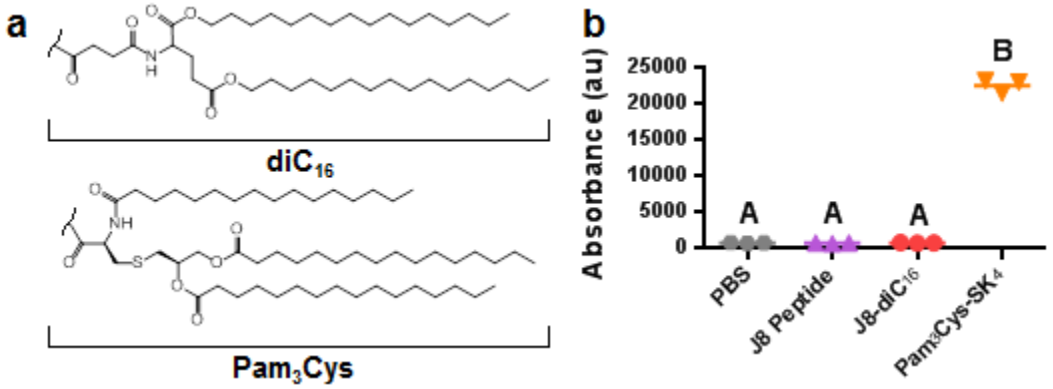

Figure 2.5 - J8-diC_{16} micelles do not stimulate TLR-2 *in vitro*. (**a**) Chemical structure similarity between diC_{16} and Pam_3Cys, a known TLR-2 stimulant. (**b**) HEK-293 cells transfected to express TLR-2 on their surface and luminesce when the TLR-2 is stimulated were incubated with PBS, 10 µM J8 peptide, 10 µM J8-diC_{16} micelles, or 1 µM Pam_3Cys. Only cells incubated with Pam_3Cys strongly fluoresced whereas cells exposed to J8-diC_{16} had similar fluorescence to the PBS and J8 peptide controls. Within a graph, groups that possess different letters have statistically significant differences in mean ($p \leq 0.05$) whereas those that possess the same letter are similar ($p > 0.05$).

2.5 Discussion

Vaccines are the most effective method for the prevention of pathogenic infections, yet a viable vaccine that protects against GAS has yet to make it to the market. While previous research employing the J8 peptide vaccine to induce a protective antibody response has been promising [9-12], common issues associated with peptide vaccines (*i.e.* immunogenicity, conformational dependence, localized high concentration delivery, and adjuvant supplementation) have kept it from moving into human clinical trials. The design of a self-adjuvanting, delivery device that overcomes these problems could yield the first commercial vaccine against GAS. While a variety of systems could be designed, micelles possess several advantages over other nanoparticle-based systems. Micelles are water soluble which makes them

easy to deliver via injection in comparison to non-soluble particles like polymeric or metallic nanoparticles that must be suspended [43, 44] increasing solution viscosity and which have a tendency to agglomerate complicating their injectability [45, 46]. Also, since the micelle delivery device is comprised of the peptide itself, it is more than 80% peptide vaccine by weight which is considerably higher than the ~25% maximum peptide loading possible with other nanoparticle delivery devices [47]. Previous research has shown peptide amphiphile micelles presenting a cytotoxic T cell epitope act as self-adjuvants that induce a strong immune response [33], so the same platform technology was tested for its ability to facilitate an antibody-mediated response.

J8-diC_{16} peptide amphiphiles were synthesized and readily formed micelles, which possessed several desirable physical characteristics. Each J8-diC_{16} micelle shown in Figure 2.1**c** traps hundreds to thousands of peptides together which greatly increases local antigen concentration upon interaction with immune cells compared to soluble peptide which can rapidly disseminate from the injection site. Also, many B cell epitopes including J8 are conformationally-dependent meaning their secondary structure is crucial for their immunogenicity [48]. A major issue with conformationally-dependent peptide antigens is that since they lack the tertiary structure restraints of their native protein they often lose their secondary structure. This is particularly evident with the J8 peptide which is highly α-helical in the context of the M1 protein [49], but possesses no α-helicity when isolated and in solution (Figure 2.1**d**). Interestingly, confining and crowding the peptide within the micelle corona gives an artificial tertiary structure that facilitates J8 peptide becoming highly α-helical (42.5%, Figure 2.1**d**) which agrees with previous research that has shown the capacity of peptide amphiphile micelles to reestablish native peptide secondary structure [50-52]. Annealing the micelles induced them to form much longer structures (Figure 2.2**a**) with even greater α-helicity (50.5%,

Figure 2.2**b**). This modulation allows for each micelle to deliver an order of magnitude greater number of peptides (thousands to tens of thousands) with nearly 20% more of them in the correct conformational state. It should also be noted that micelle peptide structure and elongation are interrelated phenomena. The increase in temperature allows for greater peptide folding into the native α-helical structure [51, 53] which compacts the peptide head group of the amphiphile decreasing the overall molecular packing parameter [54] and allowing for longer micelle lengths.

Soluble J8 peptide supplemented with the strong physical adjuvant IFA and the universal T helper epitope KLIP induced a strong IgG response in mice after prime immunization followed by three booster immunizations (Figure 2.3) While IFA has been used clinically in the past, there now exists serious concerns that it may cause autoimmunity [55, 56] and minimize the effectiveness of induced immune cells [57]. Vaccine adjuvanting systems capable of mediating similar responses to IFA have the potential to move peptide-based vaccines to the market. Annealed J8-diC_{16} micelles were found to induce a strong J8-specific antibody response comparative to the J8 + KLIP in IFA control vaccine formulation (Figure 2.3). In specific, J8-diC_{16} micelles induced the production of IgM that peaked after the 2^{nd} booster vaccination and isotype switching that led to IgG titers that increased with each round of vaccination which is characteristic of a classical antibody-mediated vaccination response [58]. Further investigation into the specific nature of the J8-diC_{16} induced IgG response revealed that it was almost entirely comprised of IgG1 subtype (Figure 2.4) which has been found to facilitate complement activation and macrophage engulfment as well as protect against GAS infections [59]. These results provide considerable evidence that peptide amphiphile micelles can act as self-adjuvants capable of inducing strong antibody responses against peptide antigens. While promising, the capacity for micelle-induced J8-specific antibodies to identify their cognate sequence within the

M1 protein has not yet been studied. Future experiments will investigate whether J8-specific antibodies can preferentially bind native M1 protein presented extracellularly on wild-type GAS bacteria.

While the mechanism responsible for micelle adjuvanticity is currently unknown, a few theories do exist. First, micellization can induce native peptide secondary structure (*e.g.* α-helicity) and increase local peptide concentration which enhances conformationally-correct antigen delivery at the injection site. Second, micelles are a nanoparticulate which may strongly interact with antigen presenting cells (APCs). In specific, hydrophobic moieties like diC_{16} can act as danger signals known as a pathogen associated molecular patterns (PAMP) which can interact with pathogen recognition receptors (PRRs) called Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs) found on the surface of APCs [13]. The importance of micellization is evident by the lack of an immune response induced when J8 peptide was delivered in solution separately from micelles (J8 + diC_{16}-SK_4, Figure 2.3 and Figure 2.4). While some adjuvants like IFA can induce antibody responses for co-delivered antigens, peptide amphiphile micelles must directly deliver the peptide antigen in order to function as an adjuvant which is similar to other self-assembled peptide vaccines [60, 61]. With regards to PRR stimulation, while diC_{16} has a chemical structure quite similar to Pam_3Cys, it was found unable to stimulate TLR-2, the cognate receptor for Pam_3Cys (Figure 2.5). This result combined with previous research that has shown a lack of TLR-2 stimulation and APC activation by peptide amphiphile micelles [33] provides convincing evidence that peptide amphiphile micelle adjuvanticity is conveyed by its ability to function as a delivery device instead of as a PRR stimulating system. However, other TLRs exist and will need to be tested in the future to confirm diC_{16}-based micelles do not act as PAMPs.

2.6 Conclusions

While other work has shown lipopeptides and self-assembled peptides can be effective vaccines, this research is the first to demonstrate that peptide amphiphile micelles can be utilized as a self-adjuvanting vaccine delivery vehicle to induce a significant peptide-specific antibody response. Micellization allows for the fabrication of cylindrical nanomaterials mostly comprised of conformationally correct (*i.e.* α-helical) J8 peptide vaccine which can be enhanced by annealing. When delivered subcutaneously to mice, J8-diC_{16} peptide amphiphile micelles induced a strong IgG1 antibody response similar to immunization with a conventional gold-standard vaccine formulation. Additional experiments revealed that micelle adjuvanticity appears to be conveyed by its peptide delivery capacity instead of through stimulation of danger signal receptors. These results provide evidence that peptide amphiphile micelles are a novel biomaterials platform that can be utilized to achieve desirable immune responses.

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3 Modular peptide amphiphile micelles improve an antibody-mediated immune response to Group A Streptococcus

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3.1 Summary

Inducing a strong and specific immune response is the hallmark of a successful vaccine. Nanoparticles have emerged as promising vaccine delivery devices to discover and elicit immune responses. Fine-tuning a nanoparticle vaccine to create an immune response with specific antibody and other cellular responses is influenced by many factors such as shape, size, and composition. Peptide amphiphile micelles are a unique biomaterials platform that can function as a modular vaccine delivery system, enabling control over many of these important factors and delivering payloads more efficiently to draining lymph nodes. In this study, the modular properties of peptide amphiphile micelles are utilized to improve an immune response against a Group A Streptococcus B cell antigen (J8). The hydrophobic/hydrophilic interface of peptide amphiphile micelles enabled the precise entrapment of amphiphilic adjuvants which were found to not alter micelle formation nor shape. These heterogeneous micelles significantly enhanced murine antibody responses when compared to animals vaccinated with non-adjuvanted micelles or soluble J8 peptide supplemented with a classical adjuvant. The heterogeneous micelle induced antibodies also showed cross-reactivity with wild-type Group A Streptococcus providing evidence that micelle-induced immune responses are capable of identifying their intended pathogenic targets.

3.2 Introduction

A growing class of therapeutics leveraging peptides are being studied for both prophylactic and post-exposure vaccine applications. Peptides are attractive candidates since they can be precisely designed to contain the minimal epitopes necessary to stimulate an immune response, avoiding common problems associated with killed or attenuated pathogen vaccines such as autoimmunity, risk of infection, and allergic reaction.[1, 2] The biggest challenge facing peptide-based vaccines is the fact that peptides alone are weak immunogens. To improve peptide immunogenicity, biomaterials-based platforms have been developed.[3-8] Despite these advances, these systems have been unable to match or exceed immune responses induced by conventional vaccination approaches employing immune-potentiating molecules termed adjuvants.[9-11] An opportunity exists to combine these technologies into a rationally designed peptide vaccine platform that precisely delivers a combination of antigens and adjuvants to better stimulate and control the nature of the immune response.

Peptide amphiphiles (PAs) are a class of peptide based biomaterials consisting of bioactive peptide head groups conjugated to hydrophobic alkyl tails which self-assemble in aqueous solution into micellar structures. The hydrophobic tails are protected from water in the core of the micelle, and a multivalent display of peptide head groups form the micelle corona. Peptide amphiphile micelles (PAMs) have previously been shown to function as a peptide delivery system for a variety of applications including cancer therapy[12-14], angiogenesis[15, 16], osteogenesis[17, 18], and atherosclerosis treatment[19, 20]. In our previous studies, micelles were used as a vaccine delivery vehicle that induced a peptide-specific antibody response.[21] Interestingly, PAMs stimulated a stronger antibody response than peptide alone without the use of an adjuvant making it a self-adjuvanting device. Furthermore, while the hydrophobic tails

enhance cell uptake by anchoring into cell membranes[22], they do not act as pathogen recognition receptor (PRR) agonists and therefore do not function as molecular adjuvants.[21] Rather, it is thought to be the self-assembled, particular-based physical nature of PAMs that affords them immunogenicity. Self-assembly of PAs has the added benefit of being able to facilitate the fabrication of multifunctional heterogeneous micelles through simple mixing of different PAs or other amphiphilic molecules.[19] The modular nature of peptide amphiphile micelles provides the opportunity to further enhance and shape their immunogenicity through precise control of their components. In specific, the incorporation of secondary molecular signals or amphiphilic adjuvants capable of simulating Th-cells or PRRs found on APCs into PAMs has the capacity to yield more robust host immune responses.

Nanoparticle physical characteristics including size and shape have been found to influence immune responses as well as *in vivo* biodistribution and clearance.[4, 23] Very small soluble particles readily diffuse and rapidly dilute after subcutaneous injection.[4] Larger, intermediate sized colloidal particles have smaller diffusion speeds and efficiently transport to the lymphatic system by convection.[4] As size increases to over about 500 nm, the particles become too large for transport and become trapped in the interstitial space.[4] Antigenic PAs previously used for vaccine applications self-assemble into long, flexible cylindrical micelles approximately 5 – 15 nm in diameter and 200 nm – 2 μm in length, after annealing.[21] Recognizing that no universal rules exist that can be applied to predict the *in vivo* behavior of all nanoparticles, it is important that the biodistribution and clearance of nanomaterials be evaluated for each new structure. Given the unique properties of this system and its inherent modularity, understanding biodistribution and clearance profiles can help to further elucidate micelle

mechanisms of adjuvanticity and inform the rational design of micelles for vaccination applications.

This paper explores the potential of peptide amphiphile micelles to serve as a modular immunotherapeutic platform. Specifically, a conformationally-dependent B cell epitope derived from the M1 surface protein of Group A Streptococcus (GAS) bacterias was used. GAS causes a range of mild to severe ailments, from simple pharyngitis ("strep throat") to necrotizing fasciitis (flesh-eating disease), as well as post-infection autoimmune diseases like rheumatic heart disease for which an effective vaccine has yet to be developed. Using straightforward chemistry and simple self-assembly, PAMs were designed, tested and optimized to raise GAS-specific antibody titers.

