

Engaging Natural Killer T Cells as ‘Universal Helpers’ for Vaccination

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Abstract Conventional vaccine adjuvants enhance peptide-specific T-cell and B-cell responses by modifying peptide stability or uptake or by binding to pattern-recognition receptors on antigen-presenting cells (APCs). This article discusses the application of a distinct mechanism of adjuvant activity: the activation of type I, or invariant, natural killer T (*i*NKT) cells to drive cellular and humoral immune responses. Using a semi-invariant T-cell receptor (TCR), *i*NKT cells recognize glycolipid antigens presented on cluster of differentiation (CD)-1d molecules. When their ligands are presented in concert with peptides, *i*NKT cells can provide T-cell help, ‘licensing’ APCs to augment peptide-specific T-cell and antibody responses. We discuss the potential benefits and limitations of exploiting *i*NKT cells as ‘universal helpers’ to enhance vaccine responses for the treatment and prevention of cancer and infectious diseases.

Key Points

Natural killer T (NKT) cells are a subset of ‘innate-like’ T lymphocytes that respond to glycolipid, rather than peptide, antigens.

NKT cell-activating glycolipids can act as adjuvants, enhancing immune responses against co-administered peptides and proteins.

Vaccine strategies for co-delivering glycolipids and peptide antigens, including nanoparticles and synthetic conjugates, are effective in vivo and are entering clinical development.

1 Harnessing the Immune System Through Vaccination

The contribution of vaccination to human health is considered second only to that provided by the supply of clean drinking water [1]. For decades, vaccines have successfully been used to elicit protective antibodies against a broad range of infectious diseases. However, traditional vaccination strategies have proven less successful for the efficient induction of cytotoxic cluster of differentiation (CD)-8⁺ T-cell responses. As these T cells recognize peptides derived from intracellular antigens, they can respond to antigens that antibodies often cannot reach, including novel intracellular proteins (neoantigens) expressed by transformed or infected cells [2]. Accordingly, considerable effort is being invested in developing adjuvants that help CD8⁺ T-cell-recruiting vaccines to target and eradicate malignant or virus-infected cells [3–7].

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Vaccine adjuvants are broadly defined as substances capable of enhancing and/or shaping antigen-specific immune responses [2]. The addition of an adjuvant to a vaccine can enhance the functionality, magnitude, or breadth of an immune response and enable dose sparing [2]. To date, clinically approved adjuvants fall broadly into two categories: (1) delivery systems that improve antigen stability, release, or cellular uptake, e.g., liposomes, virosomes, or immune-stimulating complexes (ISCOMs), or (2) immunostimulants that engage pattern recognition receptors (PRRs) to drive cytokine and chemokine release by innate immune cells leading to antigen-presenting cell (APC) activation, e.g., Toll-like receptor (TLR) ligands [8, 9]. Adjuvants currently approved for human use include aluminum salt (alum), oil-in-water emulsions (MF59 and AS03), the TLR4 ligand monophosphoryl lipid A (MPL), the TLR2 ligand Pam₃Cys, and the imidazoquinoline resiquimod (R-848), and the alum-adsorbed MPL (AS04) [10–12], all of which can enhance antibody-mediated immune responses. In contrast, the licensed vaccines best able to generate CD8⁺ T-cell-mediated immunity are live attenuated pathogens, such as the yellow fever and varicella zoster virus vaccines [13–15], which also contain intrinsic adjuvants such as unmethylated CpG sequences.

Although few therapeutic cancer vaccines have been approved for clinical use, the success of checkpoint blockade monotherapy has reinvigorated interest in this approach by providing ‘proof of principal’ that T cells can act against solid cancers [16]. While some therapeutic cancer vaccines attempt to induce antibodies against growth factors, e.g., CIMAvax-EGF, most current investigational approaches focus on generating cellular, principally CD8⁺ T-cell, responses targeted directly against malignant cells. For example, the licensed prostate cancer vaccine sipuleucel-T (Provenge®, Dendreon Corporation), which comprises autologous mononuclear cells pulsed with prostatic acid phosphatase (PAP) linked directly to the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) [3], induces modest survival benefits and elicits T-cell responses against PAP [17]. A second prostate cancer vaccine, PROSTVAC® (Bavarian Nordic), which utilizes recombinant poxviruses to express prostate-specific antigen (PSA) along with three co-stimulatory molecules (lymphocyte function-associated antigen [LFA]-3, intercellular adhesion molecule [ICAM]-1, and B7.1), has generated T-cell responses in *in vivo* mouse models and in early phase clinical trials [18, 19]; the results of a phase III study are awaited.

Despite the relative success of viral vectors as vaccines, they continue to be associated with safety and manufacturing difficulties [20]. Thus, an important goal is to develop synthetic adjuvants capable of driving potent CD8⁺ T-cell responses. While some conventional vaccine

adjuvants can help induce CD8⁺ T-cell responses in humans, including proprietary oil-in-water emulsions [21], liposomes [22, 23], and TLR agonists [24], only 3-O-deacetyl-4'-MPL has been licensed for clinical use in the prophylactic human papilloma virus (HPV) vaccine Cervarix® (GlaxoSmithKline).

In this article, we discuss the ability of glycolipid ligands of type I, or invariant, natural killer T (*i*NKT) cells to function as vaccine adjuvants. Rather than just eliciting an innate immune response in which *i*NKT cells and trans-activated natural killer (NK) cells directly lyse cancer cells, these adjuvants instead act via a unique mechanism, recruiting *i*NKT cells to act as ‘universal helper’ T cells to enhance cellular and humoral immune responses against co-administered peptide antigens. We emphasize the mechanistic and functional differences between the adjuvant activity of *i*NKT cell ligands, which act via a third-party mechanism akin to that of conventional CD4⁺ T-cell help, and the activity of conventional adjuvants. Finally, we review methods of exploiting *i*NKT cell ligands for therapeutic vaccination and their preclinical and clinical results to date.

