New Generation of Adjuvants for Protection Against Disease and to Combat Bioterrorism

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Abstract

This chapter highlights the characteristics of traditional and non-traditional adjuvants, the advantages and disadvantages of their use in vaccines. It has so served to introduce the concept of molecular and host-derived adjuvants to improve immune outcomes while minimizing toxicities. A particular emphasis was placed on the generation of conformationally-restricted, response-selective agonists of the complement component C5a. These all appear to have therapeutic, manufacturing, and commercial potential. However, the development and use of such molecular adjuvants for vaccines represent only one approach put forward to meet the modern-day threats posed by antibiotic-resistant strains of bacteria and bioterrorism. It is clear that a well-organized and concerted worldwide effort that utilizes multiple approaches to develop novel adjuvant and vaccine designs will be required to overcome these growing threats to human and animal populations.

Keywords

Antigen presenting cells • C5a • Complement • EP67 • Molecular adjuvant • Vaccines

48.1 Introduction

Vaccines remain one of the greatest advances of medical history having led to the eradication of smallpox and the near-eradication of polio. Vaccination is principally a prophylactic approach with the objective of generating long-lived humoral and cellular immune responses that provide protection against invading pathogens. Traditionally, vaccines rely on the use of live-attenuated, killed, or inactivated forms of a whole pathogen as the target antigen (Ag); i.e., that entity against which an immune response is sought. Such whole-pathogen vaccines are effective in generating protective immune responses primarily due to their close resemblance to natural infections. However, these vaccines pose certain issues including undesirable host reactions, difficulties associated with generating the target Ag in culture, the risk of pathogen reversion to virulence, and logistical challenges in maintaining a cold supply chain for vaccine distribution. These vaccines also pose significant safety issues in immunocompromised and neonate patients.

In contrast to whole-pathogen vaccines that make use of a whole attenuated pathogen as the target Ag, subunit vaccines are short, specific fragments of pathogen-associated proteins, which are safer, noninfectious, and immunologically more defined than traditional, whole pathogen vaccine approaches. Advances in Ag discovery technologies using reverse engineering of protective immune responses from pathogen-infected subjects (disease non-progressors) have heralded a new era in vaccine development for prevention of global diseases.

A prominent feature that underscores the current era of modern vaccinology is the ability to generate a huge variety of Ags by various synthetic chemistry and genetic engineering techniques. As a result, Ags now can be produced rapidly

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and in large quantities with well-defined structures and in highly purified forms—desirable attributes for their use in vaccines intended to generate Ag-specific immune responses in a diverse population. Unfortunately, the routine use of these Ags as integral components of vaccines is encumbered by their inherent lack of immunogenicity. Such vaccines, therefore, require the use of adjuvants in order to potentiate and focus the immune response to the Ag so that optimal immune outcome can be achieved. Thus, the discovery and development of new adjuvants is of growing importance to the design of vaccines capable of meeting the modern threats posed to human and animal populations by new or resurgent infectious and non-infectious diseases.

This chapter highlights recent trends in the development of adjuvants and, their current proposed mechanisms of action with an emphasis on adjuvants derived from components of the host (i.e., host-derived adjuvants) rather than the more commonly-used adjuvants derived from components of pathogens (i.e., pathogen-derived adjuvants).

48.2 Adjuvants: Vehicles and Immunomodulators

An adjuvant is any substance or any formulation of substances that enhances an immune response specific to an Ag. Adjuvants increase the magnitude, longevity and quality of Ag-specific immune responses. Apart from increasing vaccine effectiveness, adjuvants also reduce the amount and number of vaccine doses required to generate protective immune responses and thus contribute to increased patient compliance. As a general rule, adjuvants can be broadly classified into either vaccine delivery vehicles or immunomodulators. Often, both of these can be combined to further increase vaccine effectiveness. The use of adjuvants in vaccines to improve their effectiveness has traditionally been empirical. The necessity for new vaccine adjuvants arises from the shortcomings of currently approved adjuvants, which are incapable of eliciting immune responses that correlate with protection against different target pathogens. Rational design of vaccine adjuvants is essential for polarization of immune responses that correlate with protection against different pathogens and tumors.

48.2.1 Vehicles

Vaccine delivery vehicles are those adjuvants that help carry Ags to and retain them in proximity to lymphocytes and other auxiliary immune cells, particularly antigen presenting cells (APC), within various lymphoid tissues. Indeed, it is

this "depot" effect that is the defining mechanism of vehicle adjuvants. Classic examples of vehicles include liposomes, emulsions, proteosomes, and immunostimulating complexes (ISCOM) (Crouch et al. 2005). Typically, the final vaccine formulation is the Ag contained within the vehicle. Other examples of vaccines that utilize vehicles include formulations in which the Ag is covalently linked to biocompatible and biodegradable microparticles such as poly(D,L-lactide-coglycolide) (PLGA) (Diwan et al. 2004), nanoparticles, or adsorbed on the surface of carriers such as calcium and aluminum salts (phosphate or hydroxide), particularly alum, which is the only adjuvant currently approved for human use (Olive et al. 2001).

48.2.2 Immunomodulators

Immunomodulators are adjuvants defined by their ability to activate APCs and/or lymphocytes. Typically, this activation is characterized by the adjuvant-induced release of cytokines form lymphocytes and other auxiliary immune cells. Examples of immunomodulators include muramyl-dipeptide (MDP), monophosphoryl lipid A (MPL) (Ulrich and Myers 1995), lipopolysaccharide (LPS) (Johnson et al. 1956), bacterial cell membranes (Muhlradt et al. 1998), certain components of the complement system (Dempsey et al. 1996; Jacquier-Sarlin et al. 1995), cytokines (Afonso et al. 1994; Kurzawa et al. 1998), and oligonucleotide mimics of bacterial DNA. The typical vaccine formulation is one in which the Ag is admixed with the immunomodulator. Variations on this theme include those in which the Ag is covalently linked to the immunomodulator with the rationale that elements of the generalized immune response invoked by the adjuvant might be more directed to the Ag. Some examples of this approach include the incorporation of lipids, lipopolysaccharides, and lipoamino acids into peptide Ags by synthetic methods (Defoort et al. 1992; Martinon et al. 1992; Metzger et al. 1991; Olive et al. 2001; Wiesmuller et al. 1992) and the genetic fusion of chemokines/cytokines to protein Ags (Biragyn et al. 1999).

It should be pointed out that this classification of adjuvants is based more on historical observations than on strict mechanisms of action. In fact, there are several examples of adjuvants belonging to the vehicle family that act as immunomodulators. A noteworthy example of this is the saponins, which are extracted from the plant *Quillaja saponaria* and are used to create emulsions into which Ags are added. Over the years, a variety of chemical modifications of saponins have been used as adjuvants and all appear to stimulate the release of various T helper 1 (Th1) and T helper 2 (Th2) cytokines in addition to their vehicle-like mode of action (Shi et al. 2005).

Finally, there are many examples of vaccine formulations in which individual adjuvants are used in combination with others as a way of optimizing immune efficiency (efficacy) and outcome. Despite the current emphasis placed on evaluating the wide variety of adjuvants in experimental use today and the myriad of possible combinations and formulations, no particular adjuvant or adjuvant system has emerged as an ideal product. Also, given the variation within a data set in which adjuvants are employed, it appears that the effectiveness of an adjuvant or adjuvant system is best evaluated on a case-by-case basis.

