Increased Efficacy of NKT Cell-Adjuvanted Peptide Vaccines Through Chemical Conjugation

Colin M. Hayman, Ian F. Hermans and Gavin F. Painter

Abstract Through vaccination infectious diseases such as smallpox, polio, measles, and tetanus have either been eradicated or significantly restricted. However, there remain many diseases for which no effective vaccine exists, and therefore new vaccine approaches are still needed. Current vaccine approaches that generate strong immune responses are often based on ill-defined immunogens such as heat-killed or live-attenuated biological products that suffer from concerns related to safety, stability, and lengthy or complex manufacturing processes. For these reasons, there is a strong push toward vaccines that elicit immune responses to defined structures within the targeted pathogen or tissue, which can be achieved by injecting defined antigenic proteins or peptides. On their own, proteins or peptides are generally poorly immunogenic and they must be combined with immune stimulants known as adjuvants to drive antigen-specific immune responses. Recent studies have shown that the direct conjugation of adjuvant compounds to protein or peptide antigens can enhance the magnitude and quality of induced immune responses. In this chapter, we will discuss the chemical approaches our group has used to synthesize a new class of vaccines based on conjugation of peptides with lipid structures that activate innate-like T cells. The stimulatory milieu created by these structures helps drive potent T cell-mediated immune responses that can prevent infectious disease, or can act therapeutically in noncommunicable conditions as diverse as cancer and allergy.

C.M. Hayman (✉) · G.F. Painter
The Ferrier Research Institute, Victoria University of Wellington, Wellington, New Zealand
e-mail: colin.hayman@vuw.ac.nz

I.F. Hermans
Malaghan Institute of Medical Research, Wellington, New Zealand

Z.J. Witczak and R. Bielski (eds.), *Coupling and Decoupling of Diverse Molecular Units in Glycosciences*, https://doi.org/10.1007/978-3-319-65587-1_14

1 Introduction

The control or eradication of infectious disease through vaccination is arguably one of mankind's major achievements. Effective prophylactic vaccines have been developed for many indications including smallpox, polio, and measles. As illustrated in Fig. 1, the impact of vaccination can be dramatic when used widely and in the case of smallpox, the disease was declared eradicated in 1980. However, there remain a number of situations where current vaccine technologies simply do not exist or are cumbersome. For example, the Bacillus Calmette–Guérin (BCG) vaccine for tuberculosis only works in certain groups and there are no vaccines for malaria or human immunodeficiency virus (HIV). In other situations, such as influenza, vaccines show limited cross-strain reactivity creating the need for regular updates which require seasonal, strain-specific vaccines to be manufactured and administered every year. The situation arises because influenza vaccines generate antibody immune responses directed at surface protein structures that change seasonally. In contrast to this, vaccines that generate T cell responses targeted at conserved epitopes across different strains are expected to show cross-strain reactivity. However, there are currently no precisely defined vaccine technologies that drive T cell responses.

Furthermore, vaccines that generate T cell responses that can also be used therapeutically against noncommunicable diseases such as cancer and allergy are now emerging. In these situations, vaccines that generate humoral (antibody) immune responses are likely to be less effective. For example, a promising new vaccine concept for allergy that targets the antigen-presenting cells that drive allergic responses for destruction exploits T cell-mediated cytotoxicity rather than antibody-mediated mechanisms [1]. Since allergy is maintained by antibody production (IgE), vaccines that generate antibody responses may even exacerbate disease. Whether it be to treat infectious disease or noncommunicable disease, for the field to make rapid progress, it desperately needs new precisely defined vaccine technologies that drive T cell responses.

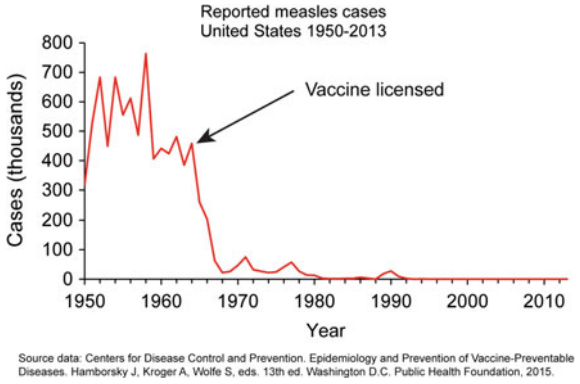


Fig. 1 The number of measles cases in the US before and after the introduction of widespread vaccination

Through the action of T cells, the immune system can recognize and respond to fragments of protein antigens, known as peptide epitopes, presented by major histocompatibility complex (MHC) molecules on the surface of infected or neoplastic (cancerous) cells through a process of antigen presentation. In the priming phase of an immune response, these antigens must first be acquired from the affected tissues by antigen-presenting cells (APCs), and then peptide epitopes presented by these cells to T cells in the lymphoid tissues. Peptides presented on MHC class I molecules are capable of stimulating $CD8^+$ T cells, whereas peptides presented on MHC class II molecules are capable of stimulating $CD4^+$ T cells. The peptide-recognition receptors expressed by T cells, called T cell receptors (TCRs), are generated through a random somatic recombination process. Within the vast repertoire of T cells generated, there is a high likelihood that some cells will recognize a given antigenic peptide structure in the context of MHC molecules. An adaptive immune response is initiated when these few antigen-specific T cells are stimulated to undergo clonal differentiation and proliferation into effector T cells that have the ability to control infection, or eliminate infected or neoplastic tissue (Fig. 2). A powerful feature of this whole process is that any abnormally expressed protein can potentially be recognized by T cells, regardless of location, be that protein derived from a pathogenic agent or resulted from genetic instability in neoplastic cells.

In contrast to T cells, B cells, the other major component of the adaptive immune system, are not cellular effectors in themselves, but secrete their antigen receptors in the form of antibodies. Antibodies can recognize extracellular antigens or components expressed on the outer surface of cells; they cannot generally recognize antigens in intracellular locations, or epitopes that are concealed within larger structures. To initiate responses to extracellular antigens, recognition is initiated through surface-expressed B cell receptors (BCRs). Once engaged, antigen-specific BCRs can trigger antigen internalization and processing resulting in peptide

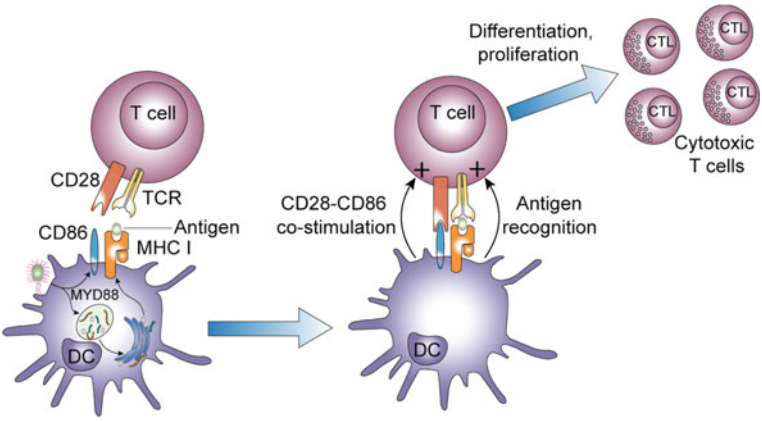


Fig. 2 MHC class I presentation and the generation of cytotoxic T lymphocytes

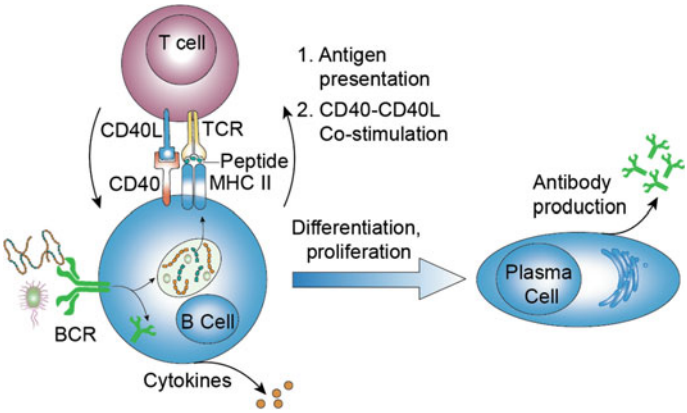


Fig. 3 B cell (humoral) immune response

fragments being presented by MHC class II molecules to $CD4^+$ T cells. This process, known as T cell help, contributes to the further activation and differentiation of B cells into plasma cells—B cells that are able to secrete large quantities of a soluble form of their BCR, namely antibodies (Fig. 3).

Our understanding of precisely what type of immune response will be most effective in certain disease situations is still rudimentary, but nonetheless it is clear that responses have to be tailored in each case; an inappropriate response can be ineffective, or even detrimental. There is therefore a corresponding need to generate new vaccines that induce specifically tailored immune responses. Modifying immune responses to whole cell, live-attenuated or vaccines that contain complex ill-defined components, is challenging and somewhat empirical. In addition to this, concerns around safety and lengthy manufacturing processes are driving a strong push toward vaccines that comprise well-defined components such as antigenic proteins, or peptides that encompass specific epitopes.

Due to their ease of manufacture and relatively simple characterization, peptide vaccines are particularly attractive vaccine candidates from a chemical manufacturing perspective [2]. However, and in contrast to whole microorganisms, a major disadvantage of peptide vaccines is their lack immunogenicity [3]. The intrinsic immunogenicity of vaccines based on microorganisms is due largely to the presence of immunostimulatory molecules that trigger pattern recognition receptors (PRRs). Expression of PRRs is largely confined to cells of the innate arm of the immune response, including most APCs. These receptors have evolved to recognize a variety conserved chemical structures that are features of microorganisms, or various tissue-derived structures associated with damage or inflammation. Examples include toll-like receptor 2 (TLR-2), which binds lipopeptide structures typically found in bacterial cell walls, and TLR-9, which binds unmethylated CpG structures typically seen in prokaryotic DNA. Once engaged, PRRs such as the TLRs activate intracellular signaling cascades that trigger immediate effector functions, such as

respiratory burst or cytokine release, or that can orchestrate downstream adaptive immune responses. Importantly, APCs positioned in infected or perturbed tissue, or in the lymphoid tissues associated with these sites, can efficiently acquire antigens in addition to receiving stimulation via their PRRs. This pattern recognition is significant in that it triggers dendritic cells (DCs), a specialized APC subpopulation, to release soluble factors such as pro-inflammatory cytokines and chemokines, and upregulates surface expression of costimulatory and antigen presentation molecules that enable effective stimulation of T cells (Fig. 4). In order to accomplish this, changes in the surface expression of adhesion molecules facilitate migration to T cell areas of the secondary lymphoid organs (e.g., draining lymph nodes or spleen) where they present antigen. Responding T cells can differentiate into effector cells that recirculate into the affected tissues, or can provide help to B cells to make antibodies.