2 Adaptive Immune Responses

Dendritic cells (DCs) are professional APCs that take up microbial products and/or infected or dying cells from peripheral tissues or the circulation and present the acquired material to T cells in the form of antigenic peptide bound to major histocompatibility complex (MHC) molecules. Given the host’s diverse repertoire of T cells, each bearing a uniquely recombined T-cell receptor (TCR), there is a high likelihood of peptide recognition for a given antigen. The ability of DCs to stimulate proliferation and differentiation of peptide-specific T cells into effector cells, which takes several days, is central to the specific and targeted eradication of pathogens and malignant cells by the adaptive immune system.

Exogenous antigens, such as those delivered in the form of a vaccine, can be processed in vacuolar compartments within DCs, with some of the peptides generated then loaded onto MHC class II molecules and presented on the cell surface to CD4⁺ T cells. Pathways involving either vacuolar presentation or proteasomal cleavage in the cytosol have been described for the ‘cross-presentation’ of exogenous antigens via MHC class I molecules to CD8⁺ T cells, which otherwise recognise only endogenous antigens. In both humans and mice, where conventional DCs have been classified into two subtypes based on expression of XCR1, evidence that XCR1⁺ DCs have a heightened capacity for cross-presentation [25] is accumulating, and these cells are therefore likely to be critical in the initiation of vaccine-induced CD8⁺ T-cell responses.

Pathogens or dead cells taken up by DCs not only contain potentially antigenic peptides but also typically contain pathogen-associated or danger-associated molecular patterns (PAMPs and DAMPs). These molecules engage cellular PRRs expressed by DCs, such as TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and cytoplasmic DNA sensors. In response, DCs up-regulate peptide presentation on MHC and express co-stimulatory molecules, enhancing the effector T-cell response against the presented peptide. Many current and proposed adjuvants exploit the activity of PRRs by serving as PAMPs or DAMPs to mimic the effect of an immunogenic infection and enhance T-cell priming by DCs. For example, R-848 activates immune cells via TLR7/8 signaling and is approved for use in topical anti-viral creams [26, 27]. Similarly, co-administration of the TLR4 ligand MPL with alum has been shown to enhance differentiation of activated T cells into cytotoxic CD8⁺ T cells in clinical trials [28, 29].

Once activated, CD8⁺ T cells move into infected or distressed tissues, where they can recognize and eliminate affected cells through recognition of antigenic peptides presented on the MHC class I molecules of target cells. Cytotoxicity is mediated by a variety of possible mechanisms, including the secretion of cytokines, targeted secretion of cytotoxins, such as perforin or granzymes [30], or Fas/Fas ligand (FasL) interactions. Activated CD4⁺ T cells exert their effector functions through interactions with MHC class II⁺ APCs in infected or malignant tissues, releasing cytokines into the local region. Alternatively, it has recently been shown that they too can have direct cytotoxic activity against MHC class II⁺ targets [31].

The expression of MHC class II is generally limited to APCs. This expression profile is fundamental to another important role of CD4⁺ T cells: that of ‘helper’ cells. In this capacity, interactions with CD4⁺ T cells can enhance the function of APCs, such as antibody production by B cells, antimicrobial activity by macrophages, and—importantly for this article—the stimulation of CD8⁺ T cells by DCs.

2.1 Help for CD8⁺ T-Cell Responses

Peptide-specific CD8⁺ T-cell responses can be enhanced by either CD4⁺ T-cell help or strong PRR stimulation. These mechanisms appear to be distinct but can cooperate. In non-inflammatory conditions, CD4⁺ T-cell help is essential for the generation of long-lived functional memory CD8⁺ T cells [32, 33]. In contrast, CD8⁺ T-cell responses can be generated against some viral infections in the absence of CD4⁺ T-cell help through sufficient early type I interferons (IFNs) invoked by PRR stimulation [34]. These systems may cooperate: APCs acquiring antigens in

conjunction with sub-maximal danger signals may seek ‘validation’ from CD4⁺ T cells to become fully activated [35]. Other factors contributing to memory CD8⁺ T-cell responses include TCR avidity, the rate of antigen clearance, and the magnitude of the effector CD8⁺ T-cell burst [36].

Upon recognition of peptides presented by DCs on MHC class II, CD4⁺ helper T cells can ‘license’ DCs to augment CD8⁺ T-cell responses against a co-presented CD8⁺ T-cell epitope [37–39]. Unlike the enhancement of CD8⁺ T-cell responses induced by PRRs, this CD4⁺ T-cell help depends upon an interaction between CD40 on the APC and CD40 ligand (CD154) on the helper T cell [40–43] (Fig. 1). A similar CD40-dependent mechanism allows CD4⁺ T cells to facilitate antibody production by B cells [44, 45].

In the case of DCs, CD4⁺ helper and CD8⁺ cytotoxic T cells must recognize their cognate peptides presented on the same cell [37, 41, 42]. This requirement for dual recognition of two peptides by discrete TCRs on the same APC may have evolved as a mechanism to avoid undesirable autoimmunity. Viral infection models in mice indicate that, while the initial activation of CD4⁺ and CD8⁺ T cells can involve antigen presentation by spatially and temporally separate DC subsets, the cross-presenting XCR1⁺ DC population ultimately provides the critical platform for both CD4⁺ T-cell help [46, 47] and generation of cytotoxic effector CD8⁺ T cells [48].

2.2 Challenges to Providing CD4⁺ T-Cell Help

While many vaccine adjuvants are able to activate PRRs within DCs, the reliable generation of CD4⁺ T-cell help in humans is more problematic. Full-length proteins can be expected to include some CD4⁺ T-cell epitopes that can attract help for CD8⁺ T cells. However, the cross-presentation of intact proteins is generally not efficient enough to generate strong CD8⁺ T-cell responses [49]. The use of peptide vaccines to circumvent this problem is hampered by the high degree of inter-individual MHC polymorphism, which narrows the range of individuals capable of responding to a given binding peptide. Moreover, an individual MHC class II binding site can accept peptides of variable lengths, complicating prediction of class II epitopes [50]. Furthermore, naïve CD4⁺ T cells specific for a given peptide are rare [51], and T-cell receptor diversity declines with age [52, 53], potentially leading to gaps in the T-cell repertoire [54, 55]. Together, this may limit opportunities to engage CD4⁺ T-cell help even if an individual’s MHC class II status is known. While ‘universal’ CD4⁺ T-cell epitopes capable of binding multiple MHC class II molecules have been identified, they have not yet shown unequivocal clinical utility [56, 57].