48.3   Adjuvant Development: General Concepts

As stated above, the principal objective for the use of an adjuvant in a vaccine is to potentiate immune response to an Ag of minimal immunogenicity. How this potentiation is achieved varies from adjuvant-to-adjuvant and in many cases, the precise mechanism of action is unknown. However, as a rule, immune potentiation is accomplished by the ability of the adjuvant to induce a variety of non-specific activities within the innate arm of the immune system. Once activated, the innate branch of immunity, particularly the complement system, orchestrates the various humoral and cell-mediated responses that operate within and between the innate and acquired arms. The result is a generalized activation and potentiation of the immune system in response to the adjuvant with the hope that this generalized immune priming will allow for a more effective processing and recognition of the Ag contained within the vaccine.

48.3.1   Pathogen-Derived Adjuvants

The manner by which an adjuvant induces this immune priming emanates from the exquisite sensitivity of the mammalian immune system to detect the presence of bacteria and bacterial components and respond accordingly in a rapid and vigorous manner. The initial response to these bacterial signals is largely a function of the innate branch of the immune system, which has evolved under continual selective pressure from pathogenic bacteria and other disease-causing microorganisms. It is not surprising, therefore, that the majority of adjuvants in use today are composed of various molecules, components, and structures derived from bacteria. The use of these adjuvants in vaccines, therefore, provides the bacterial signals to which the innate arm of the immune system vigorously reacts, which results in the priming/potentiation of the immune system.

However, this rapid and vigorous innate response to the bacteria-like signals induced by such adjuvants can result in an overly aggressive and misdirected immune response that is accompanied by undesirable side-effects such as anaphylaxis, fever, injection site granulomas or rashes, and local or systemic inflammation. Another drawback is that the adjuvant tends to induce a generalized immune response with little immune specificity directed to the Ag of interest. Also, immunity that might be directed to the Ag can be masked due to the magnitude of the innate response to the adjuvant. In an attempt to overcome these drawbacks, there is a vigorous and concerted research effort to develop modifications of bacterial-derived adjuvants such that they retain their ability to induce innate responses to bacterial signals, but minimize or eliminate the deleterious inflammatory side-effects that can accompany such signals. Notable examples of such efforts include the many chemical and structural modifications developed and tested on MPL (Ulrich and Myers 1995) the saponins (Shi et al. 2005), and cholera toxin (Lycke 2005).

48.3.2 Host-Derived Adjuvants

Against the backdrop of the various issues that accompany the use of adjuvants derived from bacterial components, a new area of adjuvant discovery focuses on the development of adjuvants derived from naturally-occurring components of host innate immunity. Such host-derived adjuvants would provide the required signaling necessary for engagement of the various cellular components of innate immunity, but with a degree of immunologic control, focus, and tolerance that may not be achieved with pathogen-derived components. In this regard, the use of cytokines as immuno-stimulatory adjuvants would qualify as host-derived adjuvants. However, the mode of action of cytokines encompasses a wide range of cellular effects within the innate and acquired arms of immunity and their use is effectively limited by issues of immunologic focus, control, and tolerance. Nonetheless, the idea of developing an adjuvant from a natural component involved in induction of host innate/acquired immunity is of keen interest. As mentioned above, the major issue will be in engineering an element of specificity such that the host-derived adjuvant induces/activates a single (or limited) pathway of immune activation and/or engages a single (or limited) population of immune cells.

48.3.3 Mucosal Adjuvants

Most of the currently approved vaccine adjuvants are intended for use in systemic immunizations. Mucosal sur-

faces, however, are the major portal for the entry of a variety of pathogens and mucosal immunization generates humoral and cellular immune responses at the mucosal surface, which restricts the entry of invading pathogens. Furthermore, generating immune response at one mucosal site is capable of generating similar immune responses at distinct mucosal sites. This phenomenon, known as the "common mucosal immune system", can be exploited to induce mucosal immune responses at inaccessible mucosal sites like the vagina and rectum, by immunizing through accessible mucosal routes such as the oral and intranasal cavity (Courtney 2010). Mucosal immunization is also the most feasible, non-invasive, and risk-free route for mass-immunization regimens.

In contrast to systemic immunization, which operates in a sterile milieu, mucosal immunization is confronted with the challenge of generating immune responses after being administered at a non-sterile site, which is constantly exposed to large quantities of harmless food and environmental antigens (Tsuji and Kosaka 2008). Mucosal immunizations also need to overcome the immune tolerance mechanisms at the mucosal site, which prevent unnecessary detrimental immune responses against non-pathogens and food antigens. While induction of immune tolerance is essential for preventing immune responses against food and environmental antigens, it necessitates the need for strong immunostimulatory components in the mucosal vaccines to generate immune responses.

Many adjuvant systems used for systemic vaccine applications such as oil-in-water emulsions and mineral salt adjuvants may not be suitable for mucosal applications. Adjuvants delivered at mucosal surfaces need to overcome the inherent tolerogenicity associated with mucosal tissues. The effective mucosal adjuvants currently being used in animal models include CpG, bacterial toxins such as cholera toxin (CT), heat-labile enterotoxins, cytokines/chemokines and VLPs (Courtney 2010; Huang et al. 2008; Lycke 2012; Vajdy and Lycke 1992; van Ginkel et al. 2005). Most of these compounds function as potent adjuvants through activation of antigen-presenting dendritic cells (DCs). Of the existing mucosal adjuvants, bacterial toxin adjuvants are most potent in generating humoral and cellular immune responses, but are very toxic and not fit for human use. CpG and other toll like receptor agonists have shown promise as mucosal adjuvants but are generally associated with inflammation. Whether pathogen-derived or host-derived, safe yet potent adjuvants that overcome mucosal tolerance and generate long-lived protective immune responses in mucosal and systemic immune responses are necessary for vaccination approaches that target pathogens, which infect through mucosal surfaces.

48.3.4 Molecular Adjuvants

The need for an adjuvant capable of inducing a robust *and* Ag-specific immune response accompanied with minimal inflammatory side-effects has given rise to a new class of adjuvants, which have come to be known as molecular adjuvants (Dempsey et al. 1996). The rationale for a molecular adjuvant is based on the notion that a single, well-defined and well-characterized molecular entity might be better in inducing an immune response more directed to the Ag with fewer non-specific side effects, particularly when the Ag is attached directly to the molecular adjuvant. Thus, a molecular adjuvant can be defined as a single molecular entity that targets an Ag to the cells of the immune system responsible for Ag processing and presentation (APCs) and/or activates specific pathways of Ag processing and presentation within these cells.

48.4 Development a Host-Derived, Molecular, and Humoral Adjuvant

As described above, "traditional" adjuvants typically are derived from bacterial components in order to provide the bacteria signals necessary for the activation and potentiation of the innate arm of the immune system. One of the first biological systems of that which becomes activated in response to these bacterial signals is the complement system. Consequently, complement and the various components that comprise the complement system provide a rich source from which a variety of host-derived adjuvants could be developed. This section describes the development of a host-derived adjuvant that was developed from a natural component of human complement known as C5a. This host-derived adjuvant embodies the attributes of a molecular adjuvant as well as a humoral adjuvant, examples of which are discussed below.

48.4.1 Complement

Complement is a plasma system comprised of interrelated proteases arranged in a cascade fashion that becomes activated in response to bacterial signals and Ags associated with bacteria. The role of complement is twofold: (1) to serve as an initial, first line of defense to invading microorganisms and (2) to enhance systemic host defense by activating and orchestrating the various humoral and cell-mediated responses within the acquired and innate arms of the immune system necessary for the effective elimination of the microorganism.

The "first line of defense" is accomplished through the generation of the membrane attack complex (MAC), which is assembled at the end of the complement cascade from the components generated by the proteolytic steps along the cascade. The MAC is directly involved in lysing the membranes of foreign microorganisms.

Secondly, the various humoral and cell-mediated responses operating at the interface of the innate and acquired arms of the immune system are activated and coordinated by pharmacologically active components that are released at certain steps along the complement cascade. These proteolytic byproducts induce the various humoral and cell-mediated aspects of the innate and acquired arms of immunity, all of which are necessary for the concerted elimination of the microorganism.