Because the triggering of PRR pathways (i.e., the TLRs) leads to increased antigen-specific immune responses, compounds that engage these pathways can be used as vaccine adjuvants. However, despite the huge potential that vaccine adjuvants of this type have to modify and shape vaccine response, there are very few products currently licensed for use in humans. Examples include the TLR-4 agonist monophosphoryl lipid A (MPL), which is incorporated into GSK's Engerix-B vaccine, Dynavax's CpG-rich motifs that are included in various products, including their vaccine for Hepatitis B (i.e., HEPLISAV-B), and the Pam_3CSK_4 series of compounds recognized via TLR-2 that were originally derived from *N*-terminally lipidated proteins isolated from the cell wall of mycobacterial species (Fig. 5).

Importantly, the addition of TLR agonists (along with other PRR agonists) serves to increase the immunogenicity of poorly immunogenic peptide vaccines [4]. Furthermore, it has recently been shown that this effect can be further enhanced by chemically conjugating adjuvant compounds directly to peptide antigens. This observation can be explained, at least in part, by the requirement of APCs to not only present peptide antigens to T cells but also be appropriately activated at the same time. For example, appropriate activation results in the provision of costimulatory molecules (e.g., CD86) providing additional T cell signals that result in its differentiation of $CD8^+$ T cells into cytotoxic T lymphocytes (CTLs) that have the capacity to kill target loaded cells (Fig. 2).

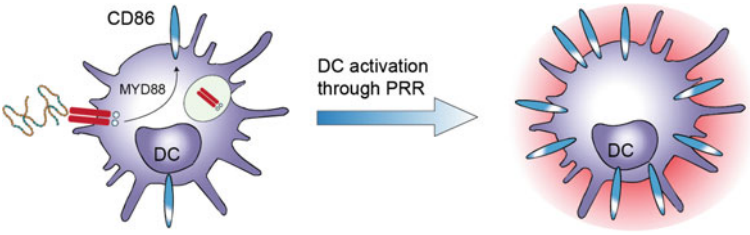
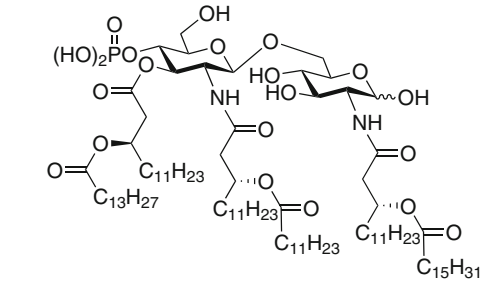


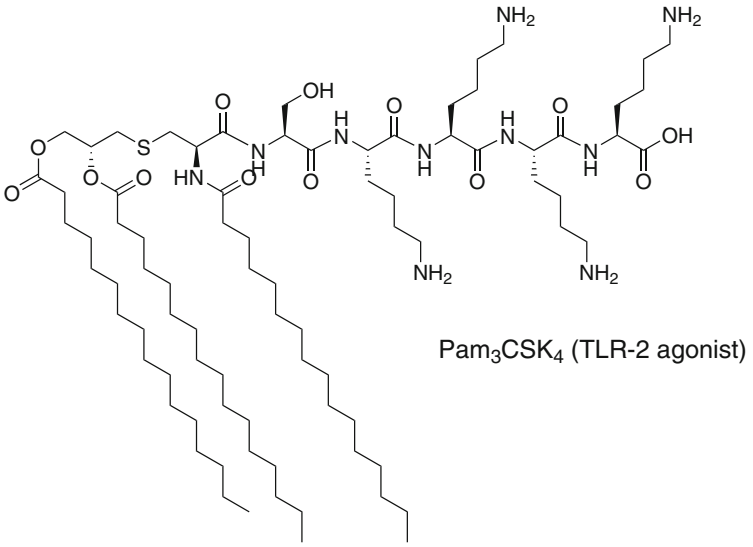
Fig. 4 Pattern recognition receptor signaling leading to DC activation (represented by cell surface CD86 upregulation)



Main active component of MPL® (TLR-4 agonist)

5'-TCGGCGC-HEG-AACGTTC-HEG-TCGGCGC-3'

CpG ODN DV230 (TLR-9 agonist)



Pam_3CSK_4 (TLR-2 agonist)

Fig. 5 Chemical structures of MPL®, DV230 and Pam_3CSK_4

In an exciting body of work, TLR ligands covalently bound to peptide antigens have been shown to increase the magnitude of either T (cellular) [5] or B (humoral or antibody) cell [6] responses. For example, conjugation has been reported to increase cellular responses through improved antigen targeting to DCs—APCs that are highly specialized in stimulating T cell responses. Conjugated compounds ultimately require cleavage within APCs, a processing step that effectively leads to the buildup of an intracellular antigen depot that supports prolonged antigen presentation and improves immune response [5, 7]. Many of these observations were

described for conjugates that utilize Pam_3CSK_4 as a TLR-2 agonist, and however conjugate vaccines have also been prepared based on a number of alternative TLR agonists such as a monoacyl TLR-2 ligand [8], the TLR-9 ligand CpG [9], and others [10–13].

The conjugation of larger sequences that include T-helper peptide epitopes can drive increased antibody production [6]. For example, a fully synthetic three-component vaccine containing the TLR-2 agonist Pam_3CSK_4, a T-helper epitope from polio virus and a B cell epitope derived from mucin was able to drive increased IgG antibody responses (Fig. 6). All three components were required to be conjugated together to induce the maximal response, and a liposomal delivery vehicle was needed.

Compared to pattern recognition receptor (PRR) agonists such as the TLR agonists, a less-explored approach to activating APCs is the use of ancillary cells that provide stimulatory signals to the APC. The most studied cells of this type are a sub-class of innate-like T cell known as type I natural killer T (NKT) cells, which are defined on the basis of a largely invariant TCR structure, expression of surface molecules in common with classic innate cells like Natural Killer (NK) cells, and a semi-activated phenotype that enables them to respond rapidly to antigen recognition [14]. Compared to classical T cells that are very rare, NKT cells are in relative abundance in many mammalian species, including humans and mice, and their TCRs structures are conserved between individuals (and across species) making the antigens they recognize attractive adjuvants for vaccine development. Whereas classical T cells recognize MHC-restricted peptide antigens (as discussed above), NKT cells respond to *lipid antigens* presented by the non-classical MHC class I molecule CD1d. The CD1d molecule contains a hydrophobic binding groove that sits between two α-helixes and above an antiparallel β-sheet that can accommodate lipids. Associated polar groups such as sugars or phosphates are situated at

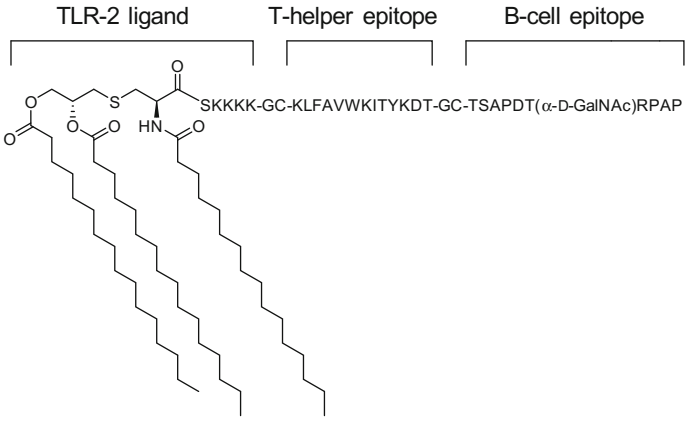


Fig. 6 The chemical structure of a TLR-2 self-adjuvanting lipopeptide vaccine (see text for details)

the surface for interaction with the TCR. A number of synthetic and natural antigens have been reported that consist of a lipid backbone and polar head group [15]. The prototypical antigen is α-galactosylceramide (α-GalCer) (Fig. 7), a synthetic glycolipid compound that was derived from structure–activity relationship studies on a class of marine sponge-derived glycolipids; it remains the most studied of all the reported compounds [16]. Its lipid chains sit deep in the CD1d hydrophobic binding pockets, whereas its polar head group forms a number of hydrogen bonds with CD1d and the TCR [17].

Lipid antigens, such as α-GalCer, presented in this fashion by APCs serve to activate NKT cells (Fig. 8). Activated NKT cells have been implicated in a variety of biological activities including antitumor and antimicrobial activities. The antitumor activity has been attributed to several activities, including NKT-mediated cytotoxicity toward $CD1d^+$ tumor cells, trans-activation of NK cells, and release of antiangiogenic cytokines like IFN-γ. In addition to this (and of direct relevance to this review), NKT cells are able to activate APCs through the provision of CD40L and soluble factors leading to the enhancement of T and B cell responses [18]. Therefore, in this context, lipid antigens such as α-GalCer can also be considered vaccine adjuvants since when added to vaccines they can drive increased antigen-specific immune responses.