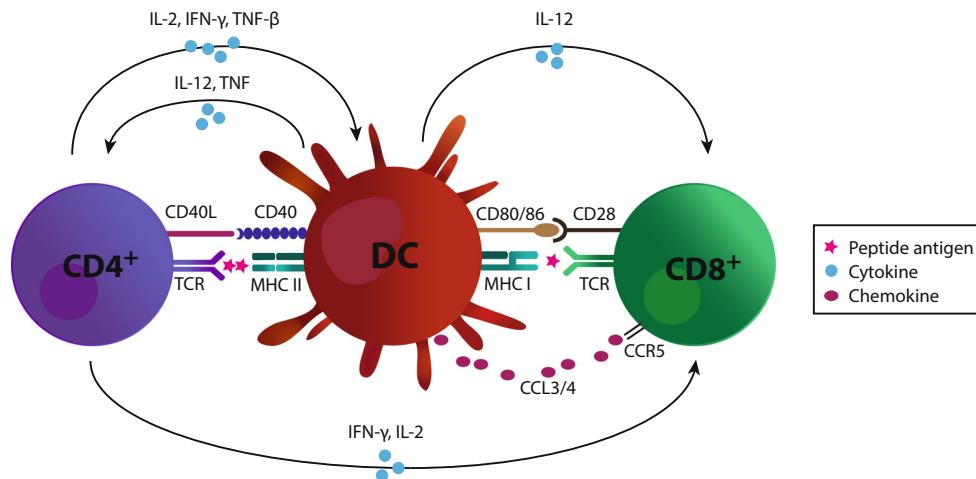


Fig. 1 Classical cluster of differentiation (CD)⁴⁺ T-cell help. CD⁴⁺ helper T cells recognize long peptides presented by dendritic cells on major histocompatibility complex class II molecules leading to the release of pro-inflammatory cytokines, such as interferon- γ , and the CD40/CD40L-dependent ‘licensing’ of dendritic cells that together augment cytotoxic CD⁸⁺ T-cell responses against co-presented CD⁸⁺

T-cell epitopes. A similar CD40-dependent mechanism also allows CD⁴⁺ T cells to facilitate antibody production by B cells (not shown). *CCL* chemokine ligand, *CCR* chemokine receptor, *IFN* interferon, *IL* interleukin, *MHC* major histocompatibility complex, *TCR* T-cell receptor, *TNF* tumor necrosis factor

Synthetic long peptides (SLPs) can be designed to contain both CD⁸⁺ and CD⁴⁺ T-cell epitopes and lead to stronger CD⁸⁺ T-cell responses than both intact proteins and minimal MHC class I-binding short peptides [49, 58]. Unlike short peptides, which can load directly onto MHC class I, SLPs require processing by DCs. This avoids peptide presentation by non-professional APCs lacking co-stimulatory molecules, which may promote tolerance. Peptide-specific T-cell responses and regression of vulvar intraepithelial neoplasia in situ has been reported in women vaccinated with human papilloma virus (HPV)-derived SLPs [59].

Other methods have been employed to overcome the difficulties in providing CD⁴⁺ T-cell help, including the administration of autologous DCs loaded ex vivo with peptides and the administration of agonistic anti-CD40 monoclonal antibodies to license DCs without the requirement for CD⁴⁺ help. However, each of these approaches has practical limitations, and vaccines that can stimulate peptide-specific CD⁸⁺ T-cell responses independently of classical CD⁴⁺ T-cell help are desirable.

2.3 Innate-Like T Cells

In addition to traditional peptide-reactive T cells, some T-cell populations bear TCRs capable of recognizing non-peptide antigens presented by molecules other than classical MHC. These include glycolipid-responsive NKT cells, phosphoantigen-responsive V γ 9V δ 2 T cells, and riboflavin-derivative-responsive mucosal-associated invariant T cells (MAITs). Unlike conventional T cells, which are restricted by highly polymorphic classical MHC molecules, these ‘innate-like’ T cells express stereotyped

TCRs that respond to defined non-peptide ligands presented by non-polymorphic molecules. As a result, defined ligands can activate innate-like T cells across entire populations, irrespective of tissue type. Indeed, the TCRs of CD1d-restricted NKT cells are so highly conserved that mouse CD1d molecules can present glycolipid antigens to human NKT cells and vice versa [60], greatly facilitating pre-clinical studies in animal models.

3 Type I or Invariant Natural Killer T (*i*NKT) Cells

NKT cells have been categorized into two principal subsets: type I and type II. The focus of this article, type I or so-called ‘invariant’, NKT cells, bear a semi-invariant TCR α chain (V α 24-J α 18 in humans, V α 14-J α 18 in mice) with a limited but not invariant TCR β -chain repertoire. These cells recognize self and foreign glycolipids presented by the non-polymorphic MHC class I-like CD1 family member, CD1d, as cognate antigens. The modified marine-sponge-derived glycolipid, α -galactosylceramide (α -GalCer), is the prototypical ligand for *i*NKT cell activation [61]. A second population of NKT cells, type II, are also CD1d-restricted but do not recognize α -GalCer and lack the semi-invariant TCR α -chain characteristic of *i*NKT cells [62, 63]. Their antigen specificity is poorly understood but includes sulfatide [64], lysophosphatidylcholine [65], lysophosphatidylethanolamine [66], phosphatidylglycerol [67, 68], and small aromatic (non-lipid) molecules [69]. Finally, some NKT cells recognize α -GalCer via a range of TCR α and β chains using alternative docking

topologies and have been termed 'atypical' NKT cells [70, 71].

As their name suggests, *i*NKT cells express surface receptors traditionally associated with NK cells, including CD161 in humans and NK1.1 and NKG2D in mice. There is evidence that these NK receptors are functional [72–74]. Most circulating human *i*NKT cells have an effector memory phenotype (CD45RO⁺, CCR7⁻, CD62L⁻) [75], which is linked with the production of cytotoxic molecules [76, 77]. Existing in a semi-activated state [78], *i*NKT cells are able to respond rapidly to self or bacterial glycolipids presented by CD1d or by pathogen-induced cytokines in the absence of direct antigenic stimulation [79].