Principally because of this latter reason, these pharmacologically active components of complement are attractive as molecular adjuvants. This is because their use has the potential of enhancing specific pathways of the innate and acquired immune processing and subsequent recognition of an Ag as opposed to the broader and less Ag-focused activation of the innate system in response to bacterial signals that come from of adjuvants derived from bacterial components.

48.4.2 Adjuvants Derived from Complement Components: Host-Derived Adjuvants

Components of complement have been used as molecular adjuvants to enhance Ag-specific immune responses to model Ags, specifically C3b, C4b (Arvieux et al. 1988; Jacquier-Sarlin et al. 1995), and C3d (Dempsey et al. 1996). These components were chosen primarily for their opsonic properties; i.e., their ability to bind to and coat the surface of a microorganism rendering it more susceptible to immune cell uptake via phagocytosis. The rationale, therefore, was that an Ag covalently attached to these complement fragments similarly would be rendered more susceptible for uptake by APCs and, consequently, would be more effectively processed and presented by the APC. In all cases, the immunogenicity of the model Ag in the Ag-complement vaccine complex was markedly enhanced relative to the Ag alone, suggesting that the complement fragment acted as a molecular adjuvant by enhancing Ag uptake by APCs and, in turn, the ability of the APC to process and present the Ag. These studies clearly demonstrate the immunologic potential of using complement components as molecular adjuvants in the design of vaccines.

48.4.3 The Anaphylatoxins

An important group of pharmacologically active byproducts of complement activation are the anaphylatoxins C3a, C4a, and C5a, which are small (74–76 residue) fragments cleaved from the larger, parent complement components C3, C4, and C5, respectively. The principal roles of the anaphylatoxins are to recruit inflammatory cells and lymphocytes to sites of tissue injury and infection and to then activate these cells' various effector responses once recruited (Hugli 1981). However, the anaphylatoxins play important roles in the activation and regulation of humoral and cell-mediated responses to Ags due to their ability to modulate various humoral and cell-mediated activities between the innate and acquired arms of the immune system (Dempsey et al. 1996; Mastellos et al. 2005; Morgan 1986). Consequently, the anaphylatoxins are attractive as molecular adjuvants, which may be capable of invoking Ag-specific humoral and/or cell-mediated immune responses.

Unfortunately, the anaphylatoxins are also potent inflammatory mediators and their use as molecular adjuvants would surely be accompanied by local and/or systemic inflammatory side effects. Also, under certain conditions, the anaphylatoxins appear to downregulate immune function (Kawamoto et al. 2004; Morgan 1986). Thus, while the anaphylatoxins have desirable immune stimulatory activities that make them attractive for use as molecular adjuvants, they carry with them the potential for adverse inflammatory side effects and immune downregulation. Therefore, immune stimulatory activities must be enhanced at the expense of its inflammatory activities and any tendency to downregulate immune response.

48.4.4 Immunostimulatory and Inflammatory Properties of C5a

C5a is a 74-residue polypeptide with pleiotropic biological functions (Fig. 48.1) including smooth muscle contraction, vascular permeability, mast cell degranulation, and chemotaxis. C5a potentiates antibody and Ag-induced T cell proliferative responses in vitro possibly through activation of T helper cells (Morgan et al. 1983). Direct stimulation of mouse and human dendritic cells with C5a enhances the expression of MHC class II, co-stimulatory molecules CD80, CD86, CD40 and CD54, and induces secretion of cytokines of Th1 phenotype (Zaal et al. 2013; Rudilla et al. 2012; Li et al. 2012). Also, C5a induces the release of a variety of immunoregulatory cytokines from APCs including IL-1, IL-6, IL-8, IL-12, TNFα, and IFNγ (Buchner et al. 1995;
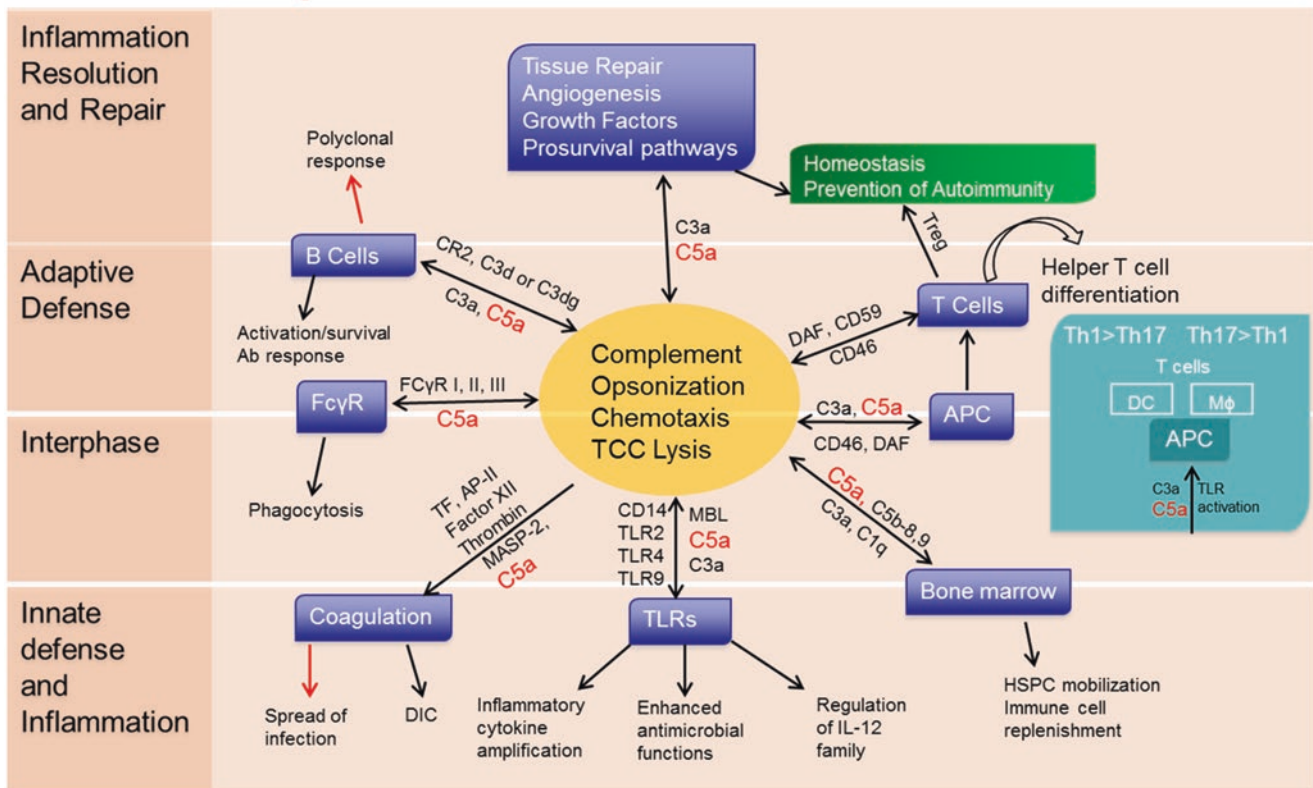
Fig. 48.1 Role of complement C5a in host defense and homeostasis. Adapted from Ricklin et al. (2010)

Goodman et al. 1982; Okusawa et al. 1987; Wetsel 1995b) (Gasque et al. 1995; Scholz et al. 1990); (Ember et al. 1994); (Floreani et al. 2007). C5a-mediated stimulation of DCs also decreased intracellular production of cAMP, which is a negative regulator of DC activation and function (Li et al. 2012). Furthermore, C5a interaction with C5aR activates PI3K, ERK1/2, NF-kB signaling pathways, which positively regulates Ag uptake, presentation, and secretion of proinflammatory cytokines by DCs (Li et al. 2012; Zaal et al. 2013). C5a was also shown to exhibit both synergistic as well as antagonistic crosstalk with toll like receptors (TLRs) in functional modulation of DCs (Li et al. 2012; Zaal et al. 2013). C5a decreased TLR4-mediated IL-12 secretion by DCs, but significantly increased secretion of other Th1 cytokines and CXC16 involved in NK cell activation. C5aR ligation with C5a was shown to be essential for generation of protective anti-viral CD8+ T cells (Kim et al. 2004). In contrast, genetic ablation or pharmacologic blocking of C5aR signaling predominantly generated Th2 and regulatory T cell responses (Kim et al. 2004). Furthermore, previous studies found that blockade of the C5aR impaired the ability of immune system to generate memory CD4+ T cells (Moulton et al. 2007). Also, DCs stimulated with C5a showed increased expression of multiple co-stimulatory molecules, migration to the drain-

ing lymph nodes, and strong interaction with antigen-specific naïve T cells (Li et al. 2012).