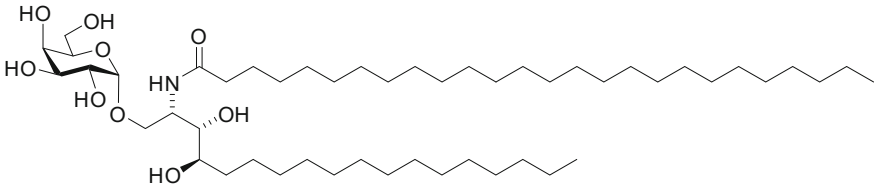


Fig. 7 α-GalCer chemical structure

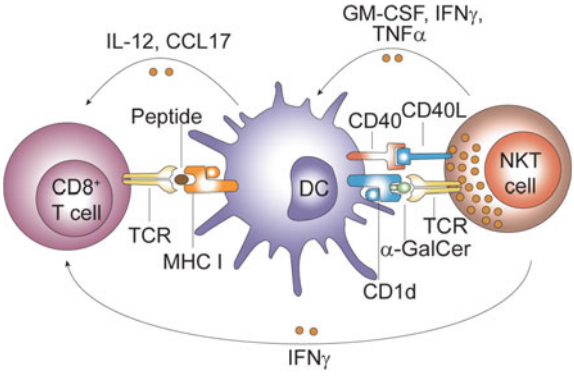


Fig. 8 Alternative APC activation through NKT cell help

2 Transforming α-GalCer into a Proadjuvant

At the heart of the vaccine technology to be described, herein is the utilization of NKT cells as "cellular adjuvants" through provision of glycolipid antigens combined with the concept that conjugation of vaccine adjuvants through covalent linkages to antigens can lead to increased adaptive immune responses and efficacy in disease challenge models. We favored the idea of utilizing NKT cells over the direct stimulation of APCs through PRRs because highly potent responses can be achieved in animal models; there was some reported adjuvant activity in in vitro cultures of human leucocytes, and evidence that this mode of APC activation can be complementary to TLR ligation, suggesting opportunities to combine with other adjuvants to maximize impact [18].

Although there are a number of highly potent NKT cell agonists (examples include 7-DW8-5 [19] and ABX196 [20]) that can drive adjuvant activity, we choose to use the original compound α-GalCer (KRN7000) because this compound has already been shown to be safe when administered in humans [21]. However, using α-GalCer as the NKT cell ligand, we required a suitable attachment site that was readily accessible and would allow the formation of stable adjuvant–antigen conjugates. Due to their high reactivity, free amines have been widely exploited as a convenient functionality for conjugation to suitably activated coupling partners; however, the only nitrogen of α-GalCer is present as an amide and is not available for functionalization. As others have done successfully, we considered exchanging the 6-hydroxyl group to an amine [22, 23] or modifying with a reactive appended group [24]; however, these approaches would not release an adjuvant compound that was known to be safe. Other potential sites for antigen conjugation to α-GalCer are the four hydroxyl groups of the galactosyl moiety which we did not consider attractive conjugations sites as they are not readily accessed in a selective manner and are less reactive than amines.

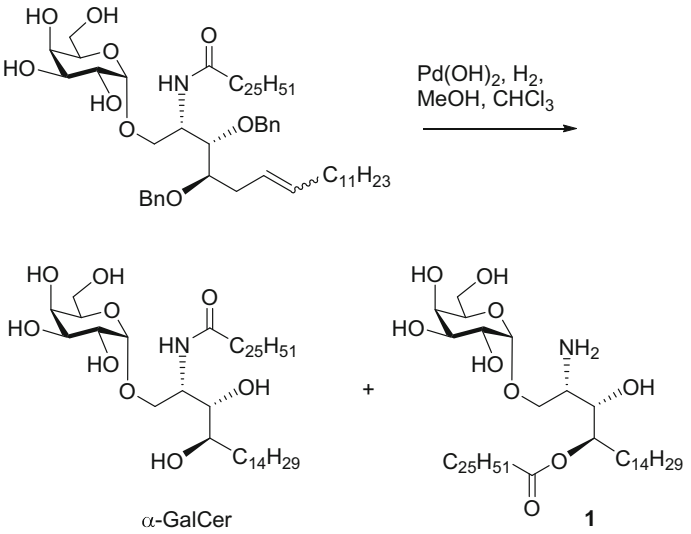
Practical access to the 2′-N atom as a synthetically useful amine was realized by carefully characterizing a reaction by-product. During an unpublished synthesis of α-GalCer, we isolated **1** as a minor component during the final deprotection step of **2** which had been accessed by Gervay-Hague's [25] glycosylation methodology (Scheme 1).

In this instance, hydrogenation of the alkene and hydrogenolysis of the two benzyl protecting groups was undertaken using Pearlman's catalyst under an atmosphere of H_2 using a mixed solvent system of chloroform/methanol. Using these conditions along with silica gel chromatography for purification, a modest yield of α-GalCer was isolated along with a significant amount of **1** as a low-R_f by-product. We reasoned that the use of chloroform as a co-solvent led to the in situ formation of HCl and promoted the $N \rightarrow O$ migration of the C26 acyl group. Strongly acidic conditions have been reported to promote the $N \rightarrow O$ migration of acyl groups; however, as far as we are aware the use of Pd-catalyzed HCl generation from chloroform had not.

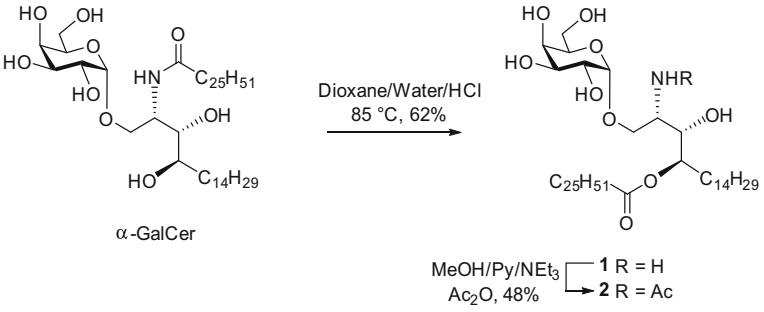
The discovery that an apparently acid-promoted $N \rightarrow O$ acyl migration gave an isolable analogue of α-GalCer with a free amine group immediately raised the intriguing possibility that **1** could form the basis of novel class of adjuvant–antigen conjugates applicable in the field of immunotherapy.

To progress this idea, we established conditions which provided ready access to **1** directly from α-GalCer using HCl in dioxane. We were also able to establish that the free amine of **1** was amenable to further modification by preparing the acetamide **2** (Scheme 2).

We had also envisaged that if **1** was released in a biological setting that the reverse $O \rightarrow N$ acyl migration would be facile under physiological conditions. A dilute solution of **1** in 99:1 PBS/DMSO was monitored over time at ambient



Scheme 1 The discovery of amine **1** during a synthesis of α-GalCer



Scheme 2 The preparation of **1** and **2**

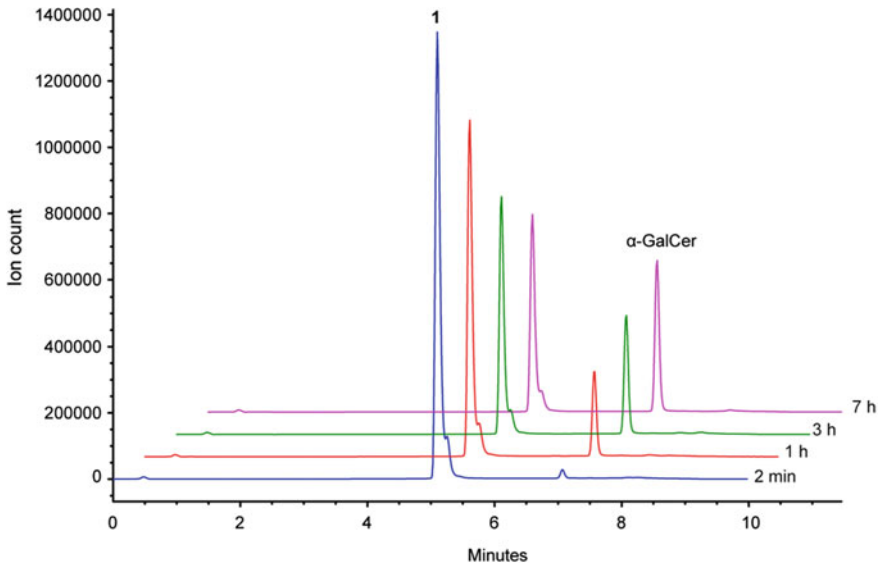


Fig. 9 HPLC-MS chromatograms of **1** in 99:1 PBS/DMSO monitored at the indicated time points

temperature by HPLC-MS. The MS was operated in single ion monitoring (SIM) mode which clearly demonstrated that **1** reverted to α-GalCer over a number of hours (Fig. 9).

With this insight into the fate of **1** at physiological pH, it was expected that its biological activity would be similar to α-GalCer and this was shown to be the case in the context of NKT cell activation in vivo. Compound **1** was able to induce the NKT cell-mediated maturation of DCs as measured by the upregulation of cell surface CD86 on DCs (Fig. 10) [26]. However, with compound **2** the $O \rightarrow N$ acyl migration is unable to take place and this correlated with an inability for **2** to activate NKT cells.

Taken together the in vivo data and the $O \rightarrow N$ acyl migration data suggested that we had a solution to a key part of the puzzle of how to make a conjugate vaccine based on α-GalCer. We proposed that capping the nitrogen atom of **1** with a stable traceless linker provided a proadjuvant which was inactive until enzymatically cleaved allowing for $O \rightarrow N$ acyl migration and generation of the active adjuvant α-GalCer. Combining the proadjuvant concept into a conjugate which comprised an antigen may give a construct which was inactive until enzymatic processing released **1** and a modified peptide antigen for further enzymatic processing to effectively deliver α-GalCer and an antigen to the same site.

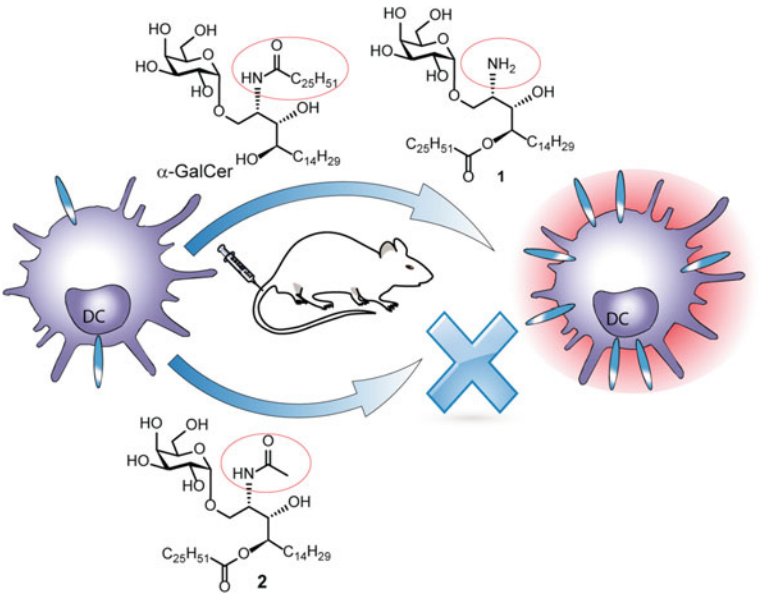


Fig. 10 α-GalCer and **1** activate DCs in vivo, whereas **2** does not

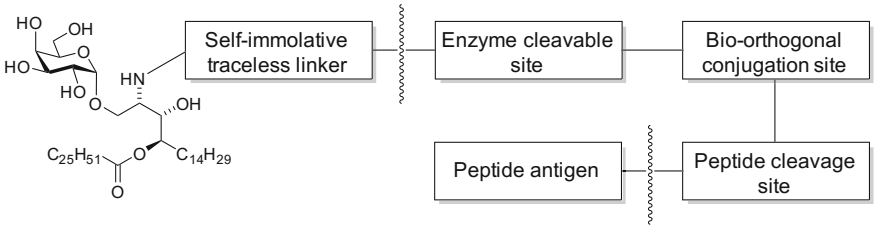


Fig. 11 The design concept for an α-GalCer proadjuvant–antigen conjugate

3 Vaccine Conjugate—Concept and Enablement

To prepare a practical proadjuvant–antigen conjugate, we need to consider a number of aspects of the overall linking strategy which would allow an antigenic peptide to be coupled to **1**. A summary of our design concept is described diagrammatically in Fig. 11.