In humans, *i*NKT cells account for a median 0.05% of T cells in the peripheral blood. However, this frequency varies widely between individuals, ranging from undetectable to over 1% [80–82], with older patients tending to have fewer circulating *i*NKT cells [83]. Although these numbers are considerably lower than in mice [84], *i*NKT cells nevertheless comprise a high proportion of T cells relative to antigen-specific CD4⁺ T cells, which can represent as few as 0.005% of the total CD4⁺ T-cell pool [85]. Moreover, circulating *i*NKT cell frequencies may not accurately reflect systemic numbers [84], with *i*NKT cells being enriched in the human liver, where they account for a median of 0.5% of T cells [86]. In outbred swine, which have a variety of *i*NKT cell frequencies similar to those seen in humans, the frequency of *i*NKT cells in blood correlates poorly with frequency in tissues/nodes and with the immune response to α -GalCer stimulation [87].

In mice, NKT cells are known to contribute to the immune response against a variety of bacterial, fungal, protozoan, and viral pathogens [88], and several microbial glycolipids that bind to CD1d have been described [89–92]. In humans, a reduction in *i*NKT cells in the peripheral blood is correlated with several autoimmune and inflammatory disorders [93–95], as well as susceptibility to graft-versus-host disease (GVHD) [96]. A protective role for *i*NKT cells in surveillance against malignancy has been reported for spontaneous tumors initiated by the chemical carcinogen methylcholanthrene (MCA) [97] and in p53-deficient mice [98]. It has been proposed that *i*NKT-cell activity is modulated by changes in lipid metabolism brought about by endoplasmic reticulum (ER) stress in cancer cells [99], which may account for this protective role. The frequency of *i*NKT cells is reduced in patients with many cancers compared with that in healthy controls, and low *i*NKT cell numbers are associated with poor clinical outcomes in head and neck cancer [100], colorectal cancer [101], and neuroblastoma [102].

Despite bearing nearly identical TCR α -chain sequences, *i*NKT cells are made up of functionally distinct CD4⁺ and CD4⁻ subsets. In humans, both CD4⁺ and CD4⁻ *i*NKT

cells are potent producers of IFN- γ , but CD4⁺ *i*NKT cells also produce T helper (T_H)-2-type cytokines, such as interleukin (IL)-4 and IL-13 [81, 103]. In addition to CD4 status, the balance of T_H1 and T_H2 cytokines released upon antigen recognition by *i*NKT cells can be influenced by (1) the lipid tail composition of CD1d ligands [104], for example, a truncated OCH ligand induces IL-4 production and a T_H2 response [105, 106]; (2) the presence of the T_H1-biasing cytokine IL-12 [107]; (3) the degree and nature of co-stimulation received from the APC [108, 109]; and (4) the presence or absence of an immunosuppressive milieu [110].

Cytokine release by *i*NKT cells influences other effectors, including antigen-specific CD8⁺ T cells, helper CD4⁺ T cells, and NK cells. For example, systemic administration of α -GalCer in mice leads to cytokine-mediated NK cell expansion and increased cytotoxicity [111, 112] that is impaired by blocking both IFN- γ and IL-12 [113]. In addition, *i*NKT cells can exhibit direct cytotoxicity against target cells presenting a potent ligand, such as α -GalCer, on CD1d. As well as expressing granzyme-B and perforin, *i*NKT cells can up-regulate expression of FasL, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), and TNF. There is evidence for involvement of each of these cytotoxicity pathways in the lysis of CD1d-expressing target cells by *i*NKT cells [81, 114–117]. The degree of cytotoxicity exhibited by *i*NKT cells correlates with both target-cell CD1d expression and the potency of the *i*NKT cell agonist [114]. However, direct cytotoxicity towards tumor cells is often hampered by a lack of CD1d expression on these cells [114].

The localization of *i*NKT cells in the spleen enables them to respond to blood-borne antigens and to shape the adaptive immune response by sensitizing lymphoid-resident cross-presenting DCs to PRR signaling [118]. In particular, the marginal zone (MZ) of the spleen contains a wide vascular bed, the marginal sinus, where the arterial blood supply terminates [119]. In mice, *i*NKT cells are widely distributed throughout the spleen during steady-state conditions and rapidly consolidate in the MZ following delivery of self or foreign glycolipid antigens [120]. Here, their activation by DCs is amplified by other bystander cells, including MZ B cells and macrophages [121, 122]. In lymph nodes, glycolipid antigens that enter the subcapsular sinus (SCS) are retained and presented to *i*NKT cells by CD169⁺ macrophages, leading to *i*NKT cell arrest within the SCS, cytokine production, and expansion [123]. Thus, the rapid activation of *i*NKT cells is a natural phenomenon that can shape immune outcomes by modulating the responses of many different cell types, including DCs [124], CD4⁺ and CD8⁺ T cells [125], and B cells [126, 127].

4 Using *i*NKT Ligands as Universal Helper Epitopes

Like CD4⁺ T cells, *i*NKT cells are capable of providing cognate help to APCs. DC-mediated presentation of α -GalCer on CD1d leads to a reciprocal interaction between *i*NKT cells and the DC, in which *i*NKT cells up-regulate CD40L and release cytokines, including GM-CSF [107, 128, 129]. This promotes DC licensing and maturation, leading to up-regulation of co-stimulatory markers, such as CD80 and CD86, and production of IL-12, which further activates *i*NKT cells as well as CD8⁺ T cells [130]. The help provided by *i*NKT cells can enhance CD8⁺ T-cell responses against co-presented peptides [131]. In a manner similar to conventional CD4⁺ T-cell help, *i*NKT-cell help depends critically on peptide and glycolipid presentation by the same APC and on the CD40/CD40L interaction [37, 124, 125]. Thus, through the recruitment of third-party *i*NKT cells, the glycolipid α -GalCer can act as a ‘universal helper’ epitope for vaccination strategies (Fig. 2).