Besides immunostimulatory properties that promote Ag presentation and functional immune responses, C5a also possesses highly proinflammatory properties. These include its potent chemotactic activities for the recruitment of inflammatory cells to sites of tissue injury and infection, its ability to induce smooth muscle contraction (Hugli et al. 1987; Shin et al. 1968), increase vascular permeability (Hugli 1990; Hugli et al. 1981; Hugli and Muller-Eberhard 1978), and induce the release of a variety of secondary inflammatory mediators such as histamine, lysosomal enzymes, and vasoactive eicosanoids from responsive cells such as mast cells, neutrophils, eosinophils, and macrophages (Drapeau et al. 1993; Goldstein et al. 1974; Johnson et al. 1975; Lundberg et al. 1987; Schorlemmer et al. 1976).

These biologic responses (inflammation and immunomodulation) are induced by the ligation of C5a to its specific, high affinity receptor (C5aR/CD88) that is expressed on the surface of the C5a-responsive cell(s). Traditionally, C5aR expression has been viewed as being limited to cells of myeloid origin such as neutrophils, monocytes, mast cells, and eosinophils (Wetsel 1995a). However, it is now known that a variety of cells of non-

myeloid origin express C5aRs. These include hepatocytes (Buchner et al. 1995), astrocytes (Gasque et al. 1995), bronchial epithelial cells (Floreani et al. 1998), epithelial cells of the gut and kidney (Wetsel 1995a), and osteoblasts (Pobanz et al. 2000).

As the inflammatory properties of C5a have been the motivation behind the development of C5a antagonists, so the immunostimulatory properties of C5a been the motivating factor for the development of agonists of C5a that can be used as surrogates of the natural factor for enhancing humoral and cell-mediated immune responses; i.e., as a molecular adjuvant. Using such agonists of C5a as a molecular adjuvant, however, requires developing response-selective agonists that are capable of invoking C5a-like immunostimulatory properties at the expense of C5a-like inflammatory properties.

48.4.5 C5a: Structure-Function Considerations

Human C5a is a 74-residue glycopolypeptide that is comprised of two important structural and functional domains. The first is the well-ordered N-terminal core domain comprised of residues 1–63, which is primarily involved in the recognition and binding of C5aRs (Mollison et al. 1989; Zuiderweg et al. 1989). The second is the C-terminal domain, residues 64–74, which extends from the N-terminal core as a finger-like projection. $C5a_{65-74}$ (ISHKDMQLGR) is a region of considerable backbone flexibility and poorly-defined structure, yet the inflammatory *and* immunostimulatory activities characteristic of natural C5a reside in this small C-terminal stretch (Ember et al. 1992, 1994; Morgan et al. 1992).

Flexibility in the C-terminal region of C5a ($C5a_{65-74}$ or ISHKDMQLGR) is a dominant feature of natural C5a. It may be that this flexibility allows the C5aR the ability to induce a unique conformation in this region of the C5a ligand that is conducive to the expression of biologic activity characteristic of that particular C5aR-bearing cell. It may be, in fact, that a biologically active conformation in this effector region of C5a responsible for activity in one type of C5aR-bearing cell may not be the ideal conformation for the expression of activity in another. This suggests that by employing a synthetic strategy in which the flexibility in this effector region of C5a is restricted, one might bias certain conformational features that are important for the expression of certain types of C5aR-mediated activities; i.e., a response-selective agonist. Such a specific and stabilized conformation, when presented to the C5aR, would be more likely to interact with those C5aRs that are capable of accommodating this particular conformation.

48.4.6 Conformationally Restricted Analogues of $C5a_{65-74}$

Over the years, our laboratory has generated a library of analogues of $C5a_{65-74}$ in which backbone flexibility was restricted by specific amino acid substitutions. This was done with the goal of biasing certain conformational features that might be helpful in the search for biologically relevant conformations responsible for the induction of C5a-like immunostimulatory activities versus those responsible for C5a-like inflammation.

Backbone flexibility was restricted by introducing three principal types of residue substitutions within $C5a_{65-74}$: (1) Pro substitutions to restrict ϕ angle flexibility and to narrow the range of sterically allowed backbone conformations in the pre-proline residue (Misicka et al. 1991), (2) Ala substitutions to evaluate the biological importance of the side-chains in the peptide, and (3) D-residue substitutions to assess the contribution of stereoisomeric arrangements. The use of such peptide modifications has an additional advantage in that Pro, Ala, and D-residues occupy well-defined regions of sterically-allowed Ramachandran space (Hruby et al. 1991). Thus, an evaluation of the changes made in the biological activity of these conformationally restricted analogues of $C5a_{65-74}$ can provide information about the specific types of backbone conformation features that are important to specific types of biological activity.

Using this approach, we showed that one peptide from this library, $C5a_{65-74}$ Y65,F67,P69,P71,D-Ala73 or YSFKPMPLaR (EP54) exhibited about 10 % of the potency of natural C5a for its ability to induce the release of spasmogenic eicosanoids from human macrophages, but only about 0.1 % of C5a activity in its ability to induce the release of β-glucuronidase from human neutrophils (Finch et al. 1997) and was reflected by a corresponding difference in binding affinity to the C5aRs expressed on these cells (Finch et al. 1997; Vogen et al. 1999).

Structural analysis of YSFKPMPLaR indicated the presence of unique conformational features that appear to be responsible for the selective accommodation by C5aRs expressed on antigen presenting cells (APC) relative to C5aRs expressed on inflammatory neutrophils (Vogen et al. 1999). These backbone conformational features identified in EP54 were then used to guide the generation of other analogues designed to enhance their biologic importance. Prominent among these new analogues is EP67 (YSFKDMP(MeL)aR), which contains an N-methylated Leu residue to enhance backbone elongation in a crucial region of the peptide, which was deemed biologically important from the NMR structure of EP54. Indeed, EP67 was shown to exhibit more potency and macrophage/dendritic cell selectivity than EP54 (Vogen et al. 2001; Taylor et al. 2001).