The first aspect of the design is that the linker attached directly to the N atom must be traceless. So enzymatic processing must either release a free amine directly or reveal an unstable moiety which in turn self-immolates under physiological conditions and collapses to reveal the free amine. Second, the linker needs to have a suitable site for conjugating an antigenic peptide of choice. For this purpose, there is a large body of work in the field of bioorthogonal chemistry where the reactive

chemical moieties couple together under benign conditions and do not react with those functional groups commonly found in biological systems that typically include peptides and proteins [27]. The third aspect we considered was a peptide cleavage sequence appended to the peptide antigen of interest which would encourage peptide release from residual linker components enabling further processing so that peptide epitopes of interest may be released and presented by MHC molecules.

3.1 *Esterase Cleavable Linker—Acyloxymethyl Carbamate*

Our first family of conjugate vaccines was based on the acyloxymethyl carbamate moiety which has precedent as a self-immolative linker system for amines that can be triggered by enzymatic activity [28]. The general mode of collapse in the context of our vaccine design is described in Fig. 12.

The acyloxymethyl carbamate contains an ester which was expected to be susceptible to enzymatic cleavage where indicated. This releases the hydroxymethyl carbamate moiety which is known to collapse to release formaldehyde and carbon dioxide, and in this case reveal the free amine **1**. To be most effective, we envisage intracellular cleavage will be required for efficacy, however, when administered in vivo this approach may allow for some extracellular cleavage to also occur. Other drugs have demonstrated successful intracellular, esterase-dependent, and linker collapse [29].

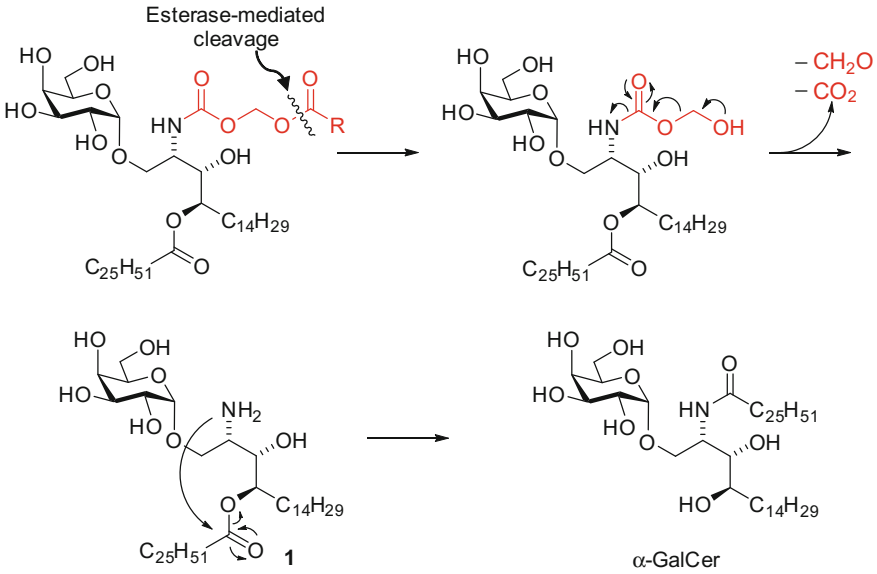
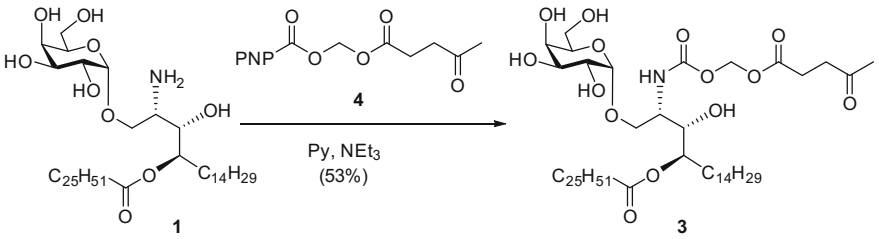


Fig. 12 The mode of collapse of the acyloxymethyl carbamate linker and the generation of α-GalCer proceeding via **1**

The specific acyloxymethyl carbamate derivative of **1** that we decided to make first was **3** where the linker is derived from levulinic acid (Scheme 3) [26].

As was the case for migrated α-GalCer compound **1** intravenous administration of **3** to C57BL/6 mice demonstrated the ability of **3** to induce DC maturation to a similar extent as α-GalCer (Fig. 13). The activity of **3** or α-GalCer was not significantly different to the PBS control when administered to CD1d knockout mice confirming the activity was dependent upon NKT cells since CD1d knockout mice do not have any NKT cells.

To test whether enzymatic processing was required to achieve this activity, an in vitro experiment was conducted whereby plate-bound CD1d was treated with **3** and tested for the ability of any potential CD1d-**3** complex to activate NKT cells (Fig. 14) [26]. Using α-GalCer as a positive control, this experiment demonstrated that **3** was not a good activator of NKT cells in the vitro experiment.



Scheme 3 The preparation of **3**

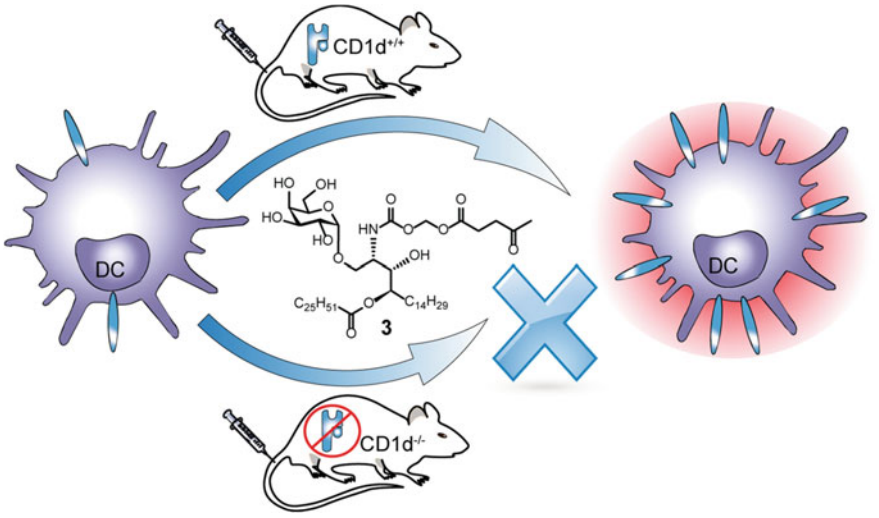


Fig. 13 Compound **3** activates DCs in a CD1d-dependent manner

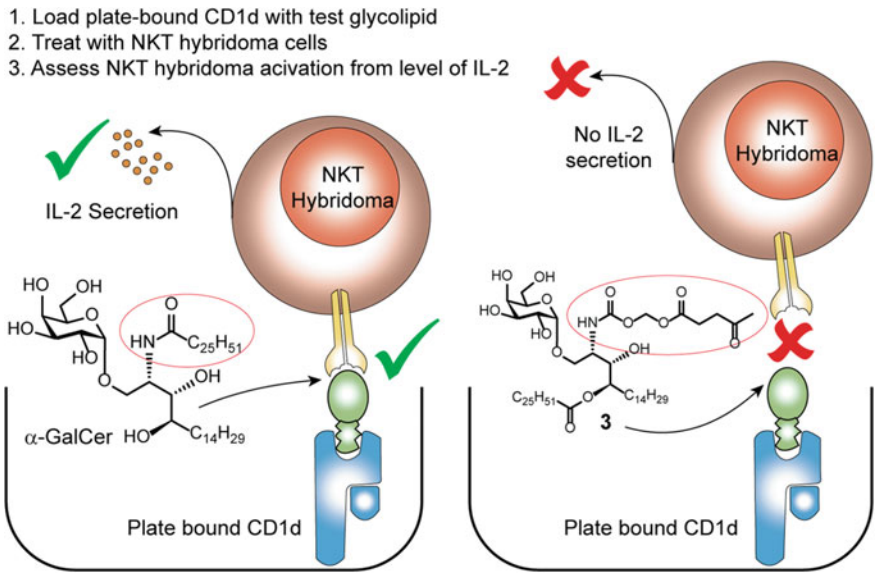


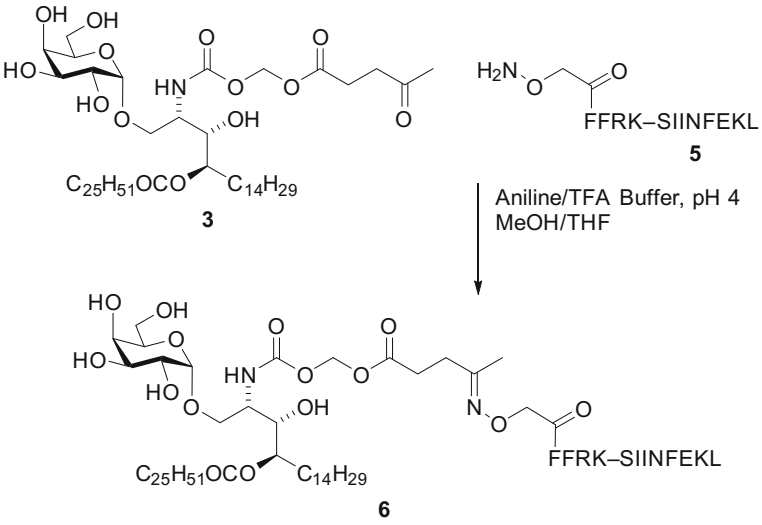
Fig. 14 Compound **3** is an inactive CD1d ligand in vitro

The observation that **3** cannot activate NKT cells in vitro whereas it is observed in whole animal studies that the injection of **3** elicits a strong NKT cell-dependent response is a good demonstration of **3** acting as a proadjuvant.