3.3 Materials and Methods

Micelle synthesis: J8 peptide (QAEDKVKQSREAKKQVEKALKQLEDKVQK) was synthesized on Rink amide MBHA resin (Novabiochem) utilizing standard Fmoc solid phase synthesis with the aid of a PS3 Peptide Synthesizer (Protein Technologies, Inc.). The N-terminus was either acetylated using 10x molar excess of acetic anhydride in DMF or covalently coupled to Rhodamine (Rho) fluorophore (Anaspec Inc) by an amidation reaction yielding Rho-J8. The resulting J8 peptides were treated using a concentrated trifluoroacetic acid solution to deprotect side groups and cleave the peptide from resin. High pressure liquid chromatography with mass spectrometry controlled fraction collection (LCMS; Shimadzu Corp.) utilizing a reversed-phase C8 column (Waters) with a gradient of acetonitrile in Milli-Q water containing 0.1% formic acid was employed to purify J8 peptide. For J8 or Rho-J8 peptide amphiphiles, the hydrophobic moiety dipalmitoylglutamic acid (diC_{16}) was synthesized by a previously established method.[24] J8 or Rho-J8 peptide was synthesized similarly to above, except the C-terminal

lysine was protected with DDE instead of Boc, which was used for the other lysines. The peptide was treated with 2% hydrazine in DMF to orthogonally deprotect the C-terminal lysine amine group which was then covalently coupled to diC_{16} by an amidation reaction yielding J8-diC_{16} and Rho-J8-diC_{16} peptide amphiphiles. These peptide amphiphiles were further processed and purified by the same methods as the peptides above. All samples were lyophilized and stored at -20°C until used. It should be noted that all peptide and peptide amphiphiles were created in a chemical synthesis laboratory using appropriate personal protective equipment to eliminate exposure to biological contaminants.

To fabricate micelles, J8-diC_{16} peptide amphiphiles were film cast by dissolving them in methanol and evaporating the solvent using nitrogen as a drying gas. The resulting film was hydrated at 70°C for 60 min in phosphate-buffered saline (PBS) and allowed to equilibrate overnight. Fluorescent micelles were assembled by dissolving Rho-J8-diC_{16} and J8-diC_{16} (25:75 molar ratio) in methanol and prepared by the same methods as J8-diC16 micelles mentioned above. Toll-Like Receptor 4 (TLR4) Agonist Monophosphoryl Lipid A (MPLA, Sigma-Aldrich) or TLR2 Agonist Pam_2Cys-SK_4 (P_2C-SK_4 Invivogen) adjuvant supplemented micelles were assembled by combining J8-diC_{16} with either adjuvant and fabricated with the film cast method as mentioned above. Each micelle formulation contained 12 nmol J8-diC_{16} and MPLA and P_2C-SK_4 were included at 10 mol%.

Micelle characterization: Micelles were characterized by previously defined methodologies[14, 19, 25] including critical micelle concentration (CMC) analysis and transmission electron microscopy (TEM). CMC was measured by fluorescent sequestration where varying concentrations of J8-diC_{16} were exposed to 1 mM 1,6-diphenyl-1,3,5-hexatriene (DPH) which greatly increases in fluorescence intensity when trapped within the micelle core.

Solutions were prepared and allowed to equilibrate for 1 h prior to fluorescent measurement utilizing a Tecan Infinite 200 plate reader (ex. 350 nm, em. 428 nm). The data were fit with two trend lines which were set equal to one another to determine the fluorescence inflection point (*i.e.* CMC). Micelle morphology was investigated using negative stain TEM. J8-diC_{16}, J8-diC_{16}/MPLA and J8-diC_{16}/P_2C micelle solutions (1 uL of 200 μM) were allowed to incubate on Formvar-coated copper grids (Ted Pella, Inc.) for 1 min after which excess liquid was wicked away with filter paper. Grids were then washed with Milli-Q water and incubated with aqueous phosphotungstic acid (1 wt%) for 1 min before the solution was wicked away. Samples were allowed to air dry and then imaged on a FEI Tecnai 12 TEM using an accelerating voltage of 120 kV. For Förster resonance energy transfer (FRET) experiments, three different micelle formulations were made. Micelles with rhodamine only were made to contain unlabeled J8-diC_{16} with 10% rhodamine labeled J8-diC_{16} and 10% unlabeled P_2C. Micelles with fluorescein only were made to contain unlabeled J8-diC_{16} and 10% fluorescein labeled P_2C. Micelles containing fluorescein and rhodamine were made to contain unlabeled J8-diC_{16} with 10% rhodamine labeled J8-diC_{16} and 10% fluorescein labeled P_2C. Micelles were excited at 490 nm and emission between 490 nm and 700 m was recorded on a Jasco FP-6500 Spectrofluorometer.

Whole animal and animal organ imaging: Female BALB/c mice 6-8 weeks old were purchased from Charles River and immunized to investigate the biodistribution and trafficking of the peptide and micelle vaccines. Mice were shaved and naired before they were anesthetized with 2% isoflurane in O_2 and subcutaneously injected at the nape of the neck with 100μL of 120 μM Rho-J8:J8 or Rho-J8-diC_{16}:J8-diC_{16} (25:75 molar ratio) suspended in PBS. Whole body fluorescence imaging was conducted at multiple time points (ex. 570 nm, em. 620 nm, IVIS 200, Xenogen, Caliper Life Sciences, Hopkinton, MA, USA). Micelles were allowed to circulate for

up to 24 hours before mice were euthanized via CO_2 overdose and cervical dislocation, after which the lymph nodes were harvested. Fluorescence imaging of organs was conducted using an IVIS 200 and quantification of the fluorescence signal was achieved via the Living Image software (Perkin Elmer, Downers Grove, IL, USA).

Murine Vaccination: Female BALB/c mice 6-8 weeks old were purchased from Charles River and immunized to investigate the capacity for various micelle formulations to induce an antibody-mediated immune response. For control groups, the potent physical adjuvant Incomplete Freund's Adjuvant (IFA) was used and purchased from Sigma-Aldrich. Micelles utilized for vaccination were fabricated by the film deposition, rehydration, and annealing method outlined above. Mice were vaccinated in the nape of the neck subcutaneously at days 0 (prime), 21 (boost 1), 28 (boost 2), and 35 (boost 3) with one of ten vaccine formulations:

PBS

J8 – 12 nmol J8 peptide

J8 + IFA – 12 nmol J8 peptide in 50 µL IFA and 50 µL PBS

J8 + MPLA – 12 nmol J8 peptide + 1.33 nmol MPLA in 100 µL PBS

J8 + P_2C-SK_4 – 12 nmol J8 peptide + 1.33 nmol P_2C-SK_4 in 100 µL PBS

J8-diC_{16} – 12 nmol J8-diC_{16} in 100 µL PBS

J8-diC_{16}/MPLA – 12 nmol J8-diC_{16} + 1.33 nmol MPLA in 100 µL PBS

J8-diC_{16}/P_2C-SK_4 – 12 nmol J8-diC_{16} + 1.33 nmol P_2C-SK_4 in 100 µL PBS

Whole blood was collected from saphenous veins pre-vaccination on days 21, 28, and 35 as well as on day 42 to analyze for J8-specific antibodies induced by the previous round of

immunization. The blood was centrifuged at 10,000 RPM for 10 min to separate out red blood cells and the supernatant serum was harvested and stored at -20°C until analysis.

Antibody Response Characterization: An enzyme-linked immunosorbent assay (ELISA) was utilized to determine J8-specific antibody titers. Flat-bottom 96-well EIA microtiter plates (Costar) were coated overnight with 100 μL of 10 μg/mL J8 peptide in sodium bicarbonate coating buffer in each well at 4°C. The wells were washed with 200 μL of 0.05% Tween 20 in PBS (PBS-T) three times and then blocked with 200 μL of assay diluent (10% FBS in PBS) for 1 h. The blocking solution was removed and 100 μL of 1:1000 diluted sera samples were added to the top row and then serially diluted two-fold with assay diluent down the plate. After 2 h incubation, wells were washed with PBS-T three times and incubated with 100 μL of 1:3000 diluted detection antibody (IgM, IgG, IgA, IgG1, IgG2a, IgG3, or IgG4; Invitrogen) for 1 h. PBS-T was used to wash wells three times after which 100 uL of Ultra TMB-ELISA substrate solution (Pierce) was added to the wells. Plates were allowed to incubate for 15 minutes in darkness and then optical density (OD) was measured for each well at 650 nm using a Tecan Infinite M200 plate reader. Endpoint antibody titers were defined as the greatest serum dilution where OD was at least twice that of normal mouse serum at the same dilution. If end-point titers are not reached with one plate then additional titrations were utilized until ODs were diluted to background.

Antibodies binding to M1 Proteins on GAS: Both wild type and Δemm 5448 GAS were generously provided by Chelsea Stewart and Partho Ghosh at UCSD. Bacteria were fixed to poly-L-lysine coated slides using 4% paraformaldehyde. After blocking the bacteria with 0.5% BSA in PBS, sera from immunized mice were added to the slides at a 1:200 dilution and incubated at room temperature for 2 hours. The slides were washed vigorously in 50 mL PBS in

a glass staining jar for 10 min using a stir bar. The bacteria were then incubated for 2 hours at room temperature with goat-anti-mouse IgG F(ab')2 conjugated to FITC to detect antibodies bound to the bacteria. Controls included secondary antibody only and sera from naïve mice (both negative). Fluorescent images were taken by a Zeiss confocal microscope. The same power, pinhole, and gain settings were used for all images.

Statistical Analysis: JMP software (SAS Institute) was used to make comparisons between groups using an ANOVA followed by Tukey's HSD test to determine pairwise statistically significant differences ($p < 0.05$). Within the figure graphs, groups that possess different letters have statistically significant differences in mean whereas those that possess the same letter are similar.

3.4 Results and Discussion

3.4.1 Micelles clear as fast as soluble peptide, but traffic to the lymph nodes more efficiently

Vaccines come in a wide variety of forms, from killed or attenuated pathogens, to recombinant subunit or virus-like particles. As the field of immunoengineering has become more sophisticated, subunit antigen and peptide vaccines have emerged as a promising solution to the weaknesses of previous generation vaccines.[1, 2, 9] Modular nanoparticle platforms, and peptide amphiphile micelles in particular, enable the control over many properties that affect vaccine-based immune responses such as size, shape, and composition. While a variety of nanoparticle vaccine systems currently exist,[4-8] micelles possess several advantages over other nanoparticle-based systems. Micelles are water soluble which makes them easy to deliver via injection, comprised of more than 80% peptide by weight, and able to deliver peptides with

native peptide secondary structure (*e.g.* α-helicity). Previous literature has shown that palmitic acid based moieties can act as PRR agonists which enhance the immunogenicity of linked peptides.[26, 27] While the exact mechanism responsible for the adjuvanticity of our micelle system is not fully understood, a previous Tirrell lab publication determined that the hydrophobic diC_{16} moiety did not activate TLR-2 *in vitro*, despite the chemical structure similarities between diC_{16} and known TLR-2 stimulants.[21] Instead, micellization itself was found to be important, since the co-delivery of J8 peptide separated from mock micelles (J8 + diC_{16}-SK_4) was unable to induce an immune response.[21] Another factor which affects the immune response is where and how immune cells interact with nanoparticles. After a subcutaneous injection, a vaccine may remain at the site of injection to act as a depot, or be quickly trafficked via the lymphatic system to interact with immune cells in the lymph nodes. Therefore, in order to further understand the nature of micelle adjuvanticity, their biodistribution and clearance was investigated and compared to free peptide.

In order to ensure that the biodistribution and clearance of micelles could be adequately imaged, 25 mol% of Rho-J8 products were incorporated into the vaccine formulations. Free J8 peptide and J8-diC_{16} micelles with 25 mol% Rho-J8 and Rho-J8-diC_{16}, respectively, were subcutaneously injected at the nape of the neck. To be consistent and relevant to the vaccine formulations used throughout the previous and current paper, a volume of 100μL and a concentration of 120μM were used. After injection, mice were immediately imaged for a zero-minute time point, followed by images at 10, 30, 60, 120, 180, 300 and 360 minutes (Figure 3.1 **A**). After 6 hours, *in vivo* imaging confirmed that the vaccine formulations fully diffused away from the initial injection site and no *in vivo* accumulation of peptide or micelles could be seen. Since no *in vivo* accumulation could be viewed, the vaccines were presumably either degraded in

the subcutaneous space or trafficked to other areas in the body at low enough concentrations to be indiscernible above background on a full mouse imaging scale.