48.4.7 Use of EP54 and EP67 as Host-Derived, Molecular and Humoral Adjuvants

48.4.7.1 Generation of Ag-Specific Humoral Immune Responses

In one study (Tempero et al. 1997), EP54 was used to induce Ag-specific antibody (Ab) responses to a B cell epitope derived from the human mucin type 1 glycoprotein (MUC1). A vaccine was generated by covalently attaching the MUC1 epitope (*YKQGGFLGL*) to the N-terminus of EP54 (*YKQGGFLGL*YSFKPMPLaR) and mice immunized with this vaccine generated high Ab titers specific for the MUC1 epitope. These anti-epitope Abs cross-reacted with MUC1 protein expressed on the surface of a transfected pancreatic cell line, indicating that the anti-*YKQGGFLGL* Abs recognized the epitope within intact whole MUC1 protein. Also, Ab isotypes generated by this EP54-containing vaccine were IgM, IgG2a, and IgG2b, contrasted with those generated by an analogous KLH construct, which were IgM and IgG1. This suggests that the EP54-containing vaccine induced an Ab class switch characteristic of a Th1-like response and, consequently, generated Ab with isotypes distinct from the traditional KLH construct. Over the years, we have used epitope-EP54 vaccine constructs to immunize rats, hamsters, rabbits, and cattle to a wide variety of epitopes of various lengths (8–35 residues) and small molecules such as nicotine (Sanderson et al. 2003) and methamphetamine (Duryee et al. 2009).

In similar fashion, EP67-containing vaccines have been used to generate Ab responses to peptide epitopes and proteins in mouse models. Mice immunized with these EP67-containing vaccines generated significant levels of Ag-specific IgM, IgG2a, and IgG2b responses (Morgan et al. 2009). Also, mice immunized with intact fungal spores from an attenuated strain of *Coccidioides posadasii* conjugated to EP67 increased the protective efficacy by a corresponding increase in Ag-specific IgG1 and IgG2a Ab responses (Hung et al. 2012).

48.4.7.2 Generation of Ag-Specific Cell-Mediated Immune Responses

Cytotoxic T cell responses (CTL) (CD4+ and CD8+) in murine models have been generated with EP54- and EP67-containing vaccines designed by their covalent attachment to peptide epitopes from the hepatitis B surface antigen (Ulrich et al. 2000), a tandem repeat region of MUC1 expressed on pancreatic cancer cells (Pisarev et al. 2005), and the gp70 glycoprotein from RAW 117-H10 lymphoma cells (Kollessery et al. 2011). Also, EP67-containing vaccines have been generated in which the target Ag is an entire protein using straightforward conjugation methods (Phillips et al. 2009). Using this approach, we have shown protective Th1 and Th17 CTL responses to *Coccidiodies posadasii* in mice immunized with vaccines in which EP67 was conjugated to intact fungal spores from an attenuated strain of *C. posadasii* (Hung et al. 2012).

48.4.7.3 Generation of Ag-Specific Mucosal Immune Responses

Simple vaccines were made by the covalent conjugation of CD8+ T cell epitopes from proteins expressed on murine cytomegalovirus (MCMV) to EP67. Intranasal immunization generated functional mucosal and systemic epitope-specific CD8+ T cells that increased protection against primary mucosal infection with MCMV. Moreover, a large proportion of these CD8+ T cells formed a pool of long-lived memory CD8+ T cells that respond strongly upon reinfection even in the absence of CD4+ Th1 help. This is supported by findings that EP67-based CTL peptides generated a higher proportion of epitope-specific mucosal (lungs) and systemic (spleen) CD8a+/CD44+ cells with cell surface phenotype (CD127+/KLRG1−) associated long-lived memory (Karuturi 2014). In addition to CD127 and KLRG1, we found that EP67-based CTL peptides generated epitope-specific CD8+ T cells with an increased expression of CD27, which is a strong predictor of proliferative recall responses upon Ag encounter (Ochsenbein et al. 2004; Xiao et al. 2008).

The immunization results in mice demonstrate the unique ability of complement C5a-derived/host-derived adjuvants for use in systemic as well as mucosal vaccine applications. In particular, EP67 because of its significantly reduced inflammatory properties may be acceptable as a universal adjuvant for various vaccine applications. More recent studies have shown the ability of EP67 in protecting against influenza and MRSA infections via induction of innate immunity from its selective engagement of C5aR-bearing APCs (Sanderson et al. 2012; Hanke et al. 2013). Taken together, these studies indicate that EP67 has significant immunostimulatory properties that are conducive to desirable innate and acquired immune outcomes (Table 48.1).

48.5 Mechanism of Action

The immune outcomes obtained from EP54- and EP67-induced humoral and cell-mediated responses suggest that these adjuvants deliver both Ag *and* stimulatory signals to C5aR-bearing APCs—events that are consistent with the mechanism shown in Fig. 48.2.

This proposed mechanism is supported by confocal microscopy where fluorescent-labeled EP54 and fluorescent-labeled B and T cell epitopes attached to EP54 were rapidly internalized by human DCs (Hegde et al. 2008). Twenty-four hours later, the internalized epitopes were presented in the context of HLA-I and HLA-II determinants on the DC surface. Thus, EP54 (and EP67) both targets and activates APCs suggesting properties of both a vehicle and an immunomodulatory adjuvant.

Table 48.1 Vaccine adjuvant properties of EP54 and EP67

| Vaccine construct	Species	Route	Immune response	Ref
Peptide epitope from MUC1 glycoprotein conjugated to EP54	C57BL6 and BALB/c mice	i.p.	Higher titers of IgG2b, IgG2c and IgM Abs in sera reactive against recombinant MUC1 and MUC1 expressing cell line	Tempero et al. (1997)
CTL peptide epitope derived from Hepatitis B surface Antigen (HBsAg) conjugated to EP54	BALB/c mice	s.c	Ag-specific CD8+ CTL responses against murine P815S target cells expressing an H-2L^d restricted CTL epitope of the HBsAg	Ulrich et al. (2000)
Nicotine hapten conjugated to EP54	Sprague–Dawley rats	i.p.	Nicotine-specific Abs capable of attenuating nicotine-induced behavioral effects	Sanderson et al. (2003)
Methamphetamine (meth) hapten conjugated to EP54	Rats	s.c/i.p.	Meth-specific Abs in sera capable of altering meth self-administration	Duryee et al. (2009)
Ovalbumin (OVA) conjugated to EP67	C57BL6 BALB/c mice	i.p.	OVA-specific Th1-like Ab class switch and OVA-specific proliferative responses in splenocytes	Morgan et al. (2009)
OVA conjugated to EP67	Young/aged mice	i.p.	Higher Ag-specific humoral responses compared to alum adjuvanted OVA	Morgan et al. (2010)
rPrp1,a protein from cell wall of *coccidioides* conjugated to EP67	Young/aged mice	i.p.	Higher Ag-specific humoral responses compared alum and CpG adjuvanted rPrp1	Morgan et al. (2010)
Peptide epitope derived from gp70 glycoprotein conjugated to EP54 and EP67	BALB/c mice	s.c	Ag-specific CTL responses and protection against metastatic RAW117-H10 lymphoma lethal challenge	Kollessery et al. (2011)
Live spores of attenuated vaccine strain of *Coccidioides posadasii* conjugated to EP67	BALB/c mice	s.c	Increased protective efficacy of live vaccine by increasing Ag-IgG1, and IgG2a, and Th1, and Th17 immune responses	Hung et al. (2012)

48.6   Review Questions

1. Describe the principal means by which a molecular adjuvant such as EP67 induces innate and/or acquired immune responses.
2. As we age, our immune response profile gradually shifts toward T helper type II (Th2) dominated responses. With this in mind, describe how a molecular adjuvant like EP67 could be advantageous in the generation of vaccines for the growing elderly population.
3. Describe how a molecular adjuvant like EP67 could accommodate the need for the rapid generation of vaccines to counter new and emerging diseases and bioterrorism attacks.