3.2 Conjugation of the Peptide Antigen—Oxime Chemistry

To complete the construction of the conjugate vaccine, we prepared the peptide antigen flanked by a peptide cleavage site which was in turn capped at the *N*-terminus with an aminooxy group as the bioorthogonal functionality (**5**). The reaction of aminooxy groups and ketones or aldehydes to form oximes is widely reported due to the reliability of the chemistry and the stability of the oxime moiety. Although oximes do hydrolyze slowly at physiological pH (half-life is measured in the order of weeks), they are well suited for bioconjugation of peptides or proteins to synthetic components free of ketones and aldehydes other than at the site of conjugation. Oxime formation is actually so efficient that solvents of high purity must be used to avoid the scavenging of the hydroxylamine component by low levels of carbonyl impurities common in many solvents [30].

The immunodominant CD8 epitope from ovalbumin, Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu (SIINFEKL) (OVA_{257}), was selected as the peptide antigen. This was appended at the *N*-terminus with the sequence Phe-Phe-Arg-Lys (FFRK) which has been shown to promote antigen presentation of CD8 epitopes via MHC class I



Scheme 4 The conjugation of ketone **3** and oxime **5** to give the vaccine **6**

molecules. The oxime-capped peptide **5** was prepared using Fmoc solid-phase peptide synthesis and reacted with ketone **3** to give the conjugate vaccine **6** (Scheme 4).

A number of features of this conjugation meant that significant optimization of the reaction conditions was required. The degree of difference in the solubilities of the glycolipid and the peptide meant that the reaction had to be carried out at a moderately high dilution (1.6 mM) which required the use of aniline as a catalyst to achieve complete reaction after 48 h. Oxime formations are also generally undertaken at mildly acidic pH to enhance the rate of reaction. In this case, we found that it was necessary to use aqueous aniline which had been buffered to pH 4. At the higher pH of 4.5–5, loss of peptide to its unreactive formaldehyde adduct was observed by LCMS which we attributed to collapse of the linker component of **3** and generation of formaldehyde in situ. With these conditions optimized, however, the reaction was monitored for loss of **3** by HPLC and purified by semi-preparative HPLC to give **6** in an isolated yield of 33%.

The biological activity of **6** was studied extensively in mice which established that it was effective as a vaccine. Proliferation of a transgenic population of peptide-specific $CD8^+$ T cells was observed by cell flow cytometry analysis of blood 7 days after injection with **6** (Fig. 15). The increase as a percentage of total $CD8^+$ T cells was a significant enhancement in the population treated with **6** compared to that treated with an admixture of α-GalCer and OVA_{257} peptide (i.e., approx. 3.5 vs. 0.5%). In addition to the proliferation of transgenic $CD8^+$ T cells, vaccination with **6** produces endogenous T cell population with a markedly enhanced cell-killing ability over the co-administration of α-GalCer and various peptide derivatives [26].

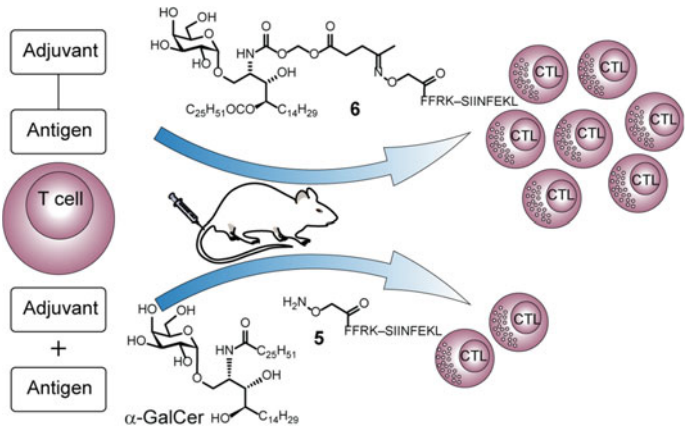


Fig. 15 Administration of conjugate vaccine **6** induces greater proliferation of antigen-specific $CD8^+$ T cells compared to admixed controls

The combined data showed a clear advantage that vaccination with a conjugated adjuvant–antigen constructs provided as compared to co-administration of the components. Intracellular processing of peptide can enhance the efficiency of MHC class I presentation through a cross-presentation mechanism; however, the advantage of the conjugate is also seen for the modified peptides which demonstrate that the effect is not simply due to modification of the peptide.

3.3 NKT Cell Conjugate Vaccine and Asthma

Allergic asthma is characterized by shortness of breath, wheezing, coughing, and airway hyperresponsiveness affecting large numbers of people—particularly in developed countries. Common treatments for the disease such as steroids target the symptoms of the disease, not the underlying cause. At a cellular level, allergen-specific $CD4^+$ T cells that produce type 2 cytokines (i.e., interleukins 4, 5, and 13), known as T_H2 cells, have an essential role in driving and maintaining allergic responses. This includes the recruitment of eosinophils and activation of B cells to secrete allergen-specific IgE antibody which in turn leads to mast cell activation [31]. It has been reported that by acquiring and presenting allergen to T_H2 cells, DCs are required in the effector phase of the inflammatory response [1]. It has also been reported that adoptively transferred allergen-specific $CD8^+$ T cells can reduce inflammation by mechanisms that potentially include CTL elimination of allergen-specific DCs [32]. For these reasons, we hypothesized that the increased T cell responses generated by our conjugated peptide vaccines might be able to suppress allergic inflammation. As reported [26], we showed our conjugate vaccine could completely suppress eosinophil infiltration, whereas admixed controls

(i.e., α-GalCer and peptide) were ineffective. Further studies are still required to determine if DC killing is the primary mechanism but the finding has opened up an exciting area of ongoing research and development.

3.4 *Protease-Cleavable Linker—Valine-Citrulline*-p-*Aminobenzyl Carbamates*

The use of esterase cleavable linkers to covalently attach α-GalCer as an adjuvant with a peptide antigen enabled us to demonstrate a clear advantage over co-administration. We decided an improved vaccine that may be able to be prepared by incorporating a traceless linker which was more directed toward intracellular enzymolysis. Valine-citrulline *p*-aminobenzyl (VC-PAB) carbamates are susceptible to enzymolysis by cathepsin B which is an abundant lysosomal enzyme in antigen-presenting cells. The VC-PAB linkage has been shown to have good plasma stability and has found application in the clinic in the field of antibody–drug conjugates [33].

The VC-PAB linkage undergoes proteolytic cleavage to reveal the amine of the PAB carbamate which in turn is self-immolative under physiological conditions to give carbon dioxide and azaquinone methide. The collapse cascade in the context of **1** is shown in Fig. 16.

To prepare a conjugate vaccine comprising the VC-PAB linker, we started with the known VC-PAB precursor **7** and capped this at the *N*-terminus with the active ester of levulinic acid **8** and subsequent formation of the activated carbonate gave **9**. This was coupled to **1** which was followed by oxime formation in a manner analogous to **6** to provide **10** as a second conjugate vaccine (Scheme 5) [34].

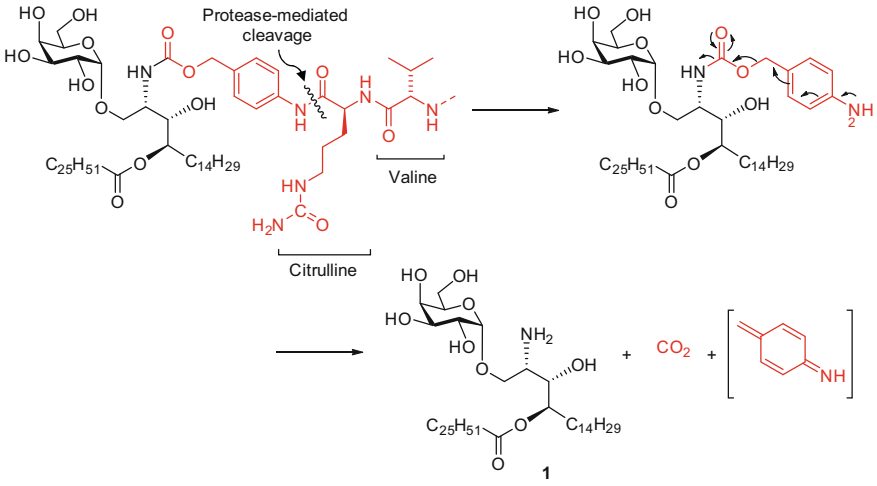
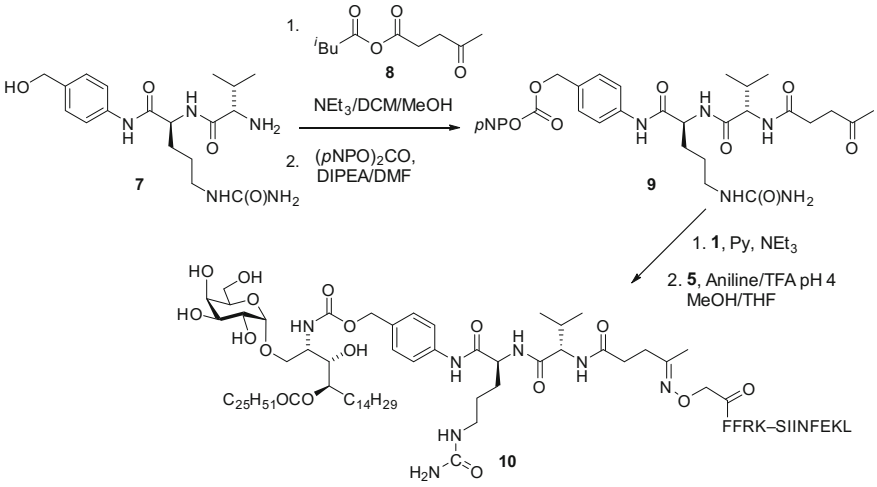


