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NKT cell costimulation: experimental progress and therapeutic promise

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Abstract

Invariant natural killer T (*i*NKT) cells are innate lymphocytes with unique specificity for glycolipid antigens and remarkable immunomodulatory properties. The role of costimulatory interactions in *i*NKT cell responses has recently come under scrutiny. Although *i*NKT cells and their prototype glycolipid agonist α-galactosylceramide (α-GalCer) have shown promise in several clinical trials conducted in patients with cancer or viral diseases, current *i*NKT cell-based therapies are far from effective. The concomitant targeting of T cell receptors (TCRs) and costimulatory molecules on *i*NKT cells represents an exciting new opportunity to optimize such therapeutic approaches. Here, we review recent advances in our understanding of *i*NKT cell costimulation and discuss potential treatment modalities based on the responsiveness of *i*NKT cells to disease-tailored glycolipids and select costimulatory ligands.

NKT cells: a brief overview

Definition, subsets and localization

NKT cells constitute a numerically minor but functionally prominent subpopulation of lymphocytes that were initially defined based on their simultaneous expression of NK cell markers (e.g. mouse NK1.1 or DX5 and human CD161) and TCRs [1,2]. Although this definition still holds true for the vast majority of NKT cells, it is no longer considered precise because certain conventional T cell populations such as $CD8^+$ T cells can also express NK cell markers upon activation [3]. In addition, the expression level of NK cell markers by NKT cells varies in accordance to their maturation and activation states [4]. NKT cells are now defined based on the unique restriction of their TCRs by CD1d, a nonpolymorphic major histocompatibility complex (MHC) class I-like glycoprotein that presents glycolipid molecules to NKT cells.

Similar to their conventional counterparts, NKT cells develop in the thymus and express an αβ TCR [2]. However, unlike conventional T cells that are positively selected by cortical thymic epithelial cells and recognize peptide:MHC complexes, the positive selection of NKT cells depends on $CD4^+CD8^+$ thymocytes that express CD1d. The identity of endogenous CD1d-restricted glycolipid antigens participating in the positive selection of NKT cells and possibly contributing to the maintenance of their partially activated

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phenotype remains elusive. Furthermore, whether cells of the NKT lineage undergo negative selection in the thymus to eliminate autoaggressive cells has yet to be established.

CD1d restriction is the cornerstone of NKT cell development and responsiveness. The advent of CD1d tetramer reagents loaded with NKT cell glycolipid ligands has allowed the accurate detection, enumeration and functional characterization of these cells [5,6]. Most CD1d-restricted NKT cells express an invariant TCRα chain exhibiting the characteristic Vα14-Jα18 and Vα24-Jα18 gene rearrangements in mice and humans, respectively [1]. This α chain pairs with a limited set of TCRβ chains (Vβ8.2, Vβ2 or Vβ7 in mice and Vβ11 in humans), thereby defining type I or "invariant" NKT (*i*NKT) cells. *i*NKT cells recognize and respond to the marine sponge-derived glycolipid α-GalCer. A smaller and relatively poorly studied subset of CD1d-restricted NKT cells, which are known as type II or variant NKT (*v*NKT) cells, express a diverse TCRαβ repertoire and fail to recognize α-GalCer [2]. In this review, we focus on *i*NKT cells and their potential for immunotherapy.

Mouse *i*NKT cells are categorized into $CD4^+CD8^-$ and $CD4^-CD8^-$ double negative (DN) subsets. An additional $CD4^-CD8^+$ subset exists in humans [7]. *i*NKT cells occur in low abundance in blood and in various tissues including the thymus, bone marrow, spleen and lymph nodes. Exceptions include the mouse liver and human omentum, which house unusually large numbers of *i*NKT cells [8]. Importantly, *i*NKT cell subsets found in different locations are functionally heterogeneous [7,9–12].

Means and modes of activation

The canonical TCR of *i*NKT cells (*i*TCR) recognizes glycolipid antigens in the context of CD1d, which is expressed by a variety of cell types including professional antigen-presenting cells (pAPCs). Certain microbial glycolipids such as those derived from *Novosphingobium* (formerly *Sphingomonas*) spp., *Ehrlichia* spp. and *Borrelia burgdorferi* induce *i*NKT cell activation in a TCR-dependent fashion [13]. However, α-GalCer is the most widely studied ligand for mouse and human *i*NKT cells. Initially found in an extract of *Agelas mauritanius*, α-GalCer might have originated from microorganisms symbiotic with this marine sponge [14]. Although α-GalCer is not a natural mammalian product, it has been employed extensively as an experimental tool to study *i*NKT cells and has also been used in clinical trials.