48.7   Answers

1. By the selective engagement and activation of C5aR-bearing antigen presenting cells.
2. This is because EP67, unlike other conventional adjuvants, drives a T helper type I (Th1) dominated immune response, which could restore an immunologically advantageous Th1/Th2 balance that is lost during the aging process.
3. EP67 can be rapidly generated in large quantities by standard peptide synthesis. Vaccines can be rapidly made in large quantities by the simple covalent conjugation of the target antigen to the N-terminus of EP67. Manufacturing of such vaccines is rapid and inexpensive, requires no
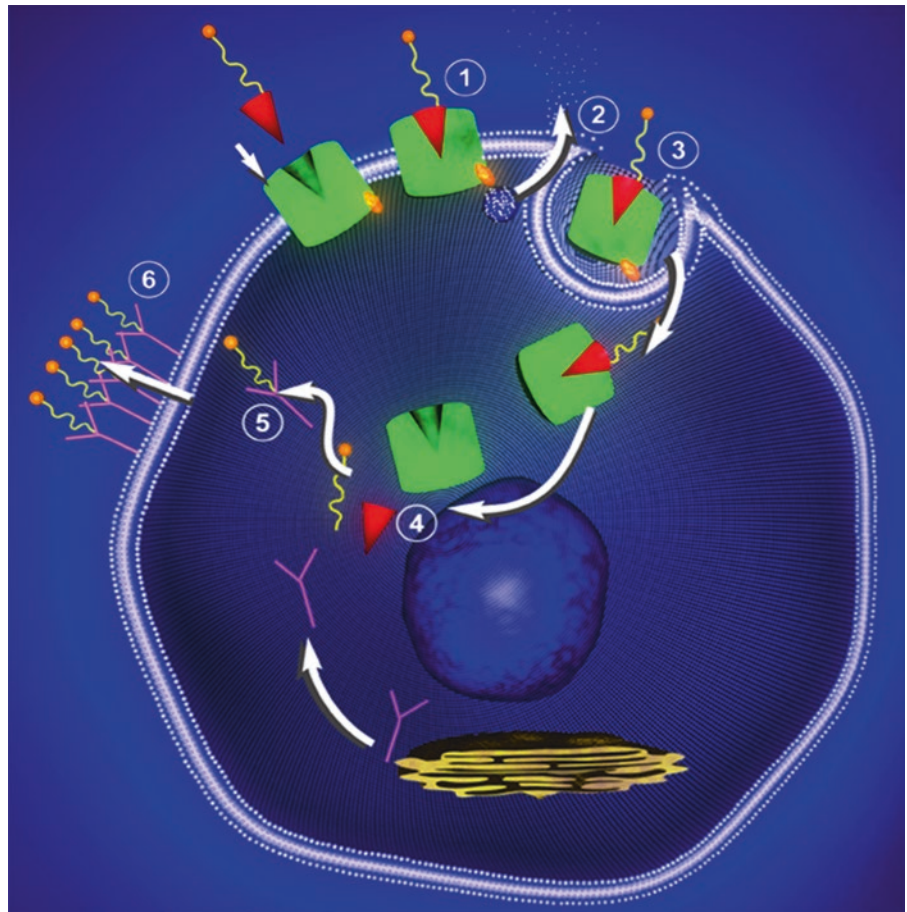
additives or preservatives, and vaccination can be accomplished on a large scale by the administration of the EP67-based vaccine dissolved merely in water.

References

Afonso LC, Scharton TM, Vieira LQ, Wysocka M, Trinchieri G, Scott P (1994) The adjuvant effect of interleukin-12 in a vaccine against Leishmania major. Science 263(5144):235–237

Arvieux J, Yssel H, Colomb MG (1988) Antigen-bound C3b and C4b enhance antigen-presenting cell function in activation of human T-cell clones. Immunology 65(2):229–235

Biragyn A, Tani K, Grimm MC, Weeks S, Kwak LW (1999) Genetic fusion of chemokines to a self tumor antigen induces protective, T-cell dependent antitumor immunity. Nat Biotechnol 17(3):253–258. doi:10.1038/6995

Buchner RR, Hugli TE, Ember JA, Morgan EL (1995) Expression of functional receptors for human C5a anaphylatoxin (CD88) on the human hepatocellular carcinoma cell line HepG2. Stimulation of acute-phase protein-specific mRNA and protein synthesis by human C5a anaphylatoxin. J Immunol 155(1):308–315

Courtney AN (2010) Characterization of alpha-galactosylceramide as a mucosal adjuvant. Dissertation Thesis

Crouch CF, Daly J, Henley W, Hannant D, Wilkins J, Francis MJ (2005) The use of a systemic prime/mucosal boost strategy with an equine

influenza ISCOM vaccine to induce protective immunity in horses. Vet Immunol Immunopathol 108(3–4):345–355. doi:10.1016/j.vetimm.2005.06.009

Defoort JP, Nardelli B, Huang W, Tam JP (1992) A rational design of synthetic peptide vaccine with a built-in adjuvant. A modular approach for unambiguity. Int J Pept Protein Res 40(3–4):214–221

Dempsey PW, Allison ME, Akkaraju S, Goodnow CC, Fearon DT (1996) C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. Science 271(5247):348–350

Diwan M, Elamanchili P, Cao M, Samuel J (2004) Dose sparing of CpG oligodeoxynucleotide vaccine adjuvants by nanoparticle delivery. Curr Drug Deliv 1(4):405–412

Drapeau G, Brochu S, Godin D, Levesque L, Rioux F, Marceau F (1993) Synthetic C5a receptor agonists. Pharmacology, metabolism and in vivo cardiovascular and hematologic effects. Biochem Pharmacol 45(6):1289–1299

Duryee MJ, Bevins RA, Reichel CM, Murray JE, Dong Y, Thiele GM, Sanderson SD (2009) Immune responses to methamphetamine by active immunization with peptide-based, molecular adjuvant-containing vaccines. Vaccine 27(22):2981–2988. doi:10.1016/j.vaccine.2009.02.105

Ember JA, Sanderson SD, Hugli TE, Morgan EL (1994) Induction of interleukin-8 synthesis from monocytes by human C5a anaphylatoxin. Am J Pathol 144(2):393–403

Ember JA, Sanderson SD, Taylor SM, Kawahara M, Hugli TE (1992) Biologic activity of synthetic analogues of C5a anaphylatoxin. J Immunol 148(10):3165–3173

Finch AM, Vogen SM, Sherman SA, Kirnarsky L, Taylor SM, Sanderson SD (1997) Biologically active conformer of the effector

region of human C5a and modulatory effects of N-terminal receptor binding determinants on activity. J Med Chem 40(6):877–884. doi:10.1021/jm960727r

Floreani AA, Gunselman SJ, Heires AJ, Hauke RJ, Tarantolo S, Jackson JD (2007) Novel C5a agonist-based dendritic cell vaccine in a murine model of melanoma. Cell Cycle 6(22):2835–2839

Floreani AA, Heires AJ, Welniak LA, Miller-Lindholm A, Clark-Pierce L, Rennard SI, Morgan EL, Sanderson SD (1998) Expression of receptors for C5a anaphylatoxin (CD88) on human bronchial epithelial cells: enhancement of C5a-mediated release of IL-8 upon exposure to cigarette smoke. J Immunol 160(10):5073–5081

Gasque P, Chan P, Fontaine M, Ischenko A, Lamacz M, Gotze O, Morgan BP (1995) Identification and characterization of the complement C5a anaphylatoxin receptor on human astrocytes. J Immunol 155(10):4882–4889

Goldstein BD, Lai LY, Cuzzi-Spada R (1974) Potentiation of complement-dependent membrane damage by ozone. Arch Environ Health 28(1):40–42

Goodman MG, Chenoweth DE, Weigle WO (1982) Induction of interleukin 1 secretion and enhancement of humoral immunity by binding of human C5a to macrophage surface C5a receptors. J Exp Med 156(3):912–917

Hanke ML, Heim CE, Angle A, Sanderson SD, Kielian T (2013) Targeting macrophage activation for the prevention and treatment of Staphylococcus aureus biofilm infections. J Immunol 190(5):2159–2168. doi:10.4049/jimmunol.1202348