Fig. 16 The mode of collapse of the valine-citrulline-*p*-aminobenzyl carbamate linker



Scheme 5 The preparation of **10**

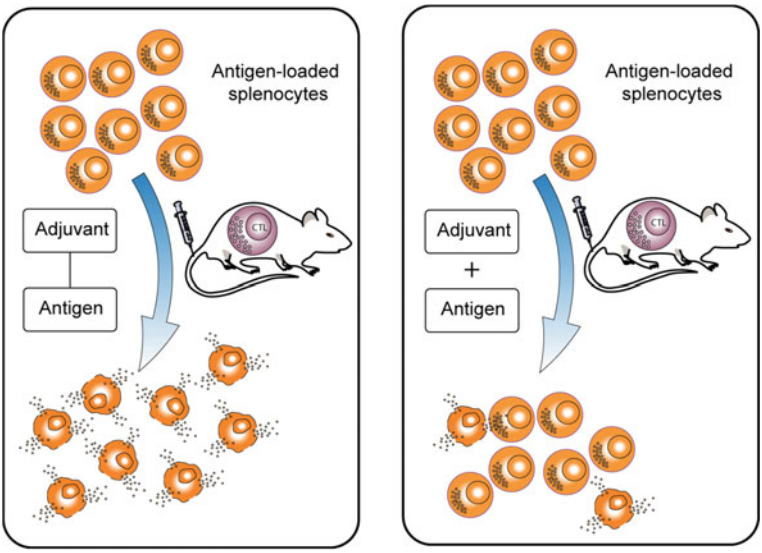


Fig. 17 Comparison of the ability of vaccination with conjugate compared to admixed controls to generate an endogenous antigen-specific cytotoxic response

The biological activity of **10** was found to be very similar to **6** in a side-by-side comparison in a cell lysis assay. The ability of **6** and **10** to elicit an endogenous antigen-specific cytolytic activity against splenocytes which had been pulsed with different concentrations of OVA_{257} peptide was measured which established a clear advantage of the conjugate vaccines over admix vaccination with α-GalCer and peptide (Fig. 17).

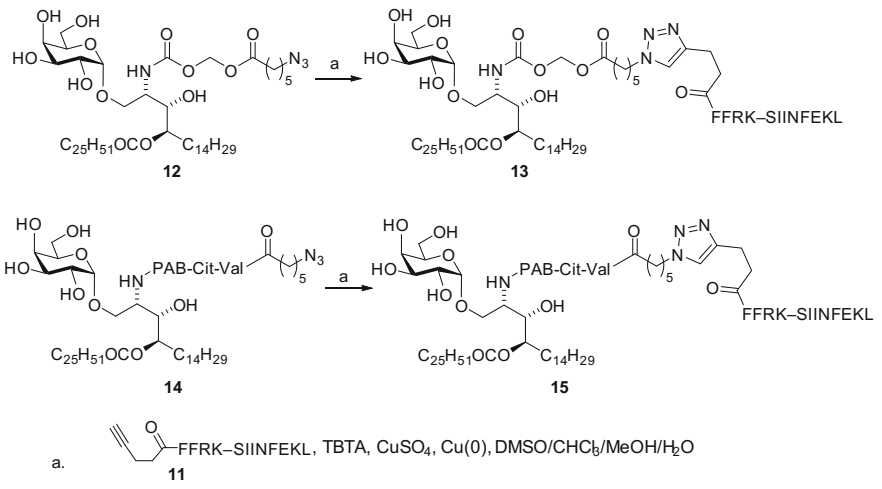
3.5 *Alternative Linkage to the Peptide—Cu(I)-Mediated Azide–Alkyne Cycloaddition*

Conjugates **6** and **10** had validated the concept of preparing a vaccine comprising covalently bound adjuvant and antigen. A limitation of oxime ligation, however, is the ease of access to a range of peptide antigens. The extreme reactivity of the aminooxy group toward adventitious aldehydes and ketones renders the preparation of aminooxy-capped peptides unreliable, and in our experience, we have found that this can limit the ability of commercial providers to readily supply them.

Therefore, we investigated the use of the Cu(I)-mediated azide–alkyne cycloaddition (CuAAC). The CuAAC reaction is one of the most widely used widely reported ligations in the area of bioconjugation. This is due to a number of reasons which include essentially complete bioorthogonality which azides and alkynes have within biological systems, the relative chemical stability of azides and alkynes, and the ease of preparation of the conjugation partners.

In the context of our vaccine conjugates, we opted to use alkyne-capped peptide **11** (Scheme 6). The glycolipid coupling partners to this were the azides **12** and **14** comprising the esterase- and protease-cleavable linkers, respectively. These were constructed in a manner analogous to the ketone-containing coupling partners above, except the linker portion was built up from 6-azidohexanoic acid instead of levulinic acid.

Similar to the development of the oxime ligations, the CuAAC chemistry required significant optimization in order for the efficient reaction of our coupling partners. Satisfactory conditions were found which used copper foil and copper sulfate as the redox couple (to generate the Cu(I)) along with the accelerating ligand



Scheme 6 Synthesis of **13** and **15**

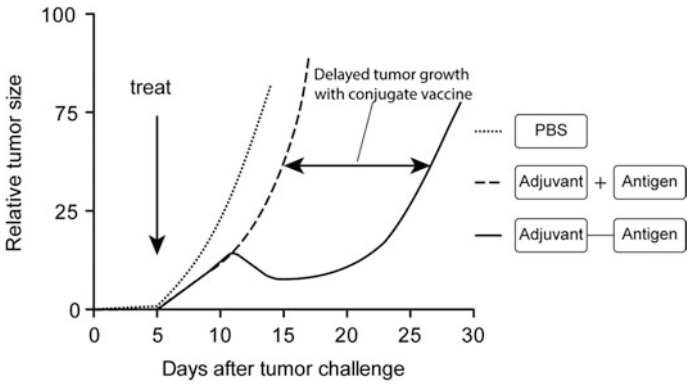


Fig. 18 Comparison of tumor growth over time of mice vaccinated with conjugate vaccines versus admixed controls

Tris-[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) to give the conjugates **13** and **15**. The disparate solubility of the reaction components again dictated the use of a complex mixture of solvents to minimize heterogeneity. We also kept the concentration of DMSO at 30% due to the detrimental effect it has on reaction rate when using TBTA as an accelerating ligand. We were unable to get the reaction to proceed using the commonly reported $CuSO_4$ with sodium ascorbate as the reductant [35].

The therapeutic activity of all four vaccine conjugates (**6**, **10**, **13** and **15**) was shown to confer significant antitumor activity against the aggressive mouse melanoma B16.OVA (Fig. 18) [34]. In this test, a single dose of candidate vaccine was administered once the tumors had become engrafted and palpable. The effect of conjugation was demonstrated by the beneficial delay in tumor outgrowth observed when animals were treated with the conjugates as compared to the co-administration of α-GalCer and either capped or uncapped OVA_{257} peptide.

A reproducible feature of the comparison of these four vaccines (i.e., **6**, **10**, **13,** and **15**) was that conjugate **13** gave a less-pronounced antitumor activity than the others. This was an interesting demonstration that the combination of the choice of linker chemistries was altering the quality of the immunological response in vivo.

4 Decoupling

The biological activity presented above demonstrates the clear advantage that the administration of the conjugated adjuvant and antigen have over the co-administration of the components. This was shown for the ability of vaccinated mice to generate a cytotoxic response against antigen-specific cells as well as vaccination having strong antitumor activity in vivo. In vitro studies have also demonstrated that constructs **2** and **3** do not induce CD1d-dependent DC activation.

However, we wanted to check if our vaccine design was releasing the active components in the way we had designed it to. For this purpose, we used the CD1d monomer NKT cell hybridoma in vitro assay (see Fig. 14).

Treating CD1d monomer-loaded plate wells with solutions of conjugate and incubating with NKT hybridoma cells resulted in no appreciable IL-2 release (i.e., no NKT cell activation), whereas the positive control, α-GalCer, induced IL-2 release as expected. In contrast, pre-treatment of the conjugate with cathepsin B at pH 5.5 recovered much of the 'α-GalCer' activity. This demonstrates that the treatment with cathepsin B generates a ligand for CD1d capable of stimulating NKT cell activity. This is consistent with the expected mode of action, whereby the cathepsin B-mediated cleavage of the VC-PAB linker generates **1** which in turn undergoes $O \rightarrow N$ acyl migration to produce α-GalCer (see Fig. 16).

This change in peptide ligation chemistry also gave vaccines with the ability to generate $CD8^+$ T cells with antigen-specific cell lysis activity comparable to the oxime-ligated conjugates **6** and **10** (data not shown).

4.1 Early Release of α-GalCer

HPLC-MSMS was used to monitor for early release of α-GalCer which may arise from hydrolysis of the self-immolative linker. Collapse of the acyloxymethyl carbamate or the VC-PAB linker would initially lead to **1** which we had already shown will give α-GalCer over time in aqueous conditions. Therefore, monitoring solutions of conjugates for the level of α-GalCer over time serves as a proxy for the susceptibility of the two linkers to nonspecific hydrolysis.

Aqueous samples of vaccines **6**, **10**, **13,** and **15** each buffered to pH 3.0, 5.0, 7.4, and 9.0 were stored at 20 °C for 8 days and monitored periodically for α-GalCer level. This study demonstrated that at pH 7.4 and 9.0 that the acyloxymethyl carbamate conjugates **6** and **13** were both susceptible to nonspecific hydrolysis, whereas no α-GalCer was detected in the solutions of the VC-PAB conjugates **10** and **15** (Fig. 19) [34].