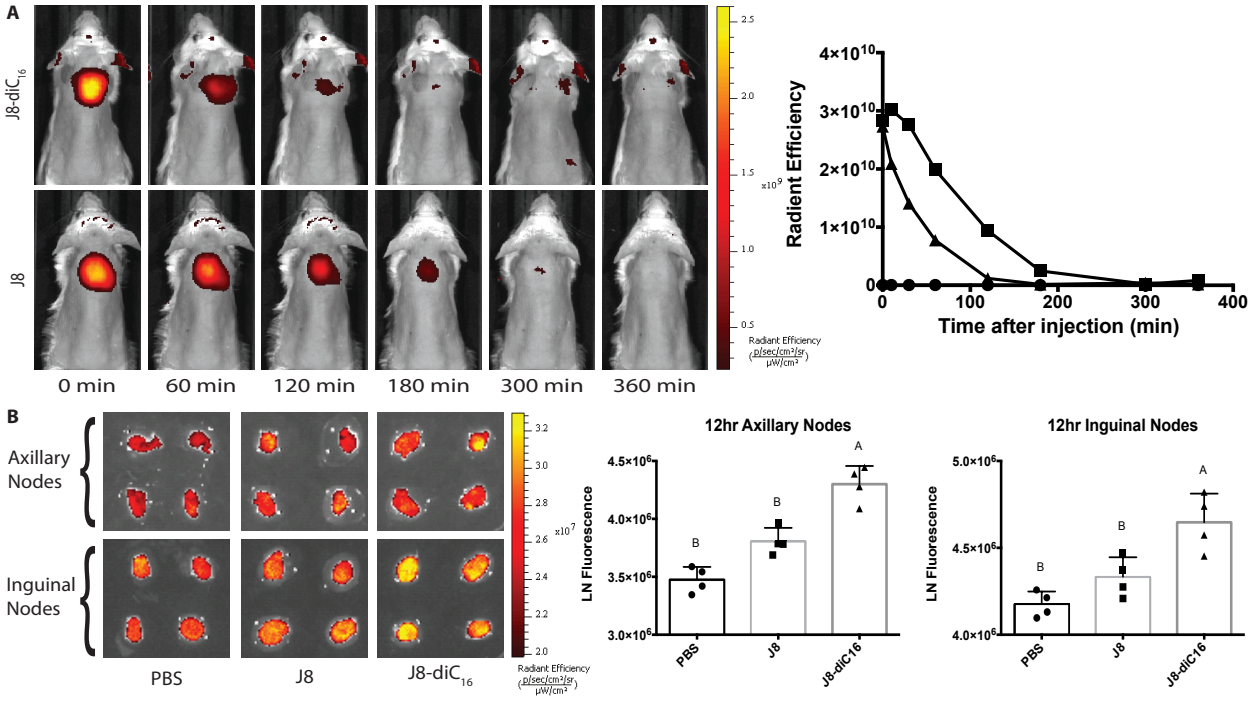


Figure 3.1 - Micelles clear on the same order as free peptide, but traffic to the lymph nodes more efficiently. (**A**) *In vivo* optical images of BALB/c mice injected with J8-diC_{16} micelles (top) and free J8 peptide (bottom) with 25 mol% Rho-J8-diC_{16} or Rho-J8, respectively. Micelle fluorescence clears approximately one hour faster than free J8 peptide (● = PBS, ■ = J8, and ▲ = J8-diC_{16}). (**B**) IVIS fluorescence imaging and quantification of excised draining axillary and inguinal lymph nodes from BALB/c mice at 12 hours (N = 4 lymph nodes per group). Within a graph, groups that possess different letters have statistically significant differences in mean ($p \leq 0.05$) whereas those that possess the same letter are similar ($p > 0.05$).

When comparing the speed with which the fluorescent signal cleared from the injection site, free peptide signal seems to last slightly longer than the micelle formulation signal. Despite this, the rate of clearance is essentially the same magnitude, approximately a few hours. As mentioned previously, a trend exists where small soluble particles readily diffuse from the injection site, intermediate particles diffuse less which allows better transport to the lymphatic system, and larger particles become trapped in the interstitial space.[4] These trends tend to hold

true for colloidal particles. The peptide amphiphile micelles formed in this research, on the other hand, self-assemble into long, flexible cylindrical micelles approximately 5 – 15 nm in diameter and 200 nm – 2μm in length. Based on this size profile and data from other nanoparticle platforms, it was hypothesized that the micelles would get trapped in the interstitial space and act as an antigen depot. Based on the live whole animal imaging data, however, it appears that micelles do not stay at the injection site any longer than its free peptide counterpart.

Since no *in vivo* accumulation could be seen from the whole mouse imaging, draining lymph nodes were imaged to assess the trafficking potential of each formulation. Vaccine formulations were injected subcutaneously and 12 hours later, draining lymph nodes were excised and kept intact to be imaged by IVIS. Figure 3.1**B** shows the distribution of the vaccines containing 25 mol% rhodamine within the inguinal and axillary lymph nodes. The micelle formulation showed significantly greater fluorescence in both sets of draining lymph nodes than in either the PBS or free peptide formulation.

Therefore, even though both vaccine formulations seemed to clear from the injection site at the same rate, micelles cleared to the lymph nodes more efficiently than free peptides. Combining this information, it suggests that the long cylindrical micelles may not primarily act as an antigen depot, but rather traffic to the lymph nodes to induce an immune response. Given the clearance timeframe in relation to free peptide and the lymph node accumulation, it could be that the micelles break down into more intermediately sized particles that can traffic to the lymphatic system. This explanation is supported by the fact that micelles are self-assembled structures held together by weak hydrophobic forces. While these micelles form long cylinders in stable solutions, they could easily reform into smaller micelles in the body. In fact, Liu *et al.* provided evidence that spherical amphiphilic micelles breakdown and traffic to the lymph nodes

by albumin hitchhiking.[28] Additionally, micelles readily interact with cells facilitating their internalization, whereas peptides do not readily internalize. Either system could account for trafficking to the lymph nodes, with micelle breakdown indicating acellular lymphatic trafficking and internalization indicating cellular lymphatic trafficking. Additional research is needed to interpret this further.

3.4.2 Design and self-assembly of mixed micelles

Conventional adjuvants and carrier proteins work, at least in part, by stimulating Th-cells and/or activating innate immune responses via stimulation of PRRs on antigen presenting cells. A strategy for peptide-based vaccines is thus to incorporate defined PRR agonists into the antigen delivery system such that immune cells can interact with both the target peptide antigen and an associated secondary signal molecule. PRR agonists can either be heterogeneously mixed in with a peptide, or conjugated directly to the peptide epitope. Previous literature has validated the conjugation approach and indicated that palmitic acid based moieties can act as TLR2 agonists which enhance the immunogenicity of linked peptides.[26, 27] While our peptide amphiphile micelle system is structurally similar, a previous Tirrell lab publication determined that the hydrophobic diC_{16} moiety did not activate TLR-2 *in vitro*.[21] Instead, micellization itself was found to be important, since the co-delivery of J8 peptide separated from mock micelles (J8 + diC_{16}-SK_4) was unable to induce an immune response.[21] Despite this result, we recognize that the exact mechanism responsible for the adjuvanticity of our peptide amphiphile system is not fully understood and further *in vivo* research is worthwhile. While this is being clarified, a parallel investigation of heterogeneous incorporation of PRR agonists to the micelle system was explored.

Co-assembling TLR agonists into micelles has many advantages compared to covalent linking. Multiple agonists and multiple peptide amphiphile antigens can be incorporated into micelles in a modular fashion. Thus, for different applications, different agonists or peptides could be incorporated by simple mixing. Depending on the application, different agonists, or amounts of agonists, could be incorporated to bias the immune response to a specific response. Taking advantage of the modular nature of PAMs, heterogeneous micelles were made that incorporate different amphiphilic adjuvants to help boost the immune response through increasing antibody titers and/or reducing the number of immunizations required. Heterogeneous J8-diC_{16} micelles were made by mixing in either MPLA or P_2C-SK_4. These adjuvants were chosen due to their amphiphilic structures, providing an opportunity to form heterogeneous micelles comprised of antigens and adjuvants. MPLA is also an approved adjuvant for human use.[29] Figure 3.2 shows the chemical structures of each micelle component. Figure 3.2**A** – Figure 3.2**C** show negative stain TEM images of J8-diC_{16} micelles, 90/10 J8-diC_{16}/MPLA, and 90/10 J8-diC_{16}/P_2C-SK_4 solutions, each incorporating 10 mol% of one of the secondary signal molecules. Heterogeneous micelles possess the long, cylindrical shape seen with pure J8-diC_{16} micelles indicating adjuvant entrapment does not affect micelle shape.

Förster resonance energy transfer (FRET) was used to demonstrate that P_2C and J8-diC_{16} reside in the same self-assembled heterogeneous micelles and do not segregate into a mixed population of different micelles. J8-diC_{16} and P_2C-SK_4 were labeled with rhodamine (Rho) and fluorescein (FL), respectively, which act as a FRET pair. When fluorescein is excited and is in close proximity to rhodamine, fluorescein non-radiatively transfers energy to rhodamine, causing rhodamine to become excited and emit light. Since the Förster distance (R_0) for fluorescein and

rhodamine is 5.5 nm, the molecules must be less than 11 nm apart ($2*R_0$), for FRET to occur which is achievable with PAMs.

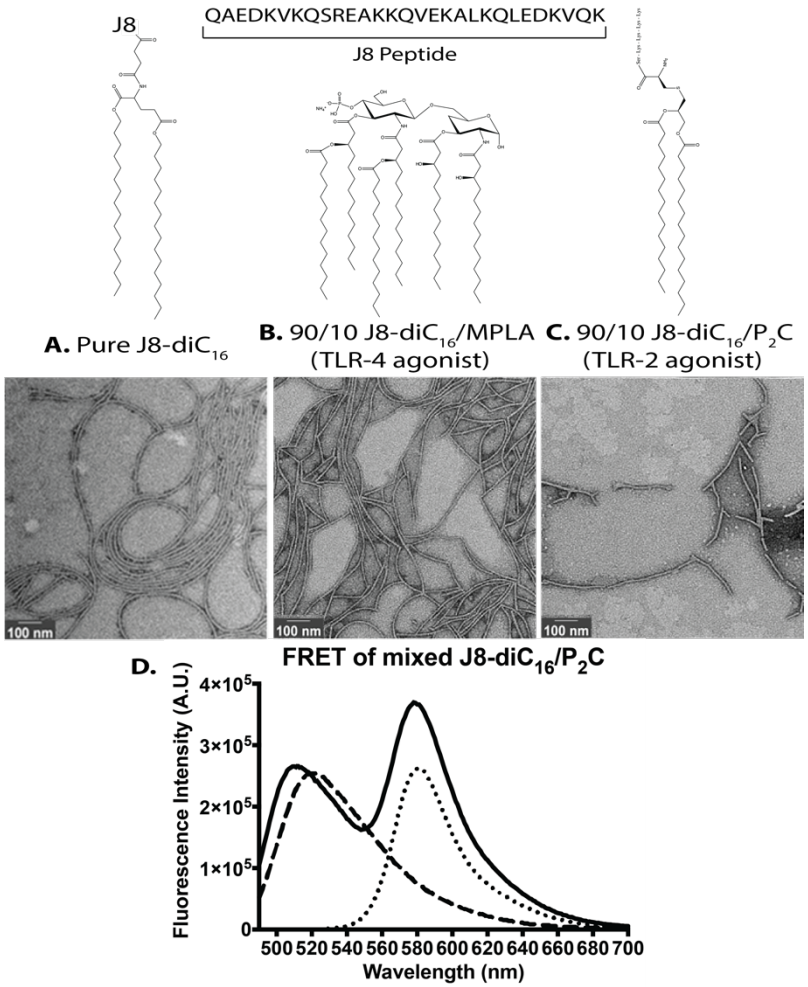


Figure 3.2 - Ampiphilic adjuvants mixed with peptide amphiphiles form heterogeneous micelles. Negative stain TEM images of (**A**) J8-diC_{16} and J8-diC_{16} mixed micelles incorporating 10 mol% of either (**B**) MPLA, or (**C**) P_2C-SK_4. Above each image is the structure of the secondary signal molecule. (**D**) Spectrophotometry revealed that Förster resonance energy transfer occurred between a rhodamine labeled J8-diC_{16} and a Fluorescin labeled P_2C-SK_4 (— = Rho-J8-diC_{16} and P_2C-SK_4-FL, -- = P_2C-SK_4-FL, ··= Rho-J8-diC_{16}). The rhodamine peak increases when fluorescein is excited in micelles that contain P_2C-SK_4-FL and Rho-J8-diC_{16} compared to micelles that contain Rho-J8-diC_{16} alone.

When P_2C-SK_4-FL is co-assembled with Rho-J8-diC_{16}, the fluorescein peak has a distinct blue shift compared to the P_2C-SK_4-FL on its own when excited at 490 nm (Figure 3.2**D**). FRET occurs more efficiently at the wavelengths that rhodamine absorbs. Since rhodamine has a peak

excitation of 552 nm, the higher wavelengths of fluorescein decrease more than the lower wavelengths, causing the blue shift in the fluorescein peak. FRET is further demonstrated by the increase in the rhodamine peak at 580 nm.

3.4.3 Heterogeneous micelles improve antibody response

To assess the ability of heterogeneous micelles to enhance antibody titers, mice were immunized with formulations comprised of 12 nmol J8-diC_{16} and 10 mol% TLR agonist. For controls, mice were also immunized with only J8-diC_{16} and mixed formulations of 12 nmol free J8 peptide and 10 mol% TLR agonist or IFA (see full formulations in Methods). Harvested serum samples were analyzed by ELISA to determine J8-specific antibody isotype titers (IgM, IgG and IgA). Figure 3.3 compares IgM and IgG titers induced by the heterogeneous micelles after each immunization with the titers of the pure J8-diC_{16} micelles and the peptides in IFA. All micelle vaccines were able to induce appreciable IgM titers which were all found to be significantly higher than J8 in IFA after the first boost. No vaccine treatment tested induced an IgA response (data not shown).

IgG titers started showing strong responses after just one immunization. The responses were particularly high with the heterogeneous micelles incorporating TLR agonists. After just one immunization with 90/10 J8-diC_{16}/MPLA, antibodies titers were a full order of magnitude higher than that of the micelles alone or soluble peptides in IFA. An even greater response was seen with heterogeneous micelles incorporating P_2C-SK_4 where a single immunization stimulated titers two full magnitudes higher than that of micelles alone or soluble peptide in IFA. Immunization with heterogeneous micelles incorporating P_2C-SK_4 stimulated the same J8 specific antibody levels as seen after two boosts with both J8 peptide in IFA and J8-diC_{16} micelles. Compared to titers after one boost with pure micelles, one boost with the heterogeneous

MPLA micelles resulted in titers that were the same order of magnitude, while titers with the heterogeneous P_2C-SK_4 micelles were one and a half times higher in magnitude. Antibody titers for heterogeneous MPLA micelles end with a max average titer slightly higher than boost-three titers of both pure micelles and J8 + IFA. Titers from mixed P_2C-SK_4 micelles leveled off after 2 boosts, with a max average titer exceeding all the other micelles by approximately one order of magnitude. Overall, the addition of either MPLA or P_2C-SK_4 to J8-diC_{16} micelles stimulates the production of higher antibody titers with fewer doses when compared to the pure J8 micelles alone and the soluble J8 peptide in IFA.