Hegde GV, Meyers-Clark E, Joshi SS, Sanderson SD (2008) A conformationally-biased, response-selective agonist of C5a acts as a molecular adjuvant by modulating antigen processing and presentation activities of human dendritic cells. Int Immunopharmacol 8(6):819–827. doi:10.1016/j.intimp.2008.01.031

Hruby VJ, Prakash O, Kazmierski W, Gehrig C, Matsunaga TO (1991) Conformational analysis of opioid receptor-selective peptides using nuclear magnetic resonance and theoretical calculations. NIDA Res Monogr 112:198–217

Huang CF, Wu TC, Chu YH, Hwang KS, Wang CC, Peng HJ (2008) Effect of neonatal sublingual vaccination with native or denatured ovalbumin and adjuvant CpG or cholera toxin on systemic and mucosal immunity in mice. Scand J Immunol 68(5):502–510. doi:10.1111/j.1365-3083.2008.02172.x

Hugli TE (1981) The structural basis for anaphylatoxin and chemotactic functions of C3a, C4a, and C5a. Crit Rev Immunol 1(4):321–366

Hugli TE (1990) Structure and function of C3a anaphylatoxin. Curr Top Microbiol Immunol 153:181–208

Hugli TE, Gerard C, Kawahara M, Scheetz ME 2nd, Barton R, Briggs S, Koppel G, Russell S (1981) Isolation of three separate anaphylatoxins from complement-activated human serum. Mol Cell Biochem 41:59–66

Hugli TE, Marceau F, Lundberg C (1987) Effects of complement fragments on pulmonary and vascular smooth muscle. Am Rev Respir Dis 135(6 Pt 2):S9–S13

Hugli TE, Muller-Eberhard HJ (1978) Anaphylatoxins: C3a and C5a. Adv Immunol 26:1–53

Hung CY, Hurtgen BJ, Bellecourt M, Sanderson SD, Morgan EL, Cole GT (2012) An agonist of human complement fragment C5a enhances vaccine immunity against Coccidioides infection. Vaccine 30(31):4681–4690. doi:10.1016/j.vaccine.2012.04.084

Jacquier-Sarlin MR, Gabert FM, Villiers MB, Colomb MG (1995) Modulation of antigen processing and presentation by covalently linked complement C3b fragment. Immunology 84(1):164–170

Johnson AG, Gaines S, Landy M (1956) Studies on the O antigen of Salmonella typhosa. V. Enhancement of antibody response to protein antigens by the purified lipopolysaccharide. J Exp Med 103(2):225–246

Johnson AR, Hugli TE, Muller-Eberhard HJ (1975) Release of histamine from rat mast cells by the complement peptides C3a and C5a. Immunology 28(6):1067–1080

Karuturi BVK (2014) Development of EP67-based mucosal vaccines against cytomegalovirus infection. Ph.D. Dissertation, Department of Pharmaceutical Sciences, College of Pharmacy, University of Nebraska Medical Center

Kawamoto S, Yalcindag A, Laouini D, Brodeur S, Bryce P, Lu B, Humbles AA, Oettgen H, Gerard C, Geha RS (2004) The anaphylatoxin C3a downregulates the Th2 response to epicutaneously introduced antigen. J Clin Invest 114(3):399–407. doi:10.1172/JCI19082

Kim AH, Dimitriou ID, Holland MC, Mastellos D, Mueller YM, Altman JD, Lambris JD, Katsikis PD (2004) Complement C5a receptor is essential for the optimal generation of antiviral CD8+ T cell responses. J Immunol 173(4):2524–2529

Kollessery G, Nordgren TM, Mittal AK, Joshi SS, Sanderson SD (2011) Tumor-specific peptide-based vaccines containing the conformationally biased, response-selective C5a agonists EP54 and EP67 protect against aggressive large B cell lymphoma in a syngeneic murine model. Vaccine 29(35):5904–5910. doi:10.1016/j.vaccine.2011.06.070

Kurzawa H, Wysocka M, Aruga E, Chang AE, Trinchieri G, Lee WM (1998) Recombinant interleukin 12 enhances cellular immune responses to vaccination only after a period of suppression. Cancer Res 58(3):491–499

Li K, Fazekasova H, Wang N, Peng Q, Sacks SH, Lombardi G, Zhou W (2012) Functional modulation of human monocytes derived DCs by anaphylatoxins C3a and C5a. Immunobiology 217(1):65–73. doi:10.1016/j.imbio.2011.07.033

Lundberg C, Gardinali M, Hugli TE (1987) Complement activation and membrane lipids in lung vascular injury. Am Rev Respir Dis 136(2):459–462. doi:10.1164/ajrccm/136.2.459

Lycke N (2005) Targeted vaccine adjuvants based on modified cholera toxin. Curr Mol Med 5(6):591–597

Lycke N (2012) Recent progress in mucosal vaccine development: potential and limitations. Nat Rev Immunol 12(8):592–605. doi:10.1038/nri3251

Martinon F, Gras-Masse H, Boutillon C, Chirat F, Deprez B, Guillet JG, Gomard E, Tartar A, Levy JP (1992) Immunization of mice with lipopeptides bypasses the prerequisite for adjuvant. Immune response of BALB/c mice to human immunodeficiency virus envelope glycoprotein. J Immunol 149(10):3416–3422

Mastellos D, Germenis AE, Lambris JD (2005) Complement: an inflammatory pathway fulfilling multiple roles at the interface of innate immunity and development. Curr Drug Targets Inflamm Allergy 4(1):125–127

Metzger J, Wiesmuller KH, Schaude R, Bessler WG, Jung G (1991) Synthesis of novel immunologically active tripalmitoyl-S-glycerylcysteinyl lipopeptides as useful intermediates for immunogen preparations. Int J Pept Protein Res 37(1):46–57

Misicka A, Lipkowski AW, Fang L, Knapp RJ, Davis P, Kramer T, Burks TF, Yamamura HI, Carr DB, Hruby VJ (1991) Topographical requirements for delta opioid ligands: presence of a carboxyl group in position 4 is not critical for deltorphin high delta receptor affinity and analgesic activity. Biochem Biophys Res Commun 180(3):1290–1297

Mollison KW, Mandecki W, Zuiderweg ER, Fayer L, Fey TA, Krause RA, Conway RG, Miller L, Edalji RP, Shallcross MA et al (1989) Identification of receptor-binding residues in the inflammatory complement protein C5a by site-directed mutagenesis. Proc Natl Acad Sci U S A 86(1):292–296

Morgan EL (1986) Modulation of the immune response by anaphylatoxins. Complement 3(3):128–136

Morgan EL, Morgan BN, Stein EA, Vitrs EL, Thoman ML, Sanderson SD, Phillips JA (2009) Enhancement of in vivo and in vitro immune functions by a conformationally biased, response-selective agonist of human C5a: implications for a novel adjuvant in vaccine design. Vaccine 28(2):463–469. doi:10.1016/j.vaccine.2009.10.029

Morgan EL, Sanderson S, Scholz W, Noonan DJ, Weigle WO, Hugli TE (1992) Identification and characterization of the effector region

within human C5a responsible for stimulation of IL-6 synthesis. J Immunol 148(12):3937–3942

Morgan EL, Thoman ML, Sanderson SD, Phillips JA (2010) A novel adjuvant for vaccine development in the aged. Vaccine 28(52):8275–8279. doi:10.1016/j.vaccine.2010.10.008

Morgan EL, Thoman ML, Weigle WO, Hugli TE (1983) Anaphylatoxin-mediated regulation of the immune response. II. C5a-mediated enhancement of human humoral and T cell-mediated immune responses. J Immunol 130(3):1257–1261