Almost all *i*NKT cell antigens have a lipid tail that is buried deep within the hydrophobic pocket of CD1d as well as a sugar head that protrudes out of CD1d and is accessed by the *i*TCRα chain [15]. Unlike conventional TCRs, whose α and β chains are both involved in cognate peptide recognition near the centre of the MHC platform, *i*TCR is rotated clockwise and pushed laterally, thereby allowing only the α chain to make contact with the galactose ring of α-GalCer; the β chain helps stabilize the *i*TCR–CD1d interaction [15]. The relative diversity of the *i*TCRβ chain allows *i*NKT cells to detect distinct structural features of various CD1d-restricted glycolipid antigens [16]. The length of both acyl and phytosphingosine chains of α-GalCer analogs controls the stability of CD1d binding [17]; however, the binding affinity of the *i*TCR for α-Gal-Cer:CD1d complexes is influenced by the length of the phytosphingosine chain [17]. This might at least partially explain the distinct cytokine responses elicited by *i*NKT cells stimulated with glycolipid antigens containing the same sugar head but different lipid tails.

*i*NKT cells can also be activated by bacteria lacking *i*TCR ligands [18]. This indirect activation mode is typically mediated by dendritic cells (DCs) secreting proinflammatory cytokines in response to bacterial components such as Toll-like receptor (TLR) ligands and might also require the engagement of *i*TCR by endogenous glycolipids. Interleukin (IL)-12 and IL-18 can also directly activate *i*NKT cells in a truly TCR-independent manner [19].

Roles in immune responses and regulation

*i*NKT cells are armed with a lethal arsenal of molecular weapons including perforin, granzymes, tumor necrosis factor (TNF)-α, Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL), and are likely to be directly involved in destroying malignant cells and clearing microbial pathogens [20–22]. The immunomodulatory properties of *i*NKT cells are mainly attributable to their ability to transactivate a wide range of downstream effector cells including DCs, macrophages and NK, T and B cells. As such, *i*NKT cells not only participate in innate host defense but also assist in adaptive immune responses [1].

*i*NKT cells contain preformed mRNAs encoding T helper (T_H)1-type cytokines typified by interferon (IFN)-γ as well as T_H2-type cytokines (e.g. IL-4 and IL-13), and quickly secrete enormous quantities of these cytokines following antigenic stimulation [23]. In mice and humans, *i*NKT cell subsets demonstrate distinct cytokine profiles. For instance, although $CD4^+$ *i*NKT cells can secrete both T_H1-and T_H2-type cytokines, DN and $CD8^+$ subsets in humans preferentially produce T_H1-type cytokines [7,9,10].

The pro- versus anti-inflammatory nature of immune responses promoted or modulated by *i*NKT cells depends on the type of cytokines they secrete, which is in turn influenced by the structure and pharmacokinetic properties of *i*NKT cell glycolipid ligands and the cell membrane location of their CD1d-mediated presentation [24], the binding affinity of *i*TCR for these ligands, the costimulatory and danger signals received by *i*NKT cells and the cytokine milieu and anatomical sites where *i*NKT cell subsets are primed.

Costimulatory interactions in *i*NKT cell responses

General concepts and functional outcomes

At least two signals are needed for conventional T cell activation leading to their proliferation, extended survival, cytokine secretion and differentiation into effector cells [25–27]. Signal 1 is antigen-specific and emanates from peptide:MHC–TCR interactions. This signal might trigger a response by effector and memory T cells but is not sufficient for the optimal activation of naïve T cells. In fact, TCR engagement on naïve T cells in the absence of a costimulatory signal (also known as signal 2) might lead to anergy or apoptotic death. Anergic T cells fail to mount productive responses to subsequent encounters with their cognate antigen even under optimal conditions.