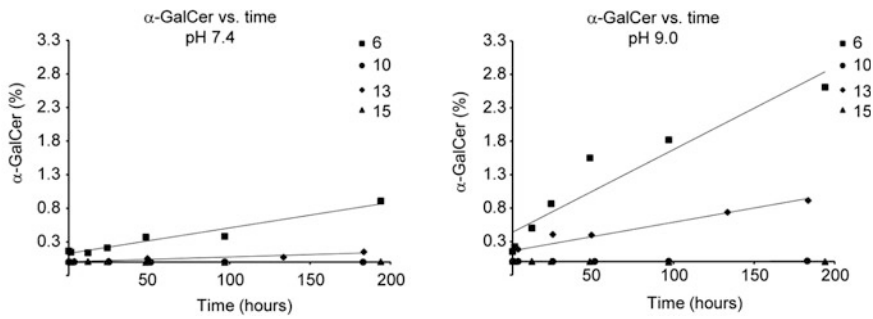


Fig. 19 α-GalCer levels monitored over time by HPLC-MSMS in dilute aqueous solutions of the conjugate vaccines **6**, **10**, **13,** and **15** at pH 7.4 and pH 9.0

4.2 Conjugates Induce Responses in Human Blood

Another important consideration in developing an NKT cell-based vaccine technology is the ability to transfer an effective vaccine response from murine models to humans. Human and mice NKT cells react to the same glycolipid antigens; however, humans have fewer NKT cells than mice. NKT cells represent 4% of the circulating T cell population in mice [36]; however, their abundance in human blood is typically <0.1% [37]. To test whether our conjugate vaccines were applicable in a human setting, we assessed the effect of a conjugate comprising a human-relevant peptide sequence from cytomegalovirus (CMV) pp65 protein [26]. The prevalence of CMV exposure in the local population enabled ready access to blood from donors which had been exposed to CMV. The peptide sequence chosen was NLVPMVATV (NLV) due to its ability to be detected in the peripheral blood mononuclear cells of HLA-A*02^{+} CMV seropositive donors. With the epitope chosen, the conjugate **16** (Fig. 20a) was prepared which made use of the more stable VC-PAB linker in combination with employing CuAAC chemistry to allow the use of a more readily available peptide coupling partner. The peptide also contained the FFRK peptide cleavage sequence designed to aid MHC class I presentation of the NLV epitope.

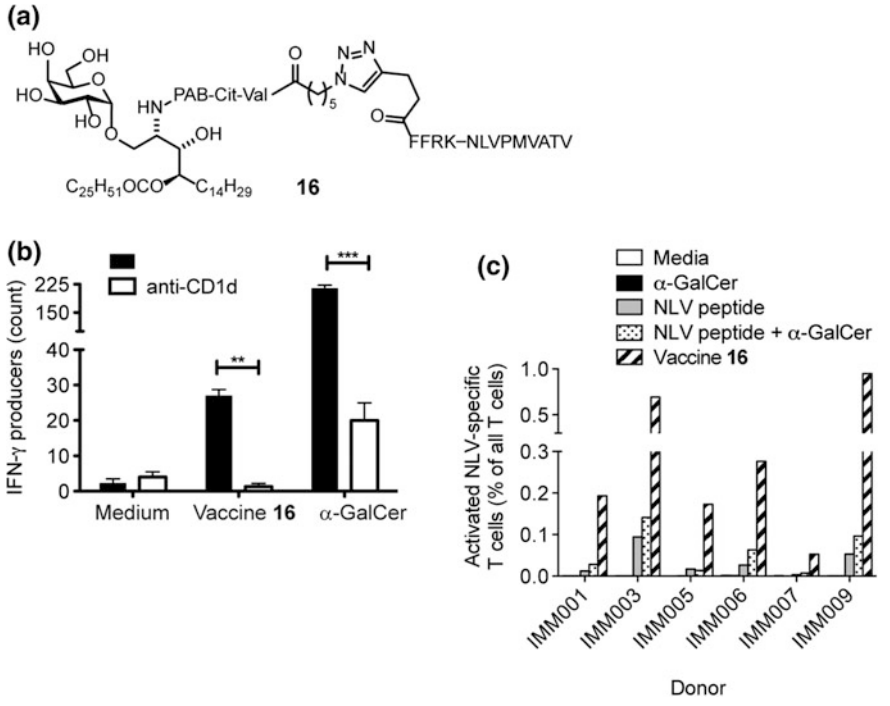


Fig. 20 **a** The chemical structure of the CMV conjugate **16**, **b** Conjugate **16** activates human NKT cells, **c** Conjugate **16** enhances peptide-specific CTL in human blood in vitro. (Reproduced from [26] with permission from the Royal Society of Chemistry)

It was initially very interesting to determine whether compound **16** was able to activate human NKT cells. To do this, **16** was incubated with PBMCs from an HLA-A*02 negative donor in the presence or absence of a CD1d blocking antibody, and the release of interferon-γ (IFN-γ) was monitored (Fig. 20b). HLA-A*02 negative donors were used for this experiment to ensure the IFN-γ was not derived from antigen-specific T cells responding to the NLV peptide. The results from this did indeed indicate that the IFN-γ release was CD1d-dependent which supports our hopes that the conjugate vaccines are active in a human setting. Further assessment of the ability of **16** to promote the proliferation of NLV antigen-specific $CD8^+$ T cells was determined by the upregulation of CD137 which is used as a marker of T cell activation. The enhancement in the percentage of antigen-specific T cells was markedly more pronounced after exposure to the conjugate vaccine **16** as opposed to α-GalCer, the NLV peptide, or co-administration of α-GalCer and NLV peptide (Fig. 20c). Importantly, this proliferation of NLV-specific $CD8^+$ T cells was observed in several donors, which is encouraging when considering the low abundance of NKT cells in humans.

5 Summary and Future Directions

In this work, we showed that treating the NKT cell agonist α-GalCer with acid caused the C26 fatty acid to 'miss-orientate' to form an inactive compound with a reactive amine handle. To stop the facile reformation of α-GalCer in physiological conditions, the amine was capped with a blocking group that was designed to be removed in vivo through enzymatic processes. The chemical design of the capping group also included provision of a chemical reporter group such as a ketone, azide, or alkyne that facilitated bioorthagonal conjugation to suitably *N*-terminally modified peptide antigens. Although both oxime ligation and copper catalyzed cycloaddition methodologies were equally efficient, the cycloaddition protocol was preferred due to the ease of synthesis of the alkyne-modified peptides versus the alkoxyamine-modified peptides. In terms of self-immolative linkers, the valine-citrulline *p*-aminobenzyl group was preferred over the acyloxy group due to increased chemical stability.

It was demonstrated that prodrugs (or proadjuvants) and conjugates were active in vivo but inactive when tested in plate-bound (CD1d monomer) assays. Importantly, NKT cell activity could be recovered in the in vitro experiments when the proteolytic enzyme cathepsin B was added. Upon conjugation with peptide antigen, increased T cell responses in terms of numbers and functional activity (i.e., antigen-specific cellular cytotoxicity) were measured in animal models. Taken together, these data provided good evidence that the conjugate vaccine activity was, at least in part, due to the designed mechanism (i.e., co-delivery of adjuvant and antigen to the same cell). Further studies are required to fully elucidate that the impact conjugation has with regard to altered biodistribution and how this contributes to biological activity. Increased T cell responses due to conjugation also

translated into better activity in animal models for allergy and cancer. Using a synthetic vaccine that induces antigen-specific cellular cytotoxicity to suppress acute allergic inflammation is a highly novel and interesting concept; however, further studies into the scope and limitations of this approach are needed.

Although it was pleasing to observe increased antitumor activity in an aggressive melanoma model, delayed tumor growth is unlikely to translate well into the clinic, so ongoing research is still needed to find therapies that induce long-term regression. There are many considerations such as repeated dosing, combination with other vaccine technologies (such as those that contain TLR agonists), the use of synthetic long peptides, analysis of T cell populations, and consideration of tumor microenvironment-induced immunosuppression. Given the stunning success in clinical trials of monoclonal antibodies (mAbs) such as nivolumab and ipilimumab [38] that block so-called checkpoint mechanisms, cancer vaccine technologies can no longer be considered in isolation. In particular, for metastatic disease cancer, vaccines must now be considered in the context of checkpoint blockade treatments and how they may enhance these. Thankfully, because checkpoint blockade treatments work by releasing T cell responses and vaccines by inducing T cell responses, the approaches are likely to be synergistic. However, much research and clinical evaluation are still required to validate this concept and other treatment combinations. Other situations where vaccination may be appropriate include pre-malignant disease [39]. This setting is attractive because vaccines tend to work better in healthier patients with lower disease burden. Furthermore, due to the potential of checkpoint blockade drugs to cause autoimmunity, these treatments are less suited to pre-malignant conditions. Finally, vaccine therapies may also work well as an adjunct therapy after tumor reduction surgery. An excellent recent review discusses these and related points in detail [40].

References

1. van Rijt LS, Jung S, Kleinjan A, Vos N, Willart M, Duez C, Hoogsteden HC, Lambrecht BN (2005) In vivo depletion of lung CD11c + dendritic cells during allergen challenge abrogates the characteristic features of asthma. J Exp Med 201(6):981–991. doi:10.1084/jem.20042311
2. Silverman E (2012) Can we afford the war on cancer? Biotechnol Healthc 9(4):13–16
3. Jones LH (2015) Recent advances in the molecular design of synthetic vaccines. Nat Chem 7 (12):952–960. doi:10.1038/nchem.2396
4. Reed SG, Orr MT, Fox CB (2013) Key roles of adjuvants in modern vaccines. Nat Med 19 (12):1597–1608. doi:10.1038/nm.3409
5. Zom GG, Khan S, Britten CM, Sommandas V, Camps MG, Loof NM, Budden CF, Meeuwenoord NJ, Filippov DV, van der Marel GA, Overkleeft HS, Melief CJ, Ossendorp F (2014) Efficient induction of antitumor immunity by synthetic toll-like receptor ligand-peptide conjugates. Cancer Immunol Res 2(8):756–764. doi:10.1158/2326-6066.CIR-13-0223
6. Ingale S, Wolfert MA, Gaekwad J, Buskas T, Boons GJ (2007) Robust immune responses elicited by a fully synthetic three-component vaccine. Nat Chem Biol 3(10):663–667. doi:10.1038/nchembio.2007.25
7. Zom GG, Filippov DV, van der Marel GA, Overkleeft HS, Melief CJ, Ossendorp F (2014) Two in one: improving synthetic long peptide vaccines by combining antigen and adjuvant in one molecule. Oncoimmunology 3(7):e947892. doi:10.4161/21624011.2014.947892