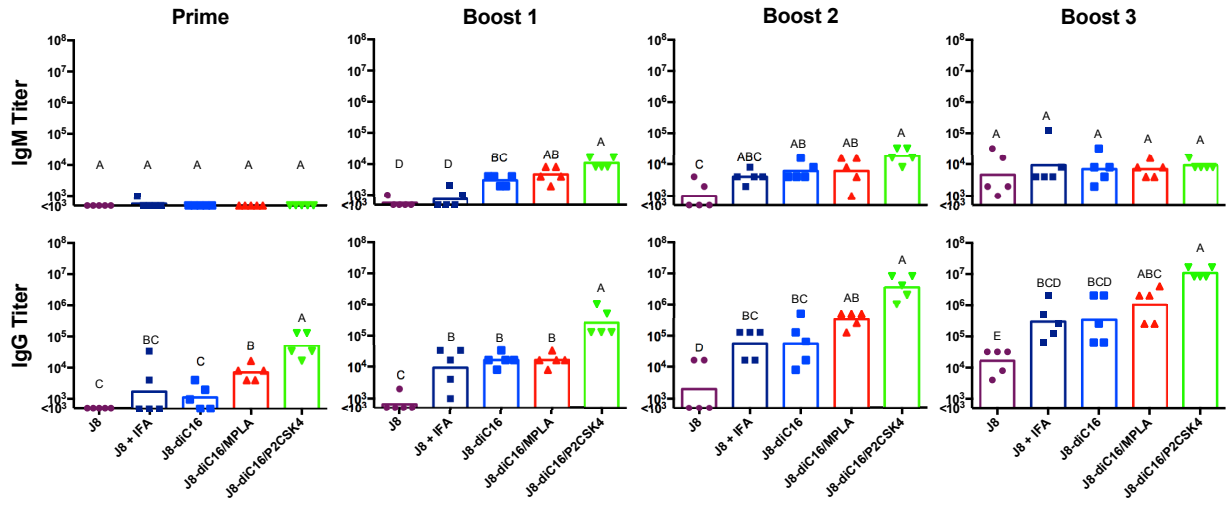


Figure 3.3 - Mixed peptide amphiphile micelles induced strong antibody isotype responses *in vivo*. J8-specific antibody titers induced by J8 + IFA and J8-diC_{16} micelles were compared to titers stimulated by mixed micelles containing 10% of either MPLA or P_2C-SK_4. Each secondary signal incorporated into the micelles resulted in an enhanced antibody response. While IgA titers were assessed, no mouse produced above background levels. Each data point represents one mouse (N = 5); bars represent the geometric mean. Within a graph, groups that possess different letters have statistically significant differences in mean ($p \leq 0.05$) whereas those that possess the same letter are similar ($p > 0.05$).

Further investigation into the specific nature of the induced IgG response revealed that it was strongly dominated by IgG1 subtype with IgG3 also being produced in response to heterogeneous micelle vaccination (Figure 3.4). A small yet significant level of IgG1 was seen

after the prime immunization for the heterogeneous micelle vaccines, but not for any other formulation. One boost with heterogeneous micelles containing P_2C-SK_4 stimulated the same J8-specific IgG1 antibody levels as seen after three boosters with the J8 peptide in IFA and J8-diC_{16} micelles. Compared to titers after one boost with J8 peptide in IFA or micelles alone, one boost with heterogeneous micelles incorporating P_2C-SK_4 resulted in IgG1 titers that were one and a half times higher in magnitude. Similar to total IgG, IgG1 antibody titers for heterogeneous P_2C-SK_4 micelles leveled off after boost two, with a max average titer exceeding that of all other micelles by approximately one order of magnitude. IgG1 subtype antibody has been found to protect against GAS infections by bacterial opsonization and macrophage engulfment.[30] Interesting IgG3 titers were produced throughout the course of the prime-boost immunization schedule. Again, the heterogeneous micelle formulations produced the highest IgG3 responses, remaining between one and three levels of magnitude higher than J8 peptide in IFA or pure micelle. The micelle with MPLA rose to a titer of approximately 10^5 after the first boost and essentially remained level through to the end of the boost schedule. IgG3 mixed micelle formulations with P_2C peaked before the end of the immunization schedule. Titers rose to approximately 10^5 after the first boost, then increased to titers greater than 10^6 after the second boost, and finally decreased back to titers of 10^5 again after the third boost. Finally, for IgG2a, while a few mice vaccinated with J8 + IFA had above background titers, J8-diC_{16} micelles induced no appreciable response. Antigen/adjuvant micelles, on the other hand, did raise small but appreciable IgG2a titers. IgG4 was also assayed for but no titers were observed (data not shown).

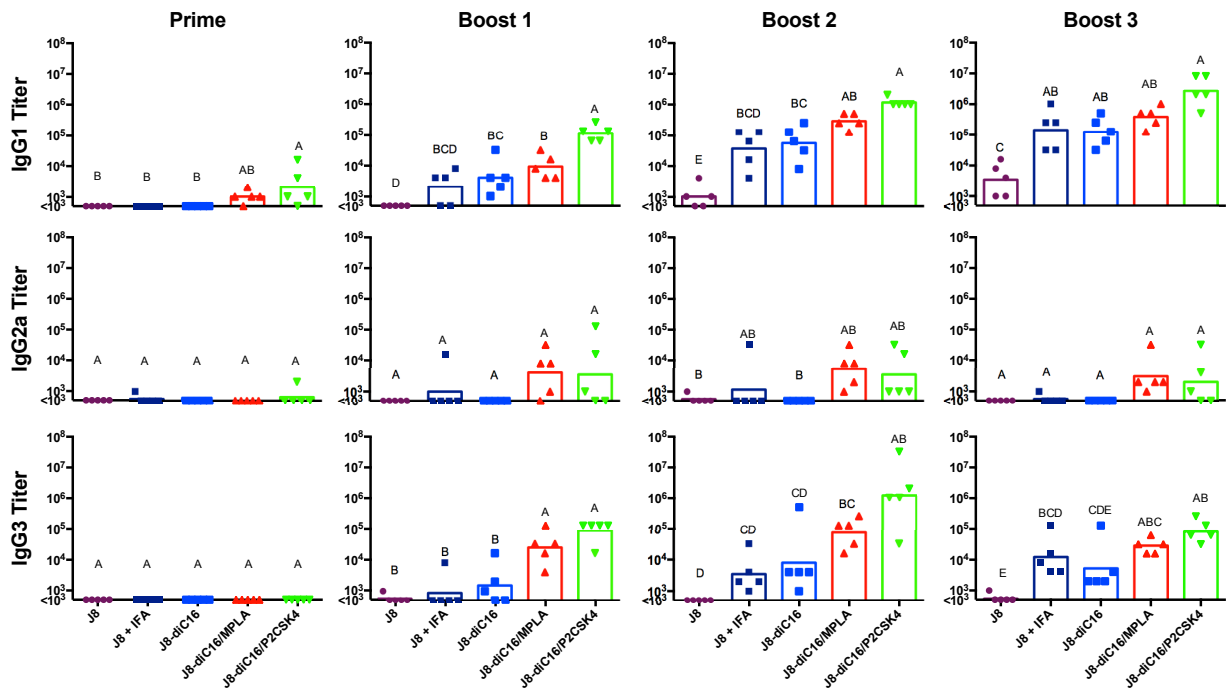


Figure 3.4 - Heterogeneous peptide amphiphile micelles induced strong IgG1 and IgG3 antibody subtype responses *in vivo*. The strong IgG response induced by J8 peptide in IFA or J8-diC_{16} vaccines was found to be predominantly comprised of the IgG1 subtype. Heterogeneous micelles, on the other hand, produced a different antibody response than the controls comprised mostly of IgG1 with some IgG3 and a small amount of IgG2a. The modular addition of adjuvants has therefore allowed us for the immune response to be controlled. Also, no mouse produced above background levels of IgG4 titers. Each point represents one mouse (N = 5); bars represent the geometric mean. Within a graph, groups that possess different letters have statistically significant differences in mean ($p \leq 0.05$) whereas those that possess the same letter are similar ($p > 0.05$).

These results provide considerable evidence that the modularity of peptide amphiphile micelles can be used to tune corresponding immune responses towards desired applications. There are eleven known TLR subtypes[31] where stimulation of different TLRs results in secretion of distinct cytokine signaling molecules influencing the ensuing immune response.[32, 33] For example, TLR4 agonists are known to promote a Th1 response which is best for intracellular viral and bacterial infections, while TLR2 agonists promote a Th2 response which is best for extracellular bacteria, parasites, and toxins.[34] It is possible that specific TLR agonists

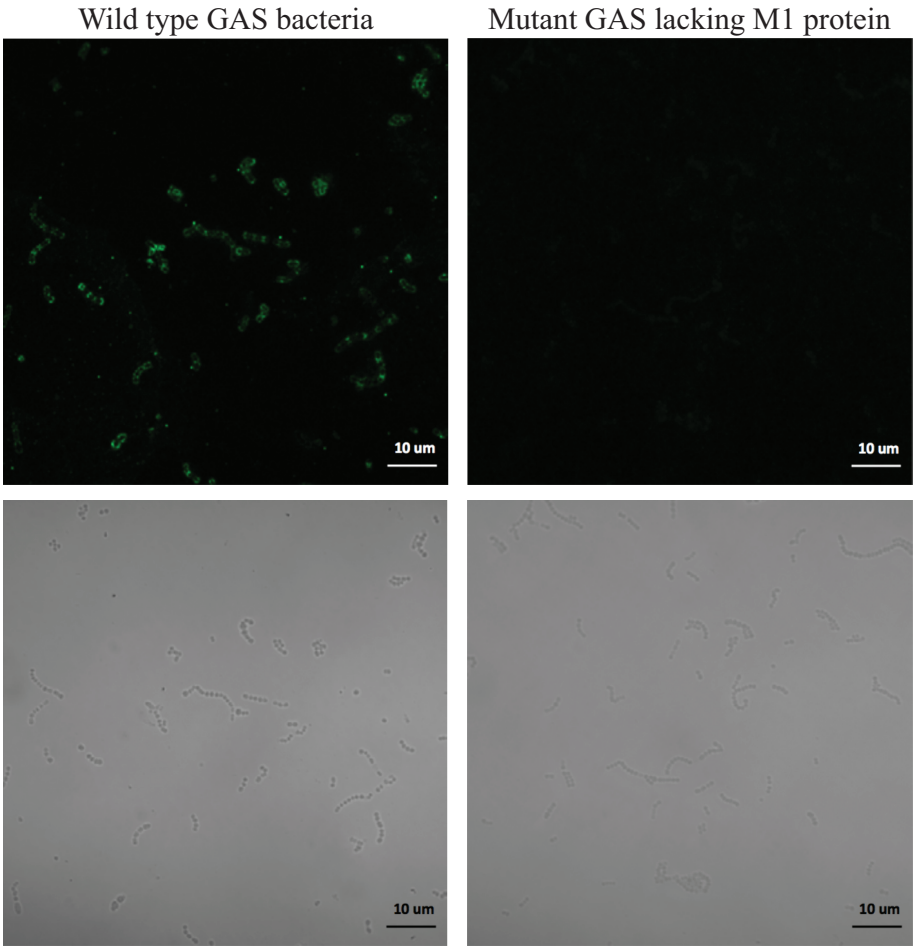
can be incorporated into a vaccine formulation for the purpose of biasing the immune response. Caution should be taken when using strong TLR agonists, however, as broad activation of innate immunity can sometimes lead to chronic inflammation and tissue damage.[35] Unlike the all-in-one peptide vaccines described above that have specific TLR agonists as part of the peptide scaffold, the PA micelle platform has the advantage of modularity.

In an attempt to explain the improved immune responses from Figure 3.3 and Figure 3.4, it may be relevant to revisit the micelle trafficking results from Figure 3.1. Combining these results, we suggest that the enhanced lymph node trafficking capacity of micelles is maintained when heterogeneous antigen/adjuvant micelles are subcutaneously injected, resulting in the co-delivery of antigens and adjuvants which dramatically improves the immune response activation in the lymph node. In fact, the importance of lymph node antigen presentation has previously been shown necessary to achieve strong and durable responses.[36]

3.4.4 Micelles induce antibodies that identify M1 protein on GAS bacteria

The ELISAs discussed above demonstrate that the antibodies stimulated by the different micelle formulations, as well as those stimulated by the J8 peptide in IFA, are capable of binding to synthetic J8 peptide. To assess the therapeutic potential of these antibodies, immunocytochemistry was used to determine the capacity of the antibodies to recognize J8 peptide within the context of M1 protein on the surface of actual GAS bacteria. Wild-type GAS bacteria (M1 strain 5448) were fixed on poly-L-lysine-coated glass slides using paraformaldehyde. After adding sera from the immunized mice to the bacteria on the slides, an anti-mouse IgG F(ab')2 fragment fluorescently labeled with FITC was used to identify antibodies from the mice that were bound to the bacteria. Similar staining was done using a mutant GAS strain (Δemm 5448) that does not express M1 protein. Figure 3.5 illustrates the

binding of antibodies from mice immunized with 90/10 J8-diC_{16}/MPLA micelles to the wild-type bacteria. Antibodies from mice immunized with all other micelle and peptide formulations were also capable of binding to the wild type GAS (data not shown). However, none of the antibodies were capable of labeling the mutant bacteria, indicating that the antibodies are specifically recognizing the native M1 protein on the surface of GAS.



Example from J8-diC_{16}/MPLA group

Figure 3.5 - Confocal images showing that antibodies from immunized mice are capable of binding to wild type GAS, but not to mutant GAS lacking surface expression of M1 protein. All images were taken with a Zeiss LSM 700. The fluorescent image is shown on top of its corresponding white light image for each sample.

Given the recent peptide amphiphile micelle success *in vivo*, it is not farfetched to believe that clinical implementation of this vaccine platform could occur in the near future. While there are many factors that affect the clinical translation of a technology, we will quickly touch on some potential advantages and limitations of the micelle platform. In addition to the already discussed advantage of platform modularity and the systemic lymph node trafficking, another advantage is the relative ease with which scale-up could occur. Since the technology required to synthesize peptides cost effectively has already been developed, the addition of a tail by the same chemistry as adding an amino acid can believably be achieved without much more effort.[37] Before clinical translation does occur, however, more basic science is needed, including testing these amphiphiles in a more genetically diverse population of mice to better represent human populations. Additionally, effects of storage conditions (time/temperature) on and kinetics of micelles should be thoroughly studied in preparation for commercial application.