Signal 2 is generated when CD28 on T cells is engaged by B7.1 and/or B7.2, which are abundantly expressed by pAPCs [25]. Cell surface molecules other than CD28 can also contribute to T cell costimulation. The ligation of costimulatory molecules, typically of CD28, can result in lipid raft aggregation and immunological synapse optimization [28,29], the upregulated expression of antiapoptotic proteins such as Bcl-xL [30], enhanced IL-2 transcription and mRNA stability [31] and increased glucose uptake and glycolysis [32]. These changes are consistent with these molecules promoting T cell survival, growth and sustained responsiveness and they help T cells meet a suddenly increased demand for energy. By contrast, several cell surface proteins function as negative costimulatory molecules (also termed coinhibitory molecules) to prevent or dampen T cell responses [33]. These coinhibitors are best exemplified by cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), programmed death (PD)-1 and B and T lymphocyte attenuator (BTLA).

Although *i*NKT cells express several costimulatory and coinhibitory molecules shared by conventional T cells, the engagement of these molecules on the two cell types might not yield similar functional outcomes. The pre-activated or “memory-like” phenotype of *i*NKT cells, which is evident even in germ-free animals [34] and human cord blood [35], together with their expression of preformed mRNAs for various cytokines [23] suggests that they

might have a lower threshold for activation compared with naïve T cells. Also importantly, anergy is defined differently in *i*NKT cells than in conventional T cells. In *i*NKT cells, a single dose of α-GalCer induces rapid cytokine secretion followed by a robust proliferative burst and then a homeostatic contraction phase when most *i*NKT cells die; the remaining cells acquire a long-term anergic state [36]. Anergic *i*NKT cells neither proliferate nor release IFN-γ upon re-exposure to α-GalCer, but they partially retain the ability to produce IL-4. Although cytokine secretion by α-GalCer-activated *i*NKT cells is likely to depend on the balanced delivery of costimulatory and coinhibitory signals [37], whether the presence of such signals during the initial glycolipid priming dictates the course and extent of subsequent *i*NKT cell anergy is unknown.

Several members of the immunoglobulin (Ig) superfamily, the TNF receptor (TNFR)/TNF superfamily and the transmembrane (or T cell) Ig and mucin domain (TIM) family costimulate *i*NKT cells either positively or negatively (Table 1 and Figure 1). Understanding how these molecules control the regulatory performance of *i*NKT cells might reveal attractive targets for therapeutic intervention.

Costimulatory molecules of the Ig superfamily

These are type I transmembrane glycoproteins containing the characteristic extracellular Ig variable-like (IgV) domain and a cytoplasmic tail that participates in signal transduction.

CD28/CTLA-4/B7 pathway—At least in mice, the development of *i*NKT cells in the thymus depends heavily on CD28–B7 interactions. CD28 knockout and B7.1/B7.2 double knockout mice exhibit a greater than 50% reduction in the frequency and absolute number of thymic *i*NKT cells, which manifests not only in early postnatal life but also in adult animals [38–40]. A similar numerical decrease in splenic and hepatic *i*NKT cells has also been noted in young $CD28^{-/-}$ and $B7^{-/-}$ mice but not in older animals [38], suggesting that homeostatic mechanisms involved in the maintenance of peripheral *i*NKT cells might be less dependent on the CD28–B7 pathway. This is further supported by the observation that *i*NKT cell-enriched thymocytes exhibit comparable homeostatic proliferation in sublethally irradiated B7-sufficient and -deficient hosts [39].

Signaling through CD28 seems to be dispensable for early lineage commitment and the positive selection of *i*NKT cells but is essential for their subsequent intrathymic maturation as judged by membrane expression levels of NK1.1, CD44, CD69 and CD122, the intracellular expression of the transcription factor T-bet and their capacity to synthesize IFN-γ [38,39]. Interestingly, transgenic over-expression of CD28 or B7 on thymocytes leads to defective rather than augmented *i*NKT cell development [38], indicating a delicate requirement for a physiological level of CD28 signaling during this process.