Although the mechanisms mirror those of classical CD4⁺ T-cell help, the nature of the help provided by *i*NKT cells differs qualitatively. While DCs licensed by CD4⁺ T cells recruit CD8⁺ T cells using CCR5 ligands [132], licensing of DCs by *i*NKT cells results in production of the chemokine CCL17, which attracts naïve CD8⁺ T cells expressing the chemokine receptor CCR4 and results in improved migration to CCL17-expressing DCs and increased DC-CD8⁺ T-cell contact time [133]. The relevance of this in terms of function and homing of the

peptide-specific CD8⁺ T cells is yet to be defined. In addition, *i*NKT cells may promote cross-talk between XCR1⁺ DCs and plasmacytoid DCs, which is reportedly important for efficient CD8⁺ memory T-cell induction [134].

By providing a universal form of T-cell help, ligands for *i*NKT cells fulfill the broad definition of an adjuvant: “a substance that enhance[s] the immunogenicity of an antigen” [135]. The adjuvant activity of *i*NKT ligands can be combined with that of conventional adjuvants that activate PRRs. Administration of TLR agonists in vitro strongly enhances the production of IFN- γ in mouse splenocytes pre-treated with α -GalCer [136]. In mice, the proliferation and effector function of antigen-specific CD8⁺ T cells is significantly improved by administration of the TLR4 ligand MPL in conjunction with α -GalCer [137]. Similarly, in human DCs, exposure to a TLR ligand and α -GalCer-activated *i*NKT cells leads to an increase in expression of maturation markers [137]. Notably, an initial α -GalCer-mediated activation of *i*NKT cells can condition multiple DC subsets to respond more effectively to TLR ligands [118], shaping the downstream CD8⁺ T-cell response.

5 Pre-Clinical and Clinical Evidence of *i*NKT Cell Help

While several studies report that systemic administration of IL-12 or α -GalCer activates *i*NKT cells and leads to tumor regression in vivo [138, 139], others report only a brief

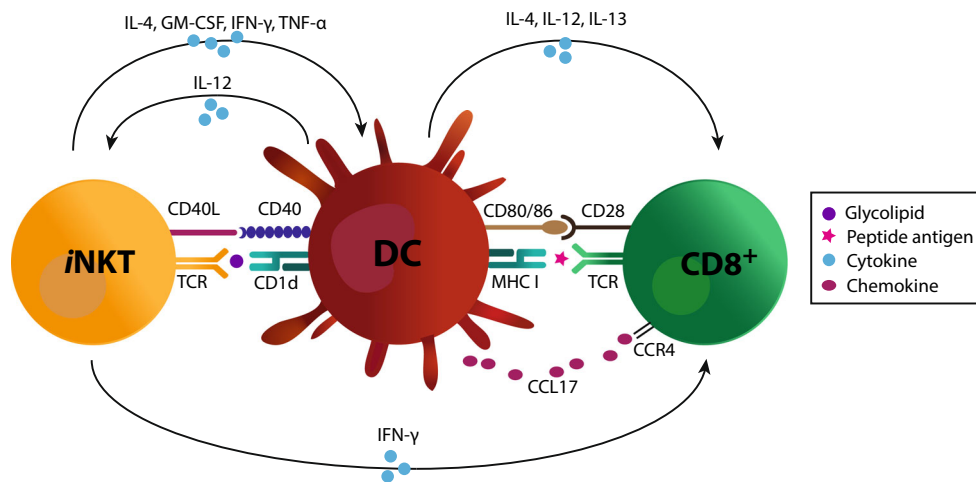


Fig. 2 Invariant natural killer T (*i*NKT) cell help. Similar to cluster of differentiation (CD)-4⁺ T cells, *i*NKT cells can provide ‘help’ to antigen-presenting cells. Presentation of α -GalCer on CD1d by dendritic cells leads to up-regulation of CD40L on *i*NKT cells and release of pro-inflammatory cytokines, such as interferon- γ and granulocyte-macrophage colony-stimulating factor, which promote dendritic cell licensing. Interleukin-12 production further activates *i*NKT cells as well as CD8⁺ T cells. The help provided by *i*NKT cells can enhance CD8⁺ T-cell

responses against co-presented peptides. In a manner similar to that of conventional CD4⁺ T-cell help, *i*NKT-cell help depends critically on peptide and glycolipid presentation by the same antigen-presenting cell and on the CD40/CD40L interaction. CD cluster of differentiation, CCL chemokine ligand, CCR chemokine receptor, GM-CSF granulocyte-macrophage colony-stimulating factor, IFN interferon, IL interleukin, *i*NKT invariant natural killer T cell, MHC major histocompatibility complex, TCR T-cell receptor, TNF tumor necrosis factor

inhibition of tumor growth and a limited impact on overall survival [140, 141]. It is likely that this approach invokes lysis of cancer cells by activated *i*NKT cells or via the downstream activation of NK cells. Moreover, sequential systematic α -GalCer administration leads to T_H2 biasing of *i*NKT cells [142] and, ultimately, a hypo-responsive state similar to anergy that may be associated with the development of an IL-10-secreting NKT cell subset (NKT₁₀) [143, 144]. This outcome may be related to α -GalCer presentation by non-professional APCs.

5.1 Co-Administration

Co-administration of α -GalCer with soluble antigens [124, 125], DNA vaccines [145], or recombinant viruses expressing a malarial antigen [146] significantly enhances peptide-specific CD8⁺ and CD4⁺ T-cell responses as well as antibody-mediated responses. The drawback of this approach is that co-administration does not necessarily ensure uptake of both antigen and adjuvant by the same APC, especially if the individual vaccine components have different pharmacokinetics. Indeed, a study in macaques demonstrated no enhancement of influenza-specific T- or B-cell immunity following intravenous co-administration of α -GalCer with live attenuated influenza virus [147]. The presentation of some of the α -GalCer by non-professional APCs may limit immune responses and provoke *i*NKT cell anergy. To fully exploit *i*NKT cell help and induce robust CD8⁺ T-cell responses, it is critical that the *i*NKT ligand and antigenic peptide are targeted to the same professional APC; this has been the goal of recent approaches [125, 133].