3.5 Conclusions

Previously, we have shown that peptide amphiphile micelles can be utilized as a self-adjuvanting vaccine delivery vehicle to induce an antigen-specific antibody response. This research expands upon the micelle vaccine concept and demonstrates how peptide amphiphile modularity can be utilized to improve corresponding responses. It was revealed that micelle formulations cleared from the injection site at a similar rate to the soluble J8 peptide, but trafficked to the lymph node more efficiently than soluble peptide. The amphiphilic and modular nature of peptide amphiphiles enabled the precise addition of amphiphilic adjuvants, which did not disrupt the formation of cylindrical micelles. When delivered subcutaneously to mice, heterogeneous micelles induced a stronger IgG1 antibody response than seen with a conventional gold-standard vaccine formulation. These experiments taken together provide convincing

evidence that heterogeneous micelles can enhance lymph node co-delivery of antigens and adjuvants leading to the dramatically improved antibody response observed.

3.6 References

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4 Preparation of long cylindrical micelle shape is important for immunogenicity of B cell epitope

4.1 Summary

Peptide amphiphile micelles are a unique biomaterials platform that have shown promise for use in immunological applications, such as vaccines and immunotherapies. The modularity of peptide amphiphiles provides seemingly endless opportunities to modify and fine-tune an immune response. However, before advancing to additional levels of complexity, systematically evaluating the effects of micelle preparation on structure and immunogenicity is a prerequisite to thoroughly understanding the platform. Here, we investigated the impact of the heat annealing and thin film casting preparation steps on the resulting immunogenicity of peptide amphiphile micelles, identifying the long cylindrical shape as an important determinant in raising strong antibody responses against B cell epitopes in both immunocompetent and athymic mice. It was found that each preparation step produced a considerably different micelle shape, leading to consistently tiered immune response results. Film only preparation created short rod-like micelles which produced significantly diminished antibody responses, but had enhanced uptake by APCs. Conversely, full film/heat preparation created long cylindrical micelles which were strongly immunogenic, but had no uptake by APCs. The remaining two preparations, no preparation and heat only preparation, created heterogeneous micelle populations which produced both middling immune responses and APC uptake levels. These findings suggest that the long cylindrical micelle shape is important for B cell epitope antibody production and T cell independent B cell activation. More importantly, these findings provide both clarity in understanding the peptide amphiphile micelle platform more completely as well as opportunity

for another lever of modularity to inform the rational design of micelles for future vaccination applications.

4.2 Introduction

Supramolecular or "self-assembling" nanomaterials are some of the most tunable, versatile platforms available to researchers. As such, they have been investigated for many applications such as tissue engineering, cell scaffolds, would repair, drug delivery, and increasingly immunological applications such as vaccines and immunotherapies.[1-14] In all of these applications, each tunable property (such as size, morphology, charge of nanoparticles, etc.) provides a way to tailor the design to improve a desired function. The broad self-assembling field has, over time, discovered useful design patterns to inform initial design parameters.[15-19] Patterns in preparation strategies such as thin film casting, heat annealing, extrusion, and sonication have also developed over the years.[20-26] However, no universal rules exist that can be applied to predict the exact impact each property or preparation strategy has on the function in any specific application. To truly understand a platform, each property must be thoroughly tested within the desired system of use. Therefore, in the present work, we sought to characterize the impact of the heat annealing and thin film casting preparation steps on the resulting immunogenicity of peptide amphiphile micelles.

Among supramolecular materials, peptide amphiphiles are a unique platform capable of delivering biologically active peptides for a wide range of biomedical applications.[1, 2, 27-33] Composed of peptide-lipid conjugates, peptide amphiphiles assemble into cylindrical, rod-like or spherical micelles under physiological conditions.[34] When the peptide is an immunogenic epitope, the micelle system is able to induce robust antigen-specific immune responses without the help of adjuvants.[3] The precise addition of adjuvants, which can also co-assemble into the

same micelle without altering micelle shape, is an effective way to tune a desired immune response.[14] Previously, it has been determined that micelle adjuvanticity requires the antigen be part of the micelle since separation of the antigen and the micelle was insufficient to induce an immune response.[3] But beyond having the epitope attached to the lipid, the impact of structure was unknown. Therefore, before advancing to additional levels of complexity, systematically evaluating the effects of micelle preparation on structure and immunogenicity is a prerequisite to thoroughly understanding the platform.

Preparation steps and design parameters used to create nanoparticles of various sizes, shapes, and particle heterogeneity are practically innumerable. To fully understand one's system, every researcher must characterize each intermediate phase of nanoparticle development and investigate the influence of each phase on cellular uptake, tissue distribution, clearance, cytotoxicity, immunogenicity and any other *in vivo* function.[35-38] For instance, liposomal size is known to be significantly impacted by the thin film casting/hydration method. Small, medium, and large sized particles each produce different immune responses, biodistribution and clearance, so understanding how thin film hydration effects formation is important.[39] Upon heating, micelles and other nanoparticles go through irreversible phase transitions. This transition can be accompanied by many structural changes, such as an increase in secondary structure; shape shortening, elongation, swelling, agglomeration or disintegration; or even just stabilization of the existing particles. Essentially, heating is a process that directs particles to their lower free energy state.[26] Again, each of these structural effects can then effect the *in vivo* function. For peptide amphiphile micelle systems, it is not known to what extent each preparation step affects immunogenicity, yet these previous reports suggest that each step may provide some impact over shape, structure and immunogenicity. Therefore, holding material composition parameters

constant, we sought to systematically compare the contribution of each preparation step to the resulting structural and immunogenic properties of the micelle. Understanding the effect of each preparation step will provide a base knowledge upon which we can expand to higher levels of modular complexity. Peptide amphiphiles containing the J8 peptide epitope have been previously shown to raise antibody responses, so we sought to further understand this system as a model for peptide amphiphiles.[3] The J8 peptide is derived from the C-terminal domain of the Group A Streptococcus (GAS) M1 protein (M-$5_{336-364}$) and provides a simple epitope for investigation since J8 contains only a conformationally-dependent B cell epitope (SREAKKQVEKAL), no T cell or mixed B cell/T cell epitope.[40, 41] We found that each preparation step produced a considerably different micelle shape, leading to consistently tiered immune response results. Film only preparation created short rod-like micelles which produced significantly diminished antibody responses, but had enhanced uptake by APCs. Conversely, the film/heat preparation created long cylindrical micelles which were strongly immunogenic, but had no uptake by APCs. The remaining two preparations, no preparation and heat only preparation, created heterogeneous micelle populations which produced both middling immune responses and APC uptake levels. These findings suggest that the long cylindrical micelle shape is important for B cell epitope antibody production and T cell independent B cell activation. More importantly, these findings provide both clarity in understanding the peptide amphiphile micelle platform more completely as well as opportunity for another lever of modularity to inform the rational design of micelles for future vaccination applications.

4.3 Materials and Methods

Peptide and peptide amphiphile synthesis: J8 peptide (QAEDKVKQSREAKKQVEKAL-KQLEDKVQK) was synthesized on Rink amide MBHA resin (Novabiochem) utilizing standard

Fmoc solid phase synthesis with the aid of a PS3 Peptide Synthesizer (Protein Technologies, Inc.). The N-terminus was either acetylated using 10x molar excess of acetic anhydride in DMF or covalently coupled to Rhodamine (Rho) fluorophore (Anaspec Inc) by an amidation reaction yielding Rho-J8. The resulting J8 peptides were treated using a concentrated trifluoroacetic acid solution to deprotect side groups and cleave the peptide from resin. High pressure liquid chromatography with mass spectrometry controlled fraction collection (LCMS; Shimadzu Corp.) utilizing a reversed-phase C8 column (Waters) with a gradient of acetonitrile in Milli-Q water containing 0.1% formic acid was employed to purify J8 peptide. For J8 or Rho-J8 peptide amphiphiles, the hydrophobic moiety dipalmitoylglutamic acid (diC_{16}) was synthesized by a previously established method.[42] J8 or Rho-J8 peptide was synthesized similarly to above, except the C-terminal lysine was protected with DDE instead of Boc, which was used for the other lysines. The peptide was treated with 2% hydrazine in DMF to orthogonally deprotect the C-terminal lysine amine group which was then covalently coupled to diC_{16} by an amidation reaction yielding J8-diC_{16} and Rho-J8-diC_{16} peptide amphiphiles. These peptide amphiphiles were further processed and purified by the same methods as the peptides above. All samples were lyophilized and stored at -20°C until used. It should be noted that all peptide and peptide amphiphiles were created in a chemical synthesis laboratory using appropriate personal protective equipment to eliminate exposure to biological contaminants.

Micelle formation: To systematically investigate the effect of each preparation step on the structure and resulting antibody response, the micelle formation/preparation steps from previous chapters were broken down into their component steps. Following HPLC purification and lyophilization, the sequence of steps for micelle vaccine preparation were to (1) dissolve powder in 3:1 chloroform:methanol, (2) film dry with nitrogen, (3) rehydrate with PBS, (4) vortex to

induce spontaneous micelle formation and, finally, (5) heat to 70ºC for 1 hour. Breaking these steps down, essentially four intermediate micelle structures can be formed. The 'formulations' are no preparation, film only preparation, heat only preparation, and film and heat preparation. The film and heat preparation is the full micelle preparation described in Chapter 2, and progresses through all the preparation steps listed above. Throughout this chapter, the film and heat preparation will be referred to as the film/heat preparation to reduce confusion. A heat only preparation is the J8-diC_{16} lyophilized powder dissolved in PBS, vortexed, and heated to 70ºC for 1 hour. A film only preparation is the micelle vaccine preparation steps (1) – (4), without the heating step (5). Finally, a no preparation formulation is just the J8-diC_{16} lyophilized powder dissolved in PBS and vortexed. Fluorescent micelles were assembled by combining Rho-J8-diC_{16} and J8-diC_{16} (25:75 molar ratio) and preparing by the same methods for each separation preparation formulation mentioned above.

Micelle characterization: Micelles were characterized by previously defined methodologies[2, 30, 43] including transmission electron microscopy (TEM) and circular dichroism (CD). The morphology of each micelle preparation was investigated using negative stain TEM. Micelle preparation solutions (1 uL of 200 μM) were allowed to incubate on Formvar-coated copper grids (Ted Pella, Inc.) for 1 min after which excess liquid was wicked away with filter paper. Grids were then washed with Milli-Q water and incubated with aqueous phosphotungstic acid (1 wt%) for 1 min before the solution was wicked away. Samples were allowed to air dry and then imaged on a FEI Tecnai 12 TEM using an accelerating voltage of 120 kV. The secondary structure of J8 peptide in solution and confined within the corona of each micelle preparation was assessed using CD. CD spectra of 30 μM solutions of each micelle preparation were measured in water at 25°C a total of 5 times and the data was averaged. The

data presented represents CD analysis performed at least 3 times per sample. Water was used alternatively to PBS since chloride ions from the PBS can interfere with CD measurements. The data were averaged and a curve from 190 nm to 250 nm was fit using a linear combination of polylysine basis spectra [44] to determine approximate α-helix, β-sheet and random coil peptide secondary structure.

Murine vaccination: Female BALB/c and Athymic mice 6-8 weeks old were purchased from Harlan Sprague – Dawley and 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 and were in compliance with the NIH Guide for the Care and Use of Laboratory Animals. Mice were vaccinated in the nape of the neck subcutaneously (100 μL) at days 0 (prime), 21 (boost 1), 28 (boost 2), and 35 (boost 3) with all the micelle preparations described above (no preparation, film only, heat only, film/heat) as well as free J8 peptide and J8 + Incomplete Freund's Adjuvant (IFA, Sigma-Aldrich). All the vaccinations constituted 12 nmol of J8 epitope peptide. Whole blood was collected from saphenous veins pre-vaccination on days 21, 28, and 35 as well as on day 42 to analyze for J8-specific antibodies induced by the previous round of immunization. The blood was centrifuged at 10,000 RPM for 10 min to separate out red blood cells and the supernatant serum was harvested and stored at -20°C until analysis. Subcutaneous injections were used to study antibody responses and T cell responses. Intraperitoneal injections were performed to study uptake by APCs. These constituted two 50 μL intraperitoneal injections in the left and right side of the abdomen.

Antibody response characterization: An enzyme-linked immunosorbent assay (ELISA) was utilized to determine J8-specific antibody titers. Flat-bottom 96-well EIA microtiter plates (Costar) were coated overnight with 100 μL of 10 μg/mL J8 peptide in sodium bicarbonate

coating buffer in each well at 4°C. The wells were washed with 200 μL of 0.05% Tween 20 in PBS (PBS-T) three times and then blocked with 200 μL of assay diluent (10% FBS in PBS) for 1 h. The blocking solution was removed and 100 μL of 1:1000 diluted sera samples were added to the top row and then serially diluted two-fold with assay diluent down the plate. After 2 h incubation, wells were washed with PBS-T three times and incubated with 100 μL of 1:3000 diluted detection antibody (IgG; Invitrogen) for 1 h. PBS-T was used to wash wells three times after which 100 uL of Ultra TMB-ELISA substrate solution (Pierce) was added to the wells. Plates were allowed to incubate for 15 minutes in darkness and then optical density (OD) was measured for each well at 650 nm using a Tecan Infinite M200 plate reader. Endpoint antibody titers were defined as the greatest serum dilution where OD was at least twice that of normal mouse serum at the same dilution. If end-point titers are not reached with one plate then additional titrations were utilized until ODs were diluted to background.