Moulton RA, Mashruwala MA, Smith AK, Lindsey DR, Wetsel RA, Haviland DL, Hunter RL, Jagannath C (2007) Complement C5a anaphylatoxin is an innate determinant of dendritic cell-induced Th1 immunity to Mycobacterium bovis BCG infection in mice. J Leukoc Biol 82(4):956–967. doi:10.1189/jlb.0206119

Muhlradt PF, Kiess M, Meyer H, Sussmuth R, Jung G (1998) Structure and specific activity of macrophage-stimulating lipopeptides from Mycoplasma hyorhinis. Infect Immun 66(10):4804–4810

Ochsenbein AF, Riddell SR, Brown M, Corey L, Baerlocher GM, Lansdorp PM, Greenberg PD (2004) CD27 expression promotes long-term survival of functional effector-memory CD8+ cytotoxic T lymphocytes in HIV-infected patients. J Exp Med 200(11):1407–1417. doi:10.1084/jem.20040717

Okusawa S, Dinarello CA, Yancey KB, Endres S, Lawley TJ, Frank MM, Burke JF, Gelfand JA (1987) C5a induction of human interleukin 1. Synergistic effect with endotoxin or interferon-gamma. J Immunol 139(8):2635–2640

Olive C, Toth I, Jackson D (2001) Technological advances in antigen delivery and synthetic peptide vaccine developmental strategies. Mini Rev Med Chem 1(4):429–438

Pisarev VM, Kirnarsky L, Caffrey T, Hanisch F-G, Sanderson SD, Hollingsworth MA, Sherman S (2005) T cells recognize PD(N/T)R motif common in a variable number of tandem repeat and degenerate repeat sequences of MUC1. Int Immunopharmacol 5:315–330

Phillips JA, Morgan EL, Dong Y, Cole GT, McMahan C, Hung CY, Sanderson SD (2009) Single-step conjugation of bioactive peptides to proteins via a self-containing succinimidyl bis-arylhydrazone. Bioconjug Chem 20:1950–1957

Pobanz JM, Reinhardt RA, Koka S, Sanderson SD (2000) C5a modulation of interleukin-1 beta-induced interleukin-6 production by human osteoblast-like cells. J Periodontal Res 35(3):137–145

Ricklin D, Hajishengallis G, Yang K, Lambris JD (2010) Complement: a key system for immune surveillance and homeostasis. Nat Immunol 11(9):785–797. doi:10.1038/ni.1923

Rudilla F, Fayolle C, Casares N, Durantez M, Arribillaga L, Lozano T, Villanueva L, Pio R, Sarobe P, Leclerc C, Prieto J, Lasarte JJ (2012) Combination of a TLR4 ligand and anaphylatoxin C5a for the induction of antigen-specific cytotoxic T cell responses. Vaccine 30(18):2848–2858. doi:10.1016/j.vaccine.2012.02.052

Sanderson SD, Cheruku SR, Padmanilayam MP, Vennerstrom JL, Thiele GM, Palmatier MI, Bevins RA (2003) Immunization to nicotine with a peptide-based vaccine composed of a conformationally biased agonist of C5a as a molecular adjuvant. Int Immunopharmacol 3(1):137–146

Sanderson SD, Thoman ML, Kis K, Virts EL, Herrera EB, Widmann S, Sepulveda H, Phillips JA (2012) Innate immune induction and influenza protection elicited by a response-selective agonist of human C5a. PLoS One 7(7), e40303. doi:10.1371/journal.pone.0040303

Scholz W, McClurg MR, Cardenas GJ, Smith M, Noonan DJ, Hugli TE, Morgan EL (1990) C5a-mediated release of interleukin 6 by human monocytes. Clin Immunol Immunopathol 57(2):297–307

Schorlemmer HU, Davies P, Allison AC (1976) Ability of activated complement components to induce lysosomal enzyme release from macrophages. Nature 261(5555):48–49

Shi B, Tang P, Hu X, Liu JO, Yu B (2005) OSW saponins: facile synthesis toward a new type of structures with potent antitumor activities. J Org Chem 70(25):10354–10367. doi:10.1021/jo051536b

Shin HS, Snyderman R, Friedman E, Mellors A, Mayer MM (1968) Chemotactic and anaphylatoxic fragment cleaved from the fifth component of guinea pig complement. Science 162(3851):361–363

Taylor SM, Sherman SA, Kirnarsky L, Sanderson SD (2001) Development of response-selective agonists of human C5a anaphylatoxin: conformational, biological, and therapeutic considerations. Curr Med Chem 8:675–684

Tempero RM, Hollingsworth MA, Burdick MD, Finch AM, Taylor SM, Vogen SM, Morgan EL, Sanderson SD (1997) Molecular adjuvant effects of a conformationally biased agonist of human C5a anaphylatoxin. J Immunol 158(3):1377–1382

Tsuji NM, Kosaka A (2008) Oral tolerance: intestinal homeostasis and antigen-specific regulatory T cells. Trends Immunol 29(11):532–540. doi:10.1016/j.it.2008.09.002

Ulrich JT, Cieplak W, Paczkowski NJ, Taylor SM, Sanderson SD (2000) Induction of an antigen-specific CTL response by a conformationally biased agonist of human C5a anaphylatoxin as a molecular adjuvant. J Immunol 164(10):5492–5498

Ulrich JT, Myers KR (1995) Monophosphoryl lipid A as an adjuvant. Past experiences and new directions. Pharm Biotechnol 6:495–524

Vajdy M, Lycke NY (1992) Cholera toxin adjuvant promotes long-term immunological memory in the gut mucosa to unrelated immunogens after oral immunization. Immunology 75(3):488–492

van Ginkel FW, Jackson RJ, Yoshino N, Hagiwara Y, Metzger DJ, Connell TD, Vu HL, Martin M, Fujihashi K, McGhee JR (2005) Enterotoxin-based mucosal adjuvants alter antigen trafficking and induce inflammatory responses in the nasal tract. Infect Immun 73(10):6892–6902. doi:10.1128/IAI.73.10.6892-6902.2005

Vogen SM, Packowski N, Kirnarsky L, Sherman SA, Taylor SM, Sanderson SD (2001) Differential activities of decapeptide agonists of human C5a: the conformational effects of backbone N-methylation. Int Immunopharmacol 1:2151–2162

Vogen SM, Prakash O, Kirnarsky L, Sanderson SD, Sherman SA (1999) Determination of structural elements related to the biological activities of a potent decapeptide agonist of human C5a anaphylatoxin. J Pept Res 54(1):74–84

Wetsel RA (1995a) Expression of the complement C5a anaphylatoxin receptor (C5aR) on non-myeloid cells. Immunol Lett 44(2–3):183–187

Wetsel RA (1995b) Structure, function and cellular expression of complement anaphylatoxin receptors. Curr Opin Immunol 7(1):48–53

Wiesmuller KH, Bessler WG, Jung G (1992) Solid phase peptide synthesis of lipopeptide vaccines eliciting epitope-specific B-, T-helper and T-killer cell response. Int J Pept Protein Res 40(3–4):255–260

Xiao Y, Peperzak V, Keller AM, Borst J (2008) CD27 instructs CD4+ T cells to provide help for the memory CD8+ T cell response after protein immunization. J Immunol 181(2):1071–1082

Zaal A, Lissenberg-Thunnissen SN, van Schijndel G, Wouters D, van Ham SM, ten Brinke A (2013) Crosstalk between Toll like receptors and C5a receptor in human monocyte derived DCs suppress inflammatory cytokine production. Immunobiology 218(2):175–180. doi:10.1016/j.imbio.2012.02.014

Zuiderweg ER, Nettesheim DG, Mollison KW, Carter GW (1989) Tertiary structure of human complement component C5a in solution from nuclear magnetic resonance data. Biochemistry 28(1):172–185