5.2 α -GalCer-Loaded Antigen-Expressing Dendritic Cells (DCs)

Administration of DCs loaded *ex vivo* with α -GalCer promotes prolonged IFN- γ production and prevents *i*NKT cell anergy, resulting in more potent anti-tumor effects [148–152]. By loading DCs with both α -GalCer and peptide, the co-presentation of a peptide CD8⁺ epitope and *i*NKT cell ligand is ensured, improving the activity of the DC vaccine. An *in vitro* study of human monocyte-derived DCs (moDCs) found that pulsing these cells with α -GalCer and a tumor-associated antigen (TAA) induced more robust expansion of TAA-specific CD8⁺ T cells than did non- α -GalCer-pulsed moDCs, suggesting that the α -GalCer adjuvant effect might be applicable to humans [153]. A limitation of this approach in clinical practice is the need for a large number of peripheral blood mononuclear cells (PBMCs) and Good Manufacturing Practice (GMP)-compliant facilities to generate the patient-specific cellular vaccines.

A related approach is to generate artificial APCs, such as α -GalCer-pulsed, CD1d-transfected embryonic kidney cells transfected with messenger RNA (mRNA) encoding the target antigen [154]. This entirely avoids the need to generate patient-specific cellular vaccines, although it does not overcome the need for GMP-compliant cell culture.

The observation that exogenous α -GalCer-pulsed DC vaccines made from CD1d^{-/-} APCs still activate *i*NKT cells *in vivo* [155] suggests that DC vaccines can simply function as a vehicle for the co-delivery of antigen and glycolipid adjuvant to an endogenous APC population. In mice, CD8⁺ T-cell responses could be generated to protein-pulsed CD1d^{-/-} DCs, with the critical endogenous DC subset involved being splenic langerin⁺ CD8 α ⁺ DCs, a subset of cross-presenting XCR1⁺ DCs [155]. If such transfer of antigen and adjuvant to resident APCs occurs in humans, the *ex vivo* generation of DCs may be unnecessary.

5.3 α -GalCer-Pulsed Whole Tumor Cell Vaccines

For the treatment of cancer, vaccines comprising irradiated tumor cells treated with α -GalCer elicit stronger protective immunity than do unpulsed tumor cells *in vivo*. In this model, resident APCs take up α -GalCer-loaded tumor cells and present TAAs alongside α -GalCer, resulting in *i*NKT cell-mediated DC licensing and enhanced CD4⁺ and CD8⁺ T-cell-dependent anti-tumor immunity [156]. An advantage of using autologous whole tumor vaccines is that they provide a broad range of patient-specific TAAs.

In animal studies, vaccines comprising whole tumor cells pulsed with α -GalCer led to effective tumor protection in various malignancies, including myeloma [157], melanoma [156, 158], murine B-cell lymphomas [159, 160], and myeloid leukemias [160, 161]. Not all of these tumors expressed CD1d, emphasizing the role of resident APCs in these responses. Nonetheless, for tumors that express low levels of CD1d, the efficacy of an α -GalCer-pulsed whole tumor vaccine could be enhanced by transfection of the tumor cell with CD1d [158], making the injected cells susceptible to *i*NKT cell-mediated lysis. The enhanced memory responses observed were attributed to tumor cell remnants being taken up and cross-presented on DCs [156, 159]; this promoted DC maturation and IL-12 production, enabling fully active DCs to recruit peptide-specific CD8⁺ and CD4⁺ T cells. In a model of intracranial glioma, the therapeutic impact of administration of irradiated glioma cells pulsed with α -GalCer was most significant when regulatory T cells were first eradicated [162].

The practical use of α -GalCer-loaded tumor cells is complicated by the need to harvest tumor cells for the generation of patient-specific vaccines, the robust precautions necessary to minimize the risk of inoculating a patient with viable tumor cells, the need for GMP-compliant

facilities to make the personalised vaccine, and the potential to induce autoimmunity. This may favor synthetic methods of delivering TAA and *i*NKT cell ligands.

5.4 Microorganisms/Micro-Particles

Nano-particulate delivery systems, such as lipo-particles or microorganisms, offer an alternative to patient-specific cellular vaccines. Co-delivery of a TAA with α -GalCer within cationic liposomes enhanced anti-tumor cytotoxic T-cell responses in vivo [163]. Vaccination with virus-like particles (VLPs) bound to α -GalCer in a composite particle activated splenic *i*NKT cells, generated antigen-specific CD8⁺ T cells, and promoted tumor rejection in vivo [164]. A recent study found that co-delivery of α -GalCer with ovalbumin (OVA) in poly(lactic-*co*-glycolic) acid (PLGA) nanoparticles elicited more powerful antigen-specific CD8⁺ T-cell responses and improved overall survival in a B16.OVA tumor model than did PLGA nanoparticles containing the TLR ligands R848 and poly I:C with the OVA antigen [165].

Exploiting intact microorganisms as a delivery vehicle may carry the additional benefit of delivering a TLR signal alongside *i*NKT ligand and antigenic peptide. Therapeutic vaccination with a *Listeria monocytogenes*-based vaccine expressing a TAA in complex with α -GalCer prevented liver metastases in a model of metastatic breast cancer [166]. This was associated with a significant increase in the percentage of active *i*NKT cells in the spleen as well as an increase in CD8⁺ T-cell responses. Similarly, incorporation of α -GalCer into a recombinant *Mycobacterium bovis* bacillus Calmette–Guèrin (rBCG) strain presenting a simian immunodeficiency viral (SIV) epitope significantly enhanced antigen-specific CD8⁺ T-cell responses following SIV re-challenge in mice [167].

Thus, nano-particulate delivery systems can be successfully employed to co-deliver antigenic peptides and *i*NKT cell ligands to APCs. Microorganism-based delivery vehicles may carry the additional advantage of activating PRRs within APCs. Disadvantages of the latter approach include the possibility of forming neutralizing antibody responses against the microorganism or eliciting off-target immunodominant CD8⁺ T-cell responses against microbial peptides.