Measurements of T cell responses: To analyze T cell activation by ELISPOT, mice were euthanized 7 days after the final booster immunization. Draining lymph nodes (inguinal, brachial, and axillary) were collected, and single-cell suspensions were prepared as previously reported.[6, 45] Briefly, 96-well ELISPOT plates (Millipore, Cat# MSIPS4510) were washed and coated overnight at 4 °C with anti-IFN-γ (BD, Cat# 51-2525KZ) or anti-IL-4 (BD, Cat# 51-1819KZ) capture antibody. After blocking with complete RPMI medium for 2 h at 37 °C, 200 μL of 2.5M/mL cells was seeded and stimulated for 48 h in a CO_2 incubator at 37 °C. Every mouse vaccination had cells stimulated with 5 μM J8 peptide. For the experimental groups, each of the four mouse vaccination preparations under investigation had their cells stimulated by the same vaccine preparation (e.g., no preparation mouse vaccination stimulated by 5 μM no preparation, film only mouse vaccination stimulated by 5 μM film only, etc.). To test that the assay was

working correctly, a naïve mouse was stimulated with Phytohemagglutinin (PHA). Plates were then washed and incubated with biotinylated anti-IFN-γ (BD, Cat# 51-1818KA) or anti-IL-4 (BD, Cat# 51-1804KZ) detection antibodies, then with streptavidin−alkaline phosphatase (Mabtech, Cat# 3310-10), and finally with the substrate Sigmafast BCIP/NBT (Sigma, Cat# B5655). Plates were allowed to dry overnight before the spots were imaged and counted using an ELISPOT reader (Cellular Technology Ltd.).

Cell uptake: Mice were euthanized 18 h after intraperitoneal (IP) immunization of fluorescently labeled micelle preparations (25:75 molar ratio of Rho-J8-diC_{16} and J8-diC_{16}). IP lavage was conducted using an intraperitoneal injection of 2 mL of Hank's balanced salt solution. The abdomen was massaged 40 times, and about 1.2−1.5 mL of lavage fluid was withdrawn from the IP space. Cells were collected by centrifugation and washed once by flow buffer (PBS containing 2% fetal bovine serum). After blocking the Fc receptor with 2.4G2 antibody (BD Bioscience, Cat# 553142), cells were stained for F4/80 (Biolegend, Cat# 123128), MHC class II (Biolegend, Cat# 107606), and CD11c (Biolegend, Cat# 117318). Cells were washed and resuspended in flow buffer with 1 μg/mL of DAPI. Flow cytometry was performed on a BD LSRII and analyzed using FlowJo software. Macrophages were gated as F4/80-positive, and dendritic cells (DC) were gated as F4/80-negative/MHC class II-high/CD11c-positive. The percentage of Rhodamine-positive cells (having internalized labeled micelles) was calculated for both macrophage and DC populations.

Dendritic cell activation: To control the doses of micelle preparations exposed to APCs, dendritic cell activation was studied *in vitro* using BMDCs. Bone (femur and tibia) marrow cells were cultured in complete RPMI medium containing 200 ng/μL of Flt-3L. Loosely bound cells and floating cells were collected on day 8 for assays. Micelle preparations (1.2 mM) were diluted

to 0.12 mM (120 μM) in complete RPMI medium. Next, 250 μL of these diluted micelle preparations were added to 250 μL of cell suspension (1×10^6 cells/mL final cell concentration) and cultured for 24 h. Control cell populations were also stimulated with 100 ng/mL lipopolysaccharides (LPS, positive), PBS (negative) and 120 μM free J8 peptide for 24 h. Cells were stained for F4/80, CD11c, and MHC class II, CD80 and CD86 for flow cytometry, as in *in vivo* studies. Again, DCs were gated as F4/80-negative/MHC class II-high/ CD11c-positive. Less than 3% of cells exhibited a macrophage phenotype (F4/80-positive), and the activation efficiency in the dendritic cell population was quantified via upregulation of CD80 and CD86.

Statistical analysis: Statistical analysis was performed in Graphpad Prism using one-way or two-way ANOVA with Dunnett's multiple comparison test as indicated in the figure legends. Means or geometric means ± standard deviations are presented unless otherwise noted.

4.4 Results

4.4.1 Preparation steps alter micelle shape and secondary structure

To systematically investigate the importance and effect of each preparation step in micelle formation, we generated a peptide amphiphile referred to as J8-diC_{16}. Synthesized using standard solid phase peptide synthesis techniques followed by purification with rpHPLC, J8-diC_{16} consists of the peptide sequence QAEDKVKQSREAKKQVEKALKQLEDKVQK and a diC_{16} tail conjugated to the epsilon amine of the C-terminal lysine residue. These particles self-assemble into micelles at low micromolar concentrations. Chapter 2 described how a combination of the thin film hydration method and heat annealing elongated the micelle structure and increased peptide α-helicity to create a self-adjuvanting vaccine delivery vehicle that induced a significant peptide-specific antibody response. However, a systematic investigation of

the effect of each preparation step on the structure and resulting antibody and broader immune response was lacking. Following HPLC purification and lyophilization, the sequence of steps for micelle vaccine preparation were to (1) dissolve powder in 3:1 chloroform:methanol, (2) film dry with nitrogen, (3) rehydrate with PBS, (4) vortex to induce spontaneous micelle formation and, finally, (5) heat to 70ºC for 1 hour. Breaking these steps down, essentially four intermediate micelle structures can be formed. The 'formulations' are referred to as: no preparation, film only preparation, heat only preparation, and film/heat preparation. The film/heat preparation is the full micelle preparation described in Chapter 2. A heat only preparation is the J8-diC_{16} lyophilized powder dissolved in PBS, vortexed, and heated to 70ºC for 1 hour. A film only preparation is the micelle vaccine preparation steps (1) – (4), without the heating step (5). Finally, a no preparation formulation is just the J8-diC_{16} lyophilized powder dissolved in PBS and vortexed.

We first characterized the micelle shape for each formulation. The film/heat preparation forms long, flexible, worm-like micelles that are several microns in length and approximately 10 nm in diameter (Figure 4.1, bottom right, as previously shown). Interestingly, the heat preparation also formed long, flexible, worm-like micelles of similar dimensions to the film/heat preparation (Figure 4.1, bottom left). Through TEM, there were no visibly discernable differences between the heat preparation vs. film/heat preparation. This despite the heat preparation undergoing none of the thin film hydration steps. Film preparation, on the other hand, shows a vastly different micelle shape. A film prepared micelle manifests as small, spherical and short rod shaped micelles (Figure 4.1, top right). Lastly, a no preparation formulation manifests as a highly heterogeneous mixture of micelle shapes and sizes, including small, spherical, and short rod shaped micelles as well as medium sized cylindrical micelles (Figure 4.1, top left).

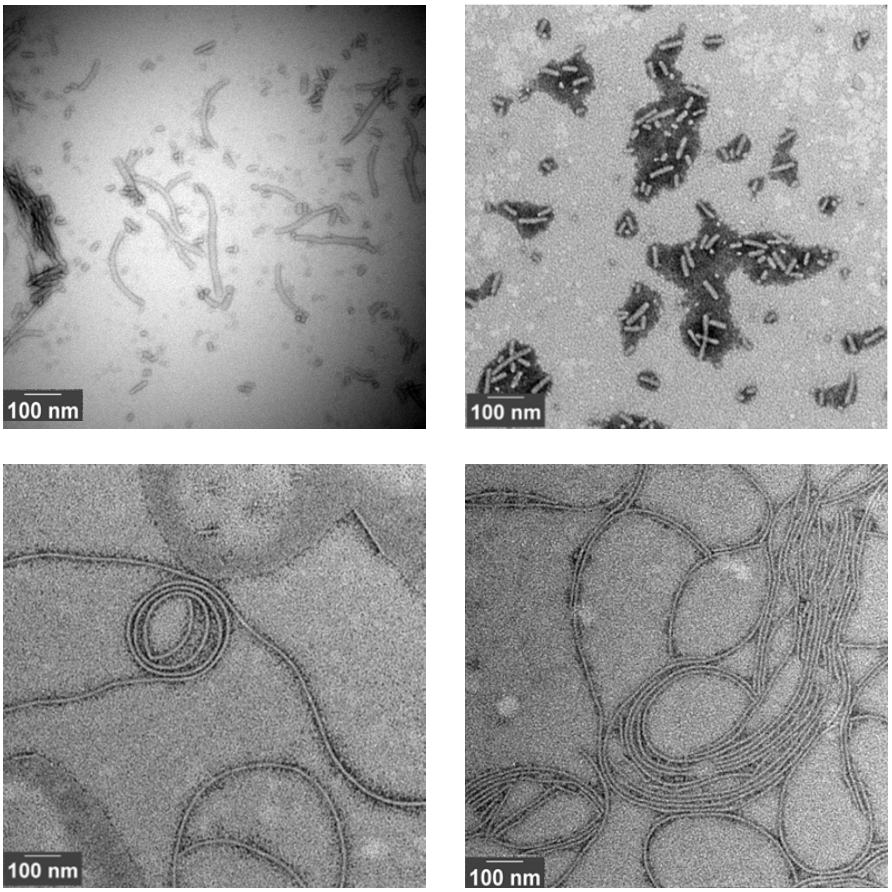

Figure 4.1 – Preparation steps alter micelle structure. Negative stain TEM images of each micelle preparation at 200 μM (scale bar: 100 nm). No preparation (top left) forms a heterogeneous population of small, spherical, and short rod shaped micelles as well as medium sized cylindrical micelles. Film only preparation (top right) forms a relatively homogeneous population of small, spherical and short rod shaped micelles. Heat only preparation (bottom left) forms long cylindrical micelles, similar in structure to the film/heat preparation. Film/heat preparation (bottom right) forms long, flexible, cylindrical/worm-like micelles.

Next, we characterized the peptide secondary structure induced by micelle formation and each preparation step. The J8 peptide alone is predominantly random coil in PBS. Even when heating or film casting the J8 peptide, the peptide remains predominantly random coil and it's α-helicity does not appreciably increase. As we know from Chapter 2, micellization of the peptide amphiphile stabilizes the secondary structure of J8 and heat annealing increases α-helical content significantly. However, the impact of each micelle preparation step individually on the peptide

secondary structure is unknown. The CD spectra of each preparation step was therefore analyzed (Figure 4.2). A micelle with no preparation is able to achieve 42.5% α-helicity, even with its heterogeneous shapes and sizes. A film prepared micelle has an α-helicity of 44.3%, followed by a heat prepared micelle at 46.9% and topped with the film/heat preparation at 50.5%.

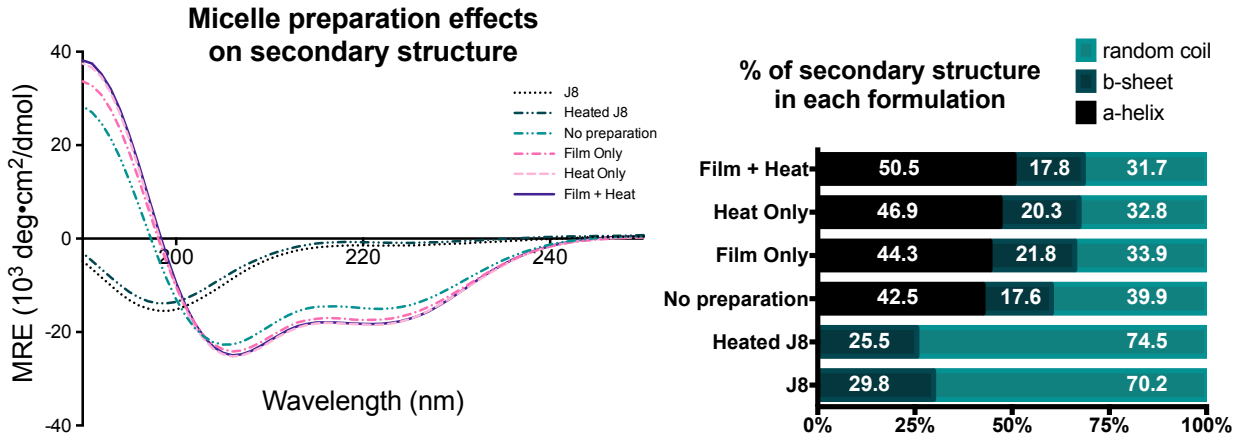

Figure 4.2 – Micelle preparations show tiered levels of secondary structure. The CD spectra of each micelle preparation are shown (left) and quantified via curve fitting (right) to determine the percent of secondary structure in each formulation. α-helix content increased for each preparation, though there is only an 8% spread between no preparation and film/heat.

Following these results, it is clear that the micelle preparation steps can act as another lever of micelle modularity. With a variety of shapes and varying levels of secondary structure development, each preparation can conceivably be used in different situations in order to tune the immune response to deliver the desired response. Therefore, it is important to evaluate the resulting effect each preparation has on the immune response.

Additionally, when taking a broader view, the structural properties of individual steps seem to tell the story of how each step contributes to micellization. In a way, it seems each preparation step is a progression from the previous step, and when combined sequentially they compile toward what we have previously characterized as the full film/heat preparation. More specifically, the no preparation step demonstrates formation of micelles as a spontaneous process

that organizes amphiphilic molecules in a favorable orientation, but as heterogeneous and randomly sized particles. The film preparation step provided uniformity of structural formation to the otherwise unstructured formation of micelles. The heat annealing step elongated the micelle structure, though presumably in a heterogeneous manner if started from no preparation. Finally, when combined, the progression is to form uniform micelles that are then elongated into the film/heat preparation previously characterized. This progression is further evidenced by the stepwise increase in α-helicity from one micelle preparation to the next. Continuing on, it'll be interesting to determine if this stepwise progression effect is revealed in the resulting immune responses.

4.4.2 Micelle-preparation-dependent antibody responses

Mice were immunized with the four preparations of J8-diC_{16} peptide amphiphiles in PBS on day 0 (prime), day 21 (boost 1), day 28 (boost 2), and day 35 (boost 3). Serum was analyzed for a specific antibody response to the J8 peptide by ELISA. Consistent with our previous reports, the film/heat preparation was able to produce an IgG antibody titer close to 10^6 after three boosts and served as a baseline for the study (Figure 4.3). Among the other preparations, without the full film/heat preparation there was a dramatic effect of significantly diminished antibody responses. In the case of the film only preparation, the antibody response was almost entirely abolished (Figure 4.3). Heat preparation and no preparation vaccines also induced diminished antibody responses, approximately two orders of magnitude lower than the baseline film/heat preparation (Figure 4.3). The antibody titer response pattern for the heat preparation and no preparation vaccines were similar throughout the vaccination schedule, essentially rising to the same levels at the same rate. The abolishment of antibody responses by the film only

preparation was complete. No mice in two independently studied groups of 5 raised more than a barely detectable titer.