8. Wright TH, Brooks AES, Didsbury AJ, MacIntosh JD, Williams GM, Harris PWR, Dunbar PR, Brimble MA (2013) Direct peptide lipidation through thiol-ene coupling enables rapid synthesis and evaluation of self-adjuvanting vaccine candidates (vol 52, pg 10616. Angew Chem Int Edit 52(45):11686. doi:10.1002/anie.201305620
9. Khan S, Bijker MS, Weterings JJ, Tanke HJ, Adema GJ, van Hall T, Drijfhout JW, Melief CJM, Overkleeft HS, van der Marel GA, Filippov DV, van der Burg SH, Ossendorp F (2007) Distinct uptake mechanisms but similar intracellular processing of two different Toll-like receptor ligand-peptide conjugates in dendritic cells. J Biol Chem 282(29):21145–21159. doi:10.1074/jbc.M701705200
10. Vecchi S, Bufali S, Uno T, Wu T, Arcidiacono L, Filippini S, Rigat F, O'Hagan D (2014) Conjugation of a TLR7 agonist and antigen enhances protection in the S. pneumoniae murine infection model. Eur J Pharm Biopharm 87(2):310–317. doi:10.1016/j.ejpb.2014.01.002
11. Jackson DC, Lau YF, Le T, Suhrbier A, Deliyannis G, Cheers C, Smith C, Zeng WG, Brown LE (2004) A totally synthetic vaccine of generic structure that targets Toll-like receptor 2 on dendritic cells and promotes antibody or cytotoxic T cell responses. Proc Natl Acad Sci USA 101(43):15440–15445. doi:10.1073/pnas.0406740101
12. Oh JZ, Kedl RM (2010) The capacity to induce cross-presentation dictates the success of a TLR7 agonist-conjugate vaccine for eliciting cellular immunity. J Immunol 185(8):4602–4608. doi:10.4049/jimmunol.1001892
13. Tighe H, Takabayashi K, Schwartz D, Marsden R, Beck L, Corbeil J, Richman DD, Eiden JJ Jr, Spiegelberg HL, Raz E (2000) Conjugation of protein to immunostimulatory DNA results in a rapid, long-lasting and potent induction of cell-mediated and humoral immunity. Eur J Immunol 30(7):1939–1947. doi:10.1002/1521-4141(200007)30:7<1939:AID-IMMU1939>3.0.CO;2-#
14. Hermans IF, Silk JD, Gileadi U, Salio M, Mathew B, Ritter G, Schmidt R, Harris AL, Old L, Cerundolo V (2003) NKT cells enhance CD4 + and CD8 + T cell responses to soluble antigen in vivo through direct interaction with dendritic cells. J Immunol 171(10):5140–5147
15. Banchet-Cadeddu A, Henon E, Dauchez M, Renault JH, Monneaux F, Haudrechy A (2011) The stimulating adventure of KRN 7000. Org Biomol Chem 9(9):3080–3104. doi:10.1039/c0ob00975j
16. Morita M, Motoki K, Akimoto K, Natori T, Sakai T, Sawa E, Yamaji K, Koezuka Y, Kobayashi E, Fukushima H (1995) Structure-activity relationship of alpha-galactosylceramides against B16-bearing mice. J Med Chem 38(12):2176–2187
17. Borg NA, Wun KS, Kjer-Nielsen L, Wilce MC, Pellicci DG, Koh R, Besra GS, Bharadwaj M, Godfrey DI, McCluskey J, Rossjohn J (2007) CD1d-lipid-antigen recognition by the semi-invariant NKT T-cell receptor. Nature 448(7149):44–49. doi:10.1038/nature05907
18. Hermans IF, Silk JD, Gileadi U, Masri SH, Shepherd D, Farrand KJ, Salio M, Cerundolo V (2007) Dendritic cell function can be modulated through cooperative actions of TLR ligands and invariant NKT cells. J Immunol 178(5):2721–2729
19. Li X, Fujio M, Imamura M, Wu D, Vasan S, Wong CH, Ho DD, Tsuji M (2010) Design of a potent CD1d-binding NKT cell ligand as a vaccine adjuvant. Proc Natl Acad Sci USA 107(29):13010–13015. doi:10.1073/pnas.1006662107
20. Tefit JN, Crabe S, Orlandini B, Nell H, Bendelac A, Deng SL, Savage PB, Teyton L, Serra V (2014) Efficacy of ABX196, a new NKT agonist, in prophylactic human vaccination. Vaccine 32(46):6138–6145. doi:10.1016/j.vaccine.2014.08.070
21. Giaccone G, Punt CJ, Ando Y, Ruijter R, Nishi N, Peters M, von Blomberg BM, Scheper RJ, van der Vliet HJ, van den Eertwegh AJ, Roelvink M, Beijnen J, Zwierzina H, Pinedo HM (2002) A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors. Clin Cancer Res Official J Am Assoc Cancer Res 8(12):3702–3709
22. Liu Y, Goff RD, Zhou D, Mattner J, Sullivan BA, Khurana A, Cantu C 3rd, Ravkov EV, Ibegbu CC, Altman JD, Teyton L, Bendelac A, Savage PB (2006) A modified alpha-galactosyl ceramide for staining and stimulating natural killer T cells. J Immunol Methods 312(1–2):34–39. doi:10.1016/j.jim.2006.02.009

23. Zhou XT, Forestier C, Goff RD, Li C, Teyton L, Bendelac A, Savage PB (2002) Synthesis and NKT cell stimulating properties of fluorophore- and biotin-appended 6″-amino-6″-deoxy-galactosylceramides. Org Lett 4(8):1267–1270
24. Pauwels N, Aspeslagh S, Vanhoenacker G, Sandra K, Yu ED, Zajonc DM, Elewaut D, Linclau B, Van Calenbergh S (2011) Divergent synthetic approach to 6”-modified alpha-GalCer analogues. Org Biomol Chem 9(24):8413–8421. doi:10.1039/c1ob06235b
25. Du W, Kulkarni SS, Gervay-Hague J (2007) Efficient, one-pot syntheses of biologically active alpha-linked glycolipids. Chem Commun 23:2336–2338. doi:10.1039/b702551c
26. Anderson RJ, Tang CW, Daniels NJ, Compton BJ, Hayman CM, Johnston KA, Knight DA, Gasser O, Poyntz HC, Ferguson PM, Larsen DS, Ronchese F, Painter GF, Hermans IF (2014) A self-adjuvanting vaccine induces cytotoxic T lymphocytes that suppress allergy. Nat Chem Biol 10(11):943–949. doi:10.1038/Nchembio.1640
27. Bertozzi CR (2011) A decade of bioorthogonal chemistry. Acc Chem Res 44(9):651–653. doi:10.1021/ar200193f
28. Sun XC, Zeckner DJ, Current WL, Boyer R, McMillian C, Yumibe N, Chen SH (2001) N-Acyloxymethyl carbamate linked prodrugs of pseudomycins are novel antifungal agents. Bioorg Med Chem Lett 11(14):1875–1879. doi:10.1016/S0960-894x(01)00333-X
29. Dinkel C, Moody M, Traynor-Kaplan A, Schultz C (2001) Membrane-Permeant 3-OH-Phosphorylated Phosphoinositide Derivatives. Angew Chem Int Ed Engl 40(16):3004–3008. doi:10.1002/1521-3773(20010817)40:16<3004:AID-ANIE3004>3.0.CO;2-O
30. Ulrich S, Boturyn D, Marra A, Renaudet O, Dumy P (2014) Oxime ligation: a chemoselective click-type reaction for accessing multifunctional biomolecular constructs. Chem-Eur J 20 (1):34–41. doi:10.1002/chem.201302426
31. Wills-Karp M (1999) Immunologic basis of antigen-induced airway hyperresponsiveness. Annu Rev Immunol 17:255–281. doi:10.1146/annurev.immunol.17.1.255
32. Wells JW, Cowled CJ, Giorgini A, Kemeny DM, Noble A (2007) Regulation of allergic airway inflammation by class I-restricted allergen presentation and CD8 T-cell infiltration. J Allergy Clin Immunol 119(1):226–234. doi:10.1016/j.jaci.2006.09.004
33. Perez HL, Cardarelli PM, Deshpande S, Gangwar S, Schroeder GM, Vite GD, Borzilleri RM (2014) Antibody-drug conjugates: current status and future directions. Drug Discov Today 19 (7):869–881. doi:10.1016/j.drudis.2013.11.004
34. Anderson RJ, Compton BJ, Tang CW, Authier-Hall A, Hayman CM, Swinerd GW, Kowalczyk R, Harris P, Brimble MA, Larsen DS, Gasser O, Weinkove R, Hermans IF, Painter GF (2015) NKT cell-dependent glycolipid-peptide vaccines with potent anti-tumour activity. Chem Sci 6(9):5120–5127. doi:10.1039/c4sc03599b
35. Yang M, Yang Y, Chen PR (2016) Transition-metal-catalyzed bioorthogonal cycloaddition reactions. Top Curr Chem 374(1):1–29. doi:10.1007/s41061-015-0001-3
36. Godfrey DI, Hammond KJ, Poulton LD, Smyth MJ, Baxter AG (2000) NKT cells: facts, functions and fallacies. Immunol Today 21(11):573–583
37. Weinkove R, Brooks CR, Carter JM, Hermans IF, Ronchese F (2013) Functional invariant natural killer T-cell and CD1d axis in chronic lymphocytic leukemia: implications for immunotherapy. Haematologica 98(3):376–384. doi:10.3324/haematol.2012.072835
38. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, Segal NH, Ariyan CE, Gordon RA, Reed K, Burke MM, Caldwell A, Kronenberg SA, Agunwamba BU, Zhang X, Lowy I, Inzunza HD, Feely W, Horak CE, Hong Q, Korman AJ, Wigginton JM, Gupta A, Sznol M (2013) Nivolumab plus ipilimumab in advanced melanoma. N Eng J Med 369(2):122–133. doi:10.1056/NEJMoa1302369
39. Beatty PL, Finn OJ (2013) Preventing cancer by targeting abnormally expressed self-antigens: MUC1 vaccines for prevention of epithelial adenocarcinomas. Ann N Y Acad Sci 1284. doi:10.1111/nyas.12108
40. van der Burg SH, Arens R, Ossendorp F, van Hall T, Melief CJ (2016) Vaccines for established cancer: overcoming the challenges posed by immune evasion. Nat Rev Cancer 16 (4):219–233. doi:10.1038/nrc.2016.16