5.5 Synthetic *i*NKT-Cell Activating Vaccine Conjugates

Given the difficulty of manufacturing autologous cellular vaccines in humans [168], more efficient and effective strategies to co-deliver *i*NKT cell-activating glycolipids and peptide antigens to the same APC are needed. Recent studies have demonstrated the efficacy of so-called ‘conjugate vaccines’, in which an antigen is conjugated, via an enzymatically cleavable linker, to a glycolipid *i*NKT cell agonist, such as α -GalCer [169] (Fig. 3). Our center has undertaken in vivo studies of synthetic vaccines comprising short peptides chemically conjugated to an α -GalCer ‘pro-drug’ via cleavable linkers. Following uptake by an APC, the conjugated components of the vaccine are cleaved enzymatically within the endosomal compartment. Incorporation of additional amino acids at the N-terminus of the peptide helps to promote proteolytic generation of the respective epitopes within APCs [170]. The antigenic peptide component of the vaccine is then cross-presented on MHC class I molecules to activate CD8⁺ T cells, while the α -GalCer prodrug spontaneously converts to active α -GalCer and is presented on CD1d molecules on the same APC to stimulate *i*NKT cells. In this way, activated *i*NKT cells provide targeted T-cell help to license DCs engaged in

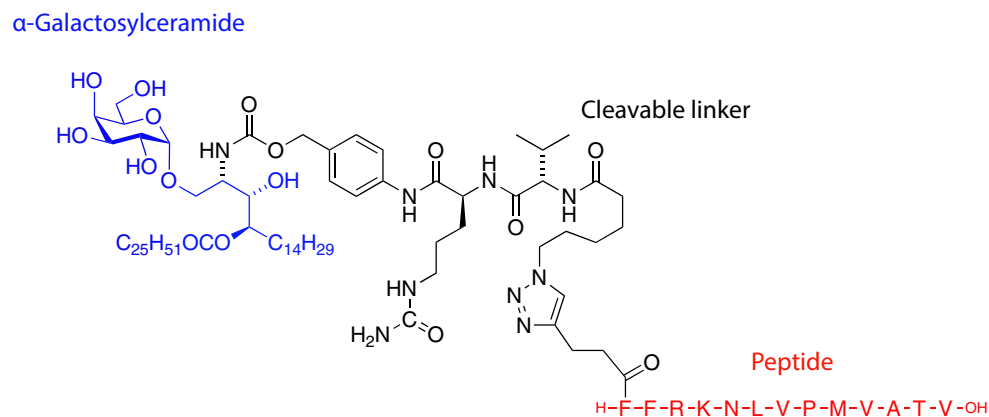


Fig. 3 Structure of a glycolipid–peptide conjugate vaccine. A prodrug form of α -GalCer is linked via a cathepsin-B-cleavable linker to a major histocompatibility complex class I-restricted peptide sequence. Combining both peptide and glycolipid in one structure

ensures uptake by the same antigen-presenting cell, facilitating co-presentation of both vaccine epitopes, which enhances the CD8⁺ T-cell response

stimulating CD8⁺ T-cell responses against the peptide. Indeed, a conjugate incorporating the immunodominant OVA peptide elicited a strong cytotoxic CD8⁺ T-cell response that suppressed OVA-induced allergic airway inflammation by killing allergen-bearing APCs [171]. A similar glycolipid–peptide conjugate vaccine, this time using a cathepsin B-cleavable linker, promoted a robust CD8⁺ T-cell-dependent immune response against established OVA-expressing B16 melanoma tumors in mice [172]. The activity was CD1d dependent and associated with cytotoxicity mediated by activated CD8⁺ T cells. Importantly, in both studies, the conjugate vaccine demonstrated biological activity superior to that of unconjugated admixed peptide and glycolipid, highlighting the importance of targeting antigen and *i*NKT cell ligand to the same APC [172, 173].

The clinical potential of these α -GalCer conjugate vaccines is supported by data demonstrating their capacity to activate both *i*NKT cells and peptide-specific CD8⁺ T cells in human cells *in vitro* [172]. Advantageously, synthetic vaccines based on established antigenic peptides are relatively easy and cost effective to manufacture [172]. This approach may therefore have utility in the treatment of cancer as well as infectious or allergic disease, although further research will be needed to investigate conjugate vaccines containing antigens associated with specific disease states. Many tumor-specific antigens have already been cataloged [174], and high-throughput gene sequencing technologies will enable identification of additional targets, the importance of cancer-specific neoantigens in tumor control being increasingly recognized [175]. To exploit this therapeutically, a highly flexible platform would be needed to manufacture such personalized vaccines.

6 Induction of Humoral Immunity by *i*NKT Cell Adjuvants

While this article focuses primarily on CD8⁺ T-cell responses, α -GalCer also promotes humoral immune responses [176]. Indeed, in the presence of α -GalCer, robust antibody responses can be elicited even in MHC class II knockout animals, which have no potential for classical CD4⁺ T-cell help [176, 177]. A cognate interaction between *i*NKT cells and CD1d on B cells can promote differentiation of *i*NKT cells into CXCR5⁺ PD-1^{hi} follicular helper NKT (NKT_{FH}) cells [178]. Co-administration of α -GalCer in conjunction with a protein vaccine results in rapid germinal center (GC) formation and class-switched antigen-specific antibody production [127, 179, 180] that is dependent on NKT_{FH} cells, CD40L [126], and the expression of CD1d on B cells [181].

Some studies have indicated that, unlike classical T_{FH} cells, NKT_{FH}-cell help does not promote long-term B-cell

memory formation [178, 182, 183]. However, two independent studies have demonstrated that inoculation with a polysaccharide–glycolipid vaccine can promote *i*NKT-dependent long-lived memory B-cell formation. Liposomal nanoparticles displaying a synthetic glycolipid and *Streptococcus pneumoniae* polysaccharide antigens elicited a direct *i*NKT–B-cell interaction *in vivo*, despite the absence of NKT_{FH}-cell differentiation [184]. The *i*NKT cells were first activated by CD1d-expressing APCs before providing cognate help to B cells that promoted prolonged antibody responses with isotype switching, affinity maturation, and long-lasting B-cell memory. In an alternative approach similar to the conjugate vaccines above, direct conjugation of an *S. pneumoniae* capsule protein to α -GalCer stimulated NKT_{FH} cells to promote generation of antigen-specific, long-lived memory B cells and plasmablasts in mice [173], leading to the development of potent anti-polysaccharide immunity and protection from pneumonia following re-challenge. Thus, analogous to the enhancement of cytotoxic CD8⁺ T-cell responses, vaccines that ensure co-delivery of a glycolipid–antigen complex have the potential to stimulate robust and long-lasting humoral immune responses.