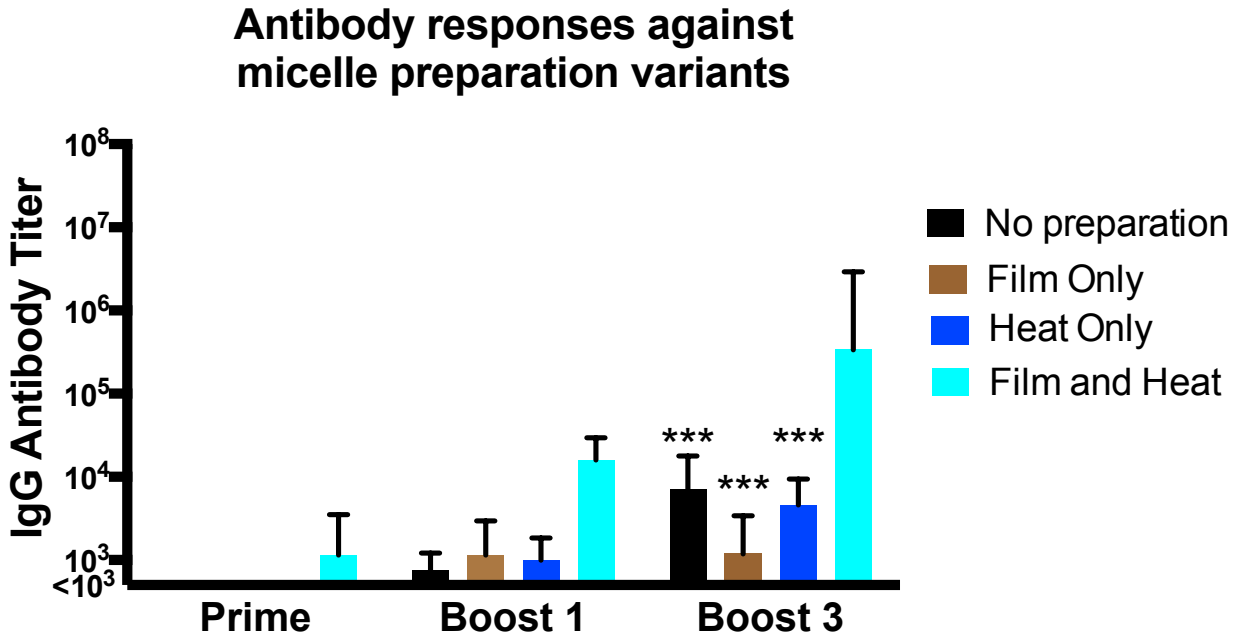

Figure 4.3 – Micelle preparations induce tiered IgG antibody responses. Compared to the film/heat preparation, all other preparations saw a dramatic effect of significantly diminished antibody responses. Film only antibody response was almost entirely abolished. Heat only preparation and no preparation antibody responses were approximately two orders of magnitude lower than the baseline film/heat preparation. Each bar represents the geometric mean ($n = 5$). Data was analyzed by one-way ANOVA and Dunnett's multiple comparison test (compared to film/heat preparation at respective time points); ***$p < 0.001$.

The analysis of antibody responses revealed the strong influence of micelle preparation on resulting immunogenicity for J8-diC_{16} particles. This research indicates that the long cylindrical micelles with full film/heat preparation raise a stronger antibody response than any other preparation scheme. Additionally, as evidenced by the tiered antibody responses, a trend has emerged where each preparation step seems to be piece of the whole possible response, consistent with the progression seen in the structural analysis.

4.4.3 J8 peptide amphiphiles – of any shape preparation – do not activate T cells or dendritic cells

The four preparations were also studied for their ability to raise CD4 T cell responses, via ELISPOT analysis (Figure 4.4). The J8 antigen was designed to contain a minimal B cell epitope but not a dominant T cell epitope from GAS (to reduce the likelihood of any untoward autoimmune response).[46, 47] The peptide alone, however, is non-immunogenic and has typically been delivered with an adjuvant such as DT or alum.[41, 48, 49] For their J8-DT vaccine platform, the Good group has demonstrated that the stimulation of a B cell response requires T cells, but these need not be memory T cells.[50] Despite this finding and because J8 is a B cell epitope, the possibility still exists for antigen specific B cell activation in a T cell independent way. Because we know J8-diC_{16} micelles can raise an antibody response without adjuvants, the peptide amphiphile micelle system may have the unique properties required to facilitate a T cell independent immune response. What remains to be tested is whether T cells are involved in a J8-diC_{16} immune response. And on top of that, if there is a T cell response, does micelle shape/preparation have an impact on the T cell response, similar to the tiered results observed for antibody responses.

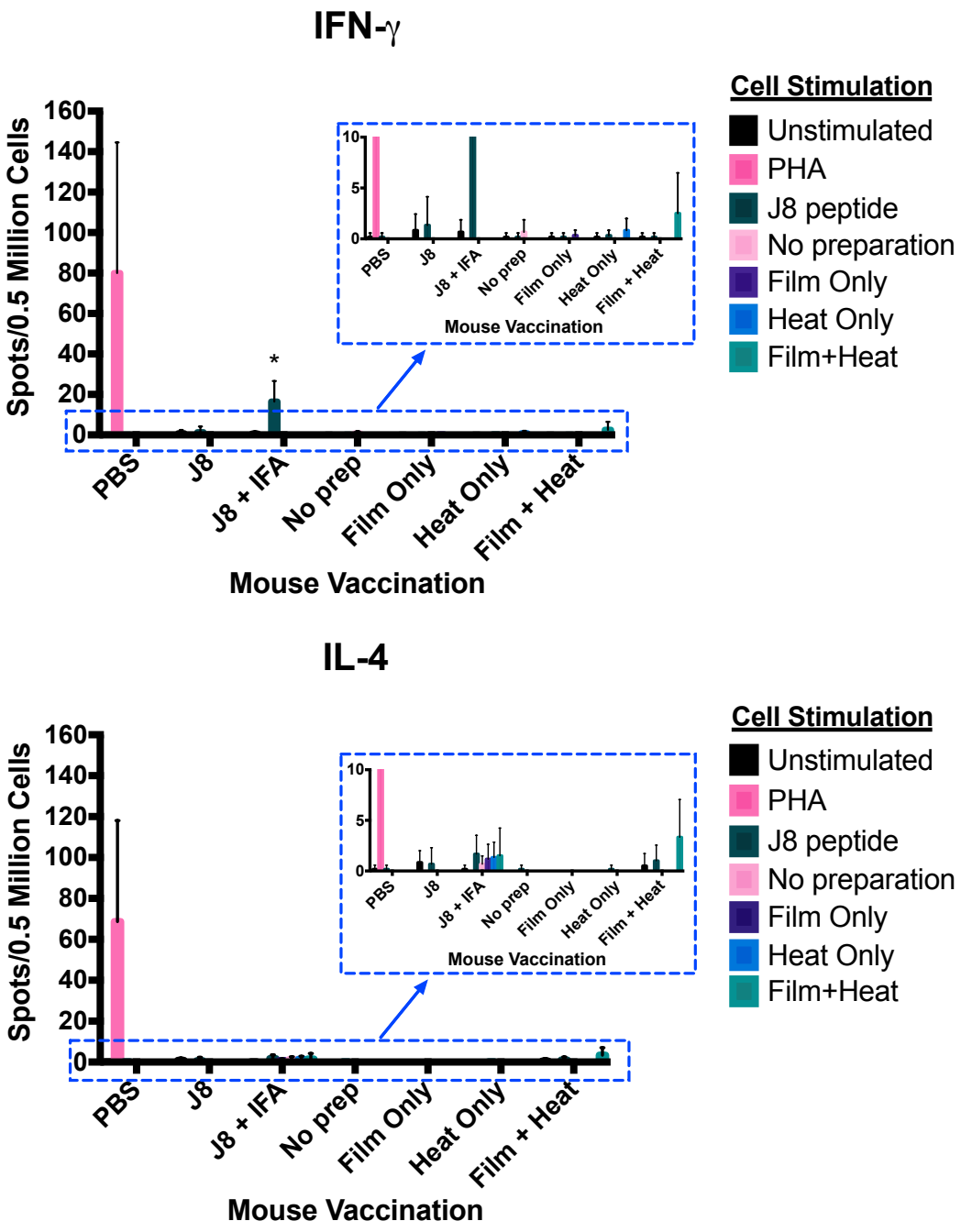

Figure 4.4 – ELISPOT analysis of T cell responses. J8-diC_{16} micelle preparations were unable to activate J8 specific T cells on the ELISPOT, indicating no J8 specific T cell responses were induced during vaccination. A naïve mouse was stimulated with Phytohemagglutinin (PHA) to indicate that the assay worked. IFN-γ or IL-4 secreting cells were quantified. The inset shows a magnified y-axis. Data were analyzed by two-way ANOVA and Dunnett's multiple comparison test (compared to unstimulated control group); *$p < 0.05$, ** $p < 0.01$.

To test CD4 T cell responses, draining lymph nodes (inguinal, axillary, and brachial) were collected seven days after the final boost of the initial immunization regimen. Cells from the lymph nodes were cultured and stimulated. Every mouse vaccination had cells stimulated with 5 μM J8 peptide. For the experimental groups, each of the four mouse vaccination preparations under investigation had their cells stimulated by the same vaccine preparation (e.g., no preparation mouse vaccination stimulated by 5 μM no preparation, film only mouse vaccination stimulated by 5 μM film only, etc.) Cells secreting IFN-γ or IL-4 were quantified. By this measure, the control group of J8 peptide in IFA was able to raise a modest T cell response, while all of the other preparations failed to induce any significant T cell responses (Figure 4.4). Given that adjuvants were not included in any formulation and that J8 is a B cell epitope, the result is slightly unsurprising.

Typically, in order to activate antigen specific T helper cells, antigens need to be internalized by APCs, proteolyzed (processed) into short T cell epitopes, loaded into class II major histocompatibility molecules (MHC class II), and presented to T cells on the APC surface. However, combining the finding that J8-diC_{16} vaccine preparations were unable to stimulate T cells with the knowledge that J8 is strictly a B cell epitope, we hypothesized that a micelle's inability to activate DCs would confirm the subsequent lack of T cell response. To test this hypothesis, we studied the activation of cultured bone marrow dendritic cells (BMDCs) via upregulation of activation markers CD80 and CD86 (Figure 4.5). Exact doses of each vaccine preparation were added to BMDC cultures, and cell activation was evaluated by flow cytometry. Results from the *in vitro* dendritic cell activation experiment indicate that no J8-diC_{16} vaccine preparation was able to activate dendritic cells (Figure 4.5). This result corresponds with the

micelles inability to activate T cells, since the activation of both cell types is inextricably linked in the lifecycle of an immune response.

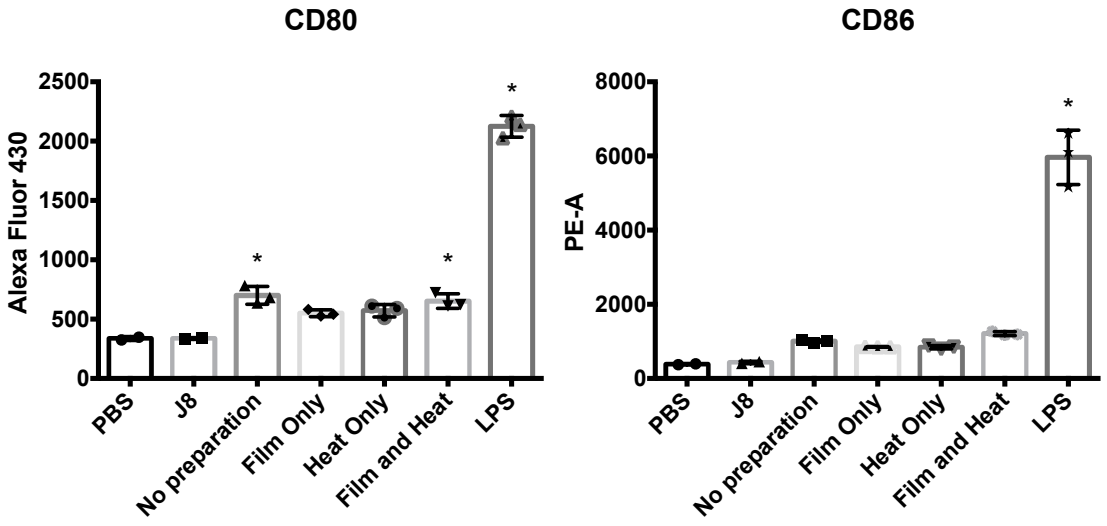

Figure 4.5 – Dendritic cells are not activated by any J8-diC_{16} micelle preparation. Despite some statistical significance for the no preparation and film/heat preparation, the activation levels are far below what is typically seen in dendritic cell activation, as indicated by the LPS positive control. Data shown were combined from two independent experiment ($n = 3$; $n = 2$ for naïve control). Data were analyzed by one-way ANOVA and Dunnett's multiple comparison test (compared to naïve control group); *$p < 0.05$.

4.4.4 Micelle preparations have differential uptake by antigen presenting cells

Having found that micelle preparation does not impact the activation of J8 specific T cells, we wanted to test whether or not the micelles are even taken up by antigen presenting cells. Without antigen presenting cell uptake, the peptide amphiphile micelles wouldn't even be afforded the opportunity to activate these cells. Therefore, we investigated whether or not micelles are taken up by antigen presenting cells, and whether or not the preparations alter uptake. Particle size is a known determinant of differing uptake rates which could result in varying T cell responses if we were delivering a T cell epitope. To test this hypothesis, we studied micelle internalization using fluorescent micelles and flow cytometry. Fluorescent micelles were assembled by combining Rho-J8-diC_{16} and J8-diC_{16} and prepared by the same

methods as the four preparations mentioned above. Rhodamine fluorescent micelles were injected intraperitoneally, and cells in the intraperitoneal lavage fluid were collected and stained for flow cytometry.

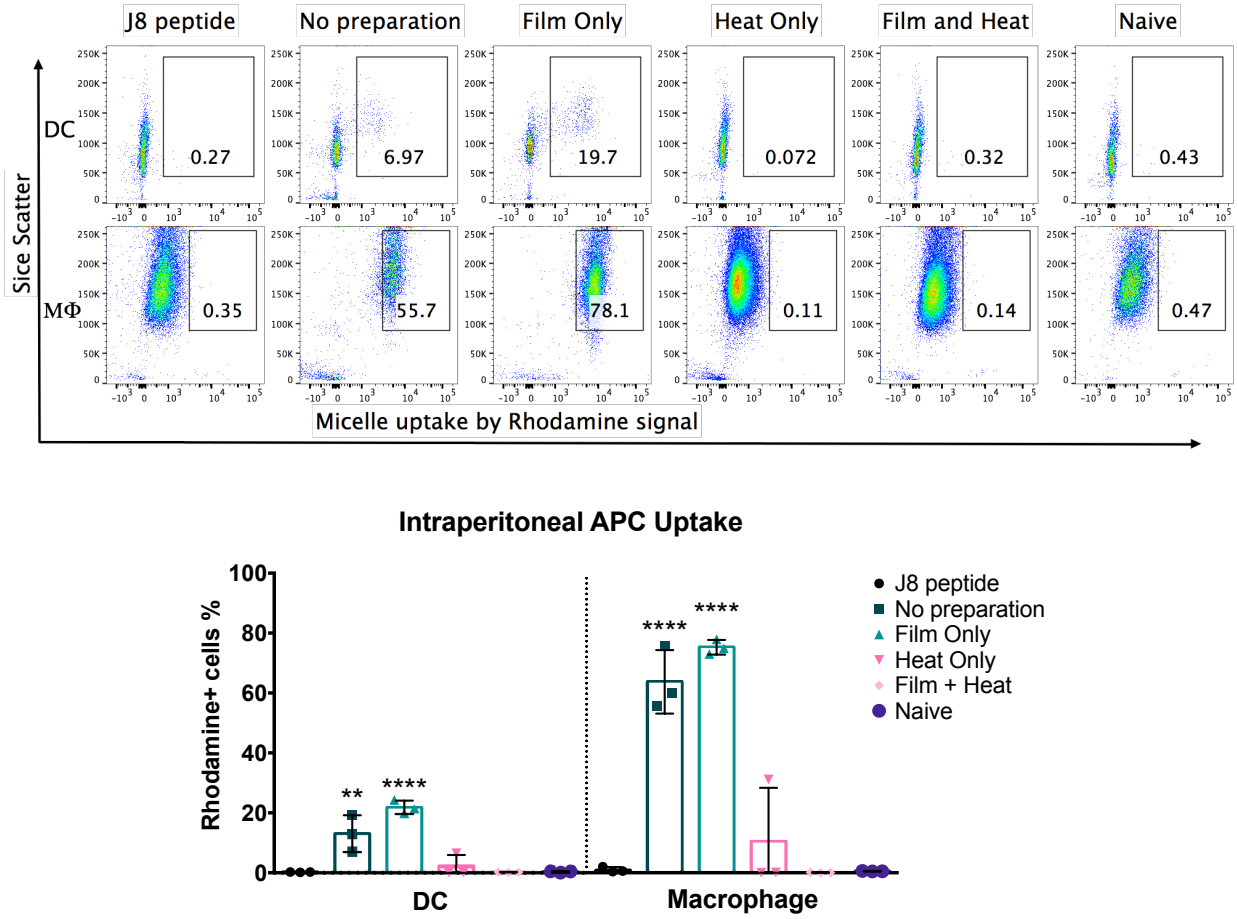

Figure 4.6 – Micelle uptake by macrophages and dendritic cells was significantly modulated by preparation shape. The long cylindrical micelles of the film/heat preparation had negligible uptake while the film only preparation with small micelles had significant internalization. The heterogeneous no preparation also showed significant internalization, though lower than the film only. The heat only preparation showed low levels of internalization, though higher than the film/heat preparation. Representative flow cytometry data for intraperitoneal injections are shown on top and quantified below. Data shown were combined from two independent experiment ($n = 3$). Data were analyzed by one-way ANOVA and Dunnett's multiple comparison test (compared to naïve control group); ** $p < 0.01$, ****$p < 0.0001$.

Dendritic cells were gated as the F4/80-negative/MHC class II-high CD11c+ population, while macrophages were gated as the F4/80-positive population. For the film only preparation, 21.7% of dendritic cells and 75.5% of macrophages were found to be Rhodamine positive; that is, these cells had acquired peptide amphiphiles (Figure 4.6). In stark contrast, negligible uptake by macrophages or DCs was observed for the film/heat preparation (Figure 4.6). For the no preparation and heat only preparation, intermediate levels of uptake were observed, falling between the two other preparations (Figure 4.6). Again, the tiered uptake results for each micelle preparation are consistent with the trend seen in earlier structural and immunological experiments.

4.4.5 Film/heat micelle preparation is able to induce antibody response in athymic mice

To further elucidate how micelle preparation could modulate immune responses and to conclusively determine if this system can activate B cells independently from T cells, our final experiment in this study investigated whether athymic mice that lack T cells can produce J8 specific antibodies (Figure 4.7). Athymic mice were immunized with the four preparations of J8-diC_{16} peptide amphiphiles as described before. Coinciding with the results from above, the film/heat micelle preparation was able to induce a modest antibody response, even without the presence of T cells or adjuvants (Figure 4.7). The other preparations were unable to raise a significant antibody response (Figure 4.7). While the antibody responses were too low to conclusively see the tiered response, it is interesting to note that both the no preparation and heat only preparation vaccinations were able to produce one weakly responding mouse. Hence, fully prepared film/heat micelles are able to raise T cell independent B cell responses and full preparation is essential to establish the strongest immune response.

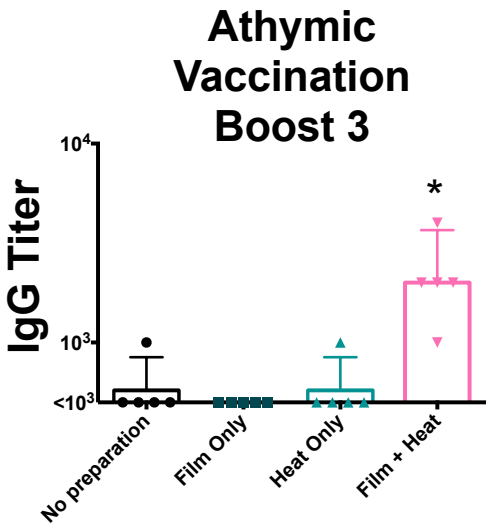

Figure 4.7 – Long cylindrical micelle shape from the film/heat preparation is required to raise T cell independent antibody response. Each data point represents one mouse (n = 5); bars represent the geometric mean. Data was analyzed by one-way ANOVA and Dunnett's multiple comparison test (compared to naïve at respective time points); *$p < 0.05$.

4.5 Discussion

It is known that preparation steps can significantly affect the structure and function of nanomaterials.[35, 36] In immunoengineering, countless examples exist where an immune response is created, destroyed or augmented based on seemingly minor nanomaterial property adjustments.[51, 52] Therefore, the impact of each preparation step on the micelle's subsequent immunogenicity is not something to be ignored. In fact, each preparation step should be viewed as a lever of modularity that needs to be characterized. With such an enormous design-space available to peptide amphiphile micelles, the importance of the present work is that it illustrates both the advantages and disadvantages of each preparation step on micelle formation and immunogenicity. The effect of each preparation step on the resulting micelle was surprisingly drastic, in both structure and immunogenic function. In this J8-diC_{16} micelle system, all measureable immune responses revealed a consistently tiered range of results. Film only

prepared micelles produced significantly diminished immune responses, but had enhanced uptake by APCs. In contrast, the film/heat prepared micelles were strongly immunogenic, but had no uptake by APCs. The remaining two preparations, no preparation and heat only preparation, produced both middling immune responses and APC uptake levels, never as good or as bad as the first two preparations (results summarized in Table 4.1).

Table 4.1 – Summary of properties and immunogenicity of vaccine preparation formulations

formulation	micelle	conformation	antibody response	DC uptake	DC activation	B cell activation	T cell activation
No preparation	short rod and medium cylinder	42% α-helix	moderate	moderate	no	yes	no
Film Only	short rod	44% α-helix	none	high	no	no	no
Heat Only	long cylinder	46% α-helix	moderate	low	no	yes	no
Film/Heat	long cylinder	50% α-helix	high	none	no	yes	no

In the present study, we did not explore in mechanistic detail the specific reason why the vaccine preparations consistently produced tiered levels of immune responses, but one can speculate why this occurred. At first look, secondary structure enhancement among preparations may have played a role in the tiered immune responses. However, because the difference in α-helicity between the four preparations is only 8%, this difference is unlikely to be the major contributor of the tiered responses. Instead, the most likely explanation would be the variations in shape each preparation produced. A secondary factor related to shape is the heterogeneity of the resulting micelle preparation. Antibody responses, both in immunocompetent BALB/c mice and immunocompromised athymic mice showed significant strength when the film/heat preparation was used. The film/heat preparation formed a long, cylindrical micelle with the highest level of α-helicity and relative homogeneity in length. On the other hand, film only prepared micelles formed a homogeneous population of short rod-like micelles which were unable to induce an antibody response. While it was previously presumed that the long

cylindrical shape played a role in the immunogenicity of J8-diC_{16} micelles, this is the first true evidence of that idea. Additionally, it seems that the long cylindrical shape is also a driving factor to allow the J8-diC_{16} micelle to induce T cell independent B cell responses, since the film/heat preparation was the only formulation able to raise an antibody response in athymic mice. This notion is supported by the observation that the two other micelle preparations (no preparation and heat only preparation) were able to raise a modest antibody response in immunocompetent mice: both higher than the film only preparation, but significantly reduced compared to the film/heat preparation. Having been formed without the film step (which provides initial structural uniformity), both of these formulations have a higher level of micelle size and shape heterogeneity. If a long cylindrical shape is required to form an immune response to J8, the no preparation and heat only preparations have some medium to long shaped micelles available to induce a response, but the small spherical, short rod-like and inconsistently sized cylindrical micelles limit the upside of a stronger immune response.

With regards to T cell activation, because J8 is a B cell epitope, the micelle preparations alone (no adjuvants) were not able to induce T cell responses during vaccination, which explains the inability to activate J8 specific T cells on the ELISPOT (because they don't exist). It seems that the control immunization J8 + IFA, however, is able to produce moderate activation of J8 specific T cells, and these cells can be subsequently stimulated with J8 peptide and activated on an ELISPOT. This despite the fact that J8 is known to be a B cell epitope. Therefore, this data indicates that with the inclusion of a strong adjuvant comes the possibility of uncovering a weak T cell epitope within the J8 sequence. Either that or the vaccine formulation had a contaminant that resulted in minor activation of J8 specific T cells.

In order for T cells to be activated, an antigen must be presented to them by an antigen presenting cell, among other signals. Although our micelle preparations are seemingly unable to activate T cells, the question remained whether antigen presenting cells are even able to take up micelles for subsequent presentation to T cells. We therefore tested micelle uptake levels, and whether or not the preparations alter antigen presenting cell uptake. It turns out micelle preparations also saw a tiered result in uptake levels, but reversed the trend we've seen thus far. In both dendritic cell and macrophage populations, film only preparations had significant uptake levels while the film/heat preparation showed negligible uptake. Intermediate levels of uptake were observed for the no preparation and heat only preparation. This result indicates that because of their shape the short rod-like micelles were scavenged by antigen presenting cells. However, this did not lead to T cell activation because of the majority B cell epitope within J8. This is further supported by the observation that even though the film only preparation micelle was taken up by APCs, DCs still showed no activation for any of the four micelle preparations. This is likely because the preparations only included a B cell epitope without a strong T cell immune adjuvant.

Finally, a determination of how these design factors effect immune responses when experimenting with a different antigen would be an important future study. If the long cylindrical shape is such an important factor when raising an immune response against a B cell epitope, will that hold true when delivering T cell epitopes or with the inclusion of adjuvants? As a specific example, if a T cell epitope were used instead of a B cell epitope, it is conceivable to predict that the short rod-like film preparation could raise a stronger immune response since their increased APC uptake could lead to more efficient presentation to T cells. But would the uptake trend hold true with a different epitope? While a tiered response rate was exposed for the J8-diC_{16} micelle

preparations used during this chapter, this discussion reminds us that it is important to remember and recognize that no universal rules exist that can be applied to predict the *in vivo* behavior of all nanoparticles and systematic evaluations of each design are necessary to confirm an assumption. Nonetheless, these findings provide both clarity in understanding the peptide amphiphile micelle system more completely as well as opportunity for another lever of modularity to inform the rational design of micelles for future vaccination applications.

4.6 Conclusions and Future Directions

We investigated the preparation effects of heat annealing and thin film casting on the structure and immunogenicity of antigen bearing self-assembling peptide amphiphile micelles. Characterization studies revealed that each preparation step produced a different shape which subsequently led to consistently tiered immune responses. Film only preparation produced short homogeneous rod-like micelles with significantly diminished immune responses, but had enhanced uptake by APCs. In contrast, the film/heat preparation produced long cylindrical micelles with strong immunogenicity, but no uptake by APCs. The remaining two preparations, (no preparation and heat only preparation) were both heterogeneous in size and shape, which lead to middling immune responses and APC uptake levels, never as good or as bad as the first two preparations. In this J8-diC_{16} micelle system, it was found that the long cylindrical shape is a driving factor of immunogenicity, since the film/heat preparation was the only formulation able to raise an antibody response in athymic mice. These findings indicate that the long cylindrical micelle shape is a unique property that can facilitate a T cell independent antibody response for B cell epitopes.

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