Activated *i*NKT cells can also provide help to B cells indirectly by boosting conventional T-cell help: DCs activated through an interaction with *i*NKT cells via CD1d have an enhanced ability to induce classical T_{FH} cells, which in turn interact with B cells [185, 186]. Co-delivery of α -GalCer and protein antigens from a number of infectious pathogens activates this ‘non-cognate’ B-cell help by *i*NKT cells to effect protective humoral responses and long-lasting B-cell memory [120, 187–190].

A clinical trial has demonstrated the adjuvant effect of the potent synthetic *i*NKT-activating glycolipid, ABX196, in humans [191]. A phase I/II dose-escalation study in healthy volunteers showed that co-administration of ABX196 with the poorly immunogenic Hepatitis B (HBs) antigen resulted in activated IFN- γ -producing *i*NKT cells in the peripheral blood as well as protective anti-HBs antibodies in 79% of the patient cohort, suggesting that ABX196 is an effective adjuvant in humans. Transient hepatotoxicity was observed in some patients who received ABX196, although most cases were mild to moderate. This might be ameliorated by using a less potent activating ligand or through changes to the mode of delivery.

7 Challenges to the Clinical Application of *i*NKT Help

A number of barriers to the exploitation of *i*NKT cell help as adjuvants may exist. In mice, strong TCR-mediated activation, coupled with co-stimulation, can induce

profound *i*NKT-cell anergy that renders these cells resistant to subsequent stimulation [143, 192]. However, anergy has most frequently been observed when *i*NKT cell ligands are administered systemically, whereas the targeting of α -GalCer to professional APCs appears to prevent or reduce this phenomenon in mice [193]. Similarly, in humans, repeated systemic injections of a glycolipid–liposomal vaccine have been reported to reduce circulating *i*NKT cell numbers as well as serum IFN- γ [191], whereas a second administration of α -GalCer-pulsed DCs led to an increase in serum IFN- γ compared with the first administration [150].

In some circumstances, *i*NKT cells also have an immunosuppressive role [94, 194]. They can protect against the development of certain autoimmune diseases, such as type I diabetes mellitus [195, 196] and experimental autoimmune encephalitis [197, 198], and can impede CD8⁺ T cell responses to viral proteins in the skin [199]. In the skin, an immunosuppressive population of *i*NKT cells plays a role in a number of pathologies [200], including oncogene-driven skin hyperplasia [201]. *i*NKT cells are also vital for the establishment of tolerance to antigens introduced into an immunologically privileged site [202]. Although questions remain surrounding how *i*NKT cells are programmed to promote different immune responses, it does appear that potent activating ligands, like α -GalCer, generate predominantly pro-inflammatory T_H1-biased responses as opposed to suppression [203, 204]. Nonetheless, a recent report did show that *i*NKT cells stimulated with α -GalCer in vivo can develop characteristics of regulatory cells, including production of the immunomodulatory cytokine IL-10 [144]. Thus, further investigation of this phenomenon is warranted.

The liver is rich in *i*NKT cells, and—in mice—hepatotoxicity has been observed in response to systemically administered α -GalCer, although not when administered in the context of a bacterial delivery system [166]. Hepatotoxicity has not been reported in phase I/II clinical trials of systemically administered α -GalCer or α -GalCer-pulsed APCs [140, 205–207]; however, a phase I clinical trial using a more potent *i*NKT-cell agonist (ABX196) indicated that stronger ligands can lead to hepatotoxicity in humans [191]. This, in conjunction with the potential to stimulate an immunosuppressive *i*NKT cell population detailed above, underscores the importance of careful selection of glycolipid and mode of delivery for vaccine development.

The lower, and highly variable, frequency of *i*NKT cells in humans compared with mice could impact the effectiveness of *i*NKT cell-based immunotherapies. However, even in humans, *i*NKT cells are enriched in tissues such as the liver [86], and the frequency of *i*NKT cells is likely to be far greater than that of a CD4⁺ T cell with a given peptide specificity. Inter-individual variations in *i*NKT frequency may not be critical: α -GalCer was an effective

adjuvant when administered with ultraviolet (UV)-killed swine influenza (SI) virus in outbred swine, which have similarly disparate *i*NKT cell frequencies to humans [208], with no reported correlation between *i*NKT-cell frequencies and the immune response in individual pigs. Finally, the cytokine and antibody responses observed in early clinical trials of *i*NKT cell ligands suggest that humans have sufficient functional *i*NKT cells to trigger immunologic responses [140, 150, 191]. Ultimately, only the results of randomized controlled trials of *i*NKT cell-activating vaccines will establish their efficacy in humans.

8 Concluding Remarks and Perspectives

Optimal cytotoxic CD8⁺ T-cell responses require cognate T-cell help for the APC and are not optimally induced by the adjuvants traditionally used in the clinic. In practice, issues of peptide selection and specificity complicate the reliable induction of CD4⁺ T-cell help. In contrast, *i*NKT cells can be specifically and universally activated by defined glycolipids, irrespective of an individual's tissue type, and provide a universal form of T-cell help that differs both qualitatively and quantitatively from conventional CD4⁺ T-cell help. Critically, the effective use of *i*NKT cell ligands as adjuvants requires co-presentation of peptide and glycolipid antigens on the same APC. A variety of approaches may achieve this, of which the most practical for clinical application may be their synthetic combination, either via liposome/microparticle packaging or chemical conjugation.

Preclinical data suggest that *i*NKT-cell help can be employed to enhance both cellular and humoral immune responses against malignancies and infections. The experience accumulated from clinical trials to date demonstrates that *i*NKT cell agonists are immunologically active in humans and have good safety profiles [140, 149, 209]. Nonetheless, informed selection of *i*NKT cell agonist, target antigen, delivery system, and dosing schedule will be necessary to minimize the risks of toxicity and to maximize the chances of eliciting an effective targeted and sustained immune response.

We conclude that *i*NKT cell ligands can provide a universal form of T-cell help to enhance peptide-specific CD8⁺ T-cell and antibody responses. Synthetic approaches to the co-delivery of peptide antigens and innate-like T-cell ligands to DCs represent a promising strategy to improve vaccine responses against cancer and infectious diseases.

Compliance with Ethical Standards

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