CD28 costimulation is crucial for the optimal responses of *i*NKT cells to glycolipid antigens. CD28 is constitutively expressed on peripheral *i*NKT cells and is thus readily available to B7.1 and/or B7.2 [41,42]. The disruption of CD28–B7 interactions impairs various effector functions elicited by *i*NKT cells. In combination, blocking monoclonal antibodies (mAbs) to B7.1 and B7.2 partially inhibit IFN-γ and IL-4 responses to α-GalCer *in vitro* and *in vivo* [42]. Similarly, diminished levels of IFN-γ and IL-4 have been detected in cultures of α-GalCer-stimulated $CD28^{-/-}$ splenocytes in comparison with wild-type splenocytes incubated with the same glycolipid antigen [41]. Furthermore, treatment with α-GalCer induces lower serum levels of IFN-γ and IL-4 in $CD28^{-/-}$ mice compared with wild-type animals [41]. Interestingly, the production of IFN-γ by α-GalCer-stimulated splenocytes was inhibited to a greater extent than that of IL-4 when B7.2 but not B7.1 was blocked [43], implicating the CD28–B7.2 costimulation axis in T_H1-skewed responses to α-GalCer.

Splenic and hepatic mononuclear cells obtained from α-GalCer-primed mice exert substantial cytotoxic activities against both NK cell-sensitive and -resistant target cells [20,41]. α-GalCer administration also reduces lung metastasis in a mouse melanoma model [20]. These are examples of *i*NKT cell-mediated T_H1-type responses that are abrogated or greatly reduced in the absence of CD28 signaling [41,42]. Importantly, experimental autoimmune encephalomyelitis (EAE), a widely accepted mouse model of multiple sclerosis, can be suppressed by α-GalCer-pulsed, B7.2-blocked APCs that favor a T_H2-type response [43].

Although the above studies demonstrate a requirement for CD28 signaling in α-GalCer-induced responses, it is not clear to what extent the direct ligation of CD28 on *i*NKT cells contributes to these responses. CD28 costimulation enhances cytokine production by anti-CD3-stimulated splenic NKT cells *in vitro* [44]. Interestingly, however, *in vivo* disruption of the CD28–B7 pathway in α-GalCer-treated mice leads to little or no decrease in intracellular cytokine levels in *i*NKT cells, whereas serum cytokine concentrations can drop substantially [37,45]. Nevertheless, from a therapeutic standpoint, the established role of CD28 costimulation in α-GalCer-mediated downstream effector responses should remain a primary focus.

The CD28 homolog CTLA-4 is a coinhibitory receptor that binds to B7.1 and B7.2 and inhibits T cell activation [25]. Temporal differences in the expression of CD28 and CTLA-4 by conventional T cells orchestrate their timely participation in signal transduction. With the exception of naturally occurring regulatory T (nTreg) cells, resting T cells do not express CTLA-4. The expression of CTLA-4 is, however, induced following T cell activation. CTLA-4 has a high binding avidity for B7 and efficiently outcompetes CD28 in this respect. The consequent low availability of B7 for binding to CD28, together with a T cell-intrinsic negative signal transmitted through CTLA-4, ensures the suppression of excessive and unnecessarily persistent T cell responses.

CTLA-4 is not detectable on the surface or within the cytoplasm of resting NKT cells [41,45,46]. α-GalCer-stimulated *i*NKT cells also fail to express detectable CTLA-4. In light of these observations, it is puzzling that an anti-CTLA-4 mAb inhibited *i*NKT cell proliferation induced by α-GalCer-pulsed DCs [14]. Responder cells in this study were obtained from the spleens of $RAG^{-/-}$Vα14 transgenic mice, which have many *i*NKT cells but no T, B or NK cells. Whether CTLA-4 is expressed by and operates in the various NKT cell subsets located in different tissues under physiological and pathological conditions warrants comprehensive investigation.

Taken together, the CD28–B7 pathway is essential for the normal thymic development of mouse *i*NKT cells but apparently not for their peripheral maintenance. While optimal *i*NKT cell responses to glycolipid antigens depend on CD28–B7 interactions, the contribution, if any, of CTLA-4–B7 interactions to these responses remains unclear.

ICOS/ICOSL pathway—Unlike naïve conventional T cells, resting *i*NKT cells constitutively express inducible costimulator (ICOS) [39,42,47]. Mouse $CD4^+$ *i*NKT cells express higher levels of ICOS in comparison with DN cells [47], suggesting a more critical role for ICOS costimulation in the former subset. ICOS levels are further upregulated on *i*NKT cells upon stimulation with α-GalCer. Unlike conventional T cells, the expression of ICOS in *i*NKT cells is independent of CD28 signaling and vice versa such that *i*NKT cells isolated from $CD28^{-/-}$ mice have normal levels of ICOS, and *i*NKT cells found in $ICOS^{-/-}$ animals show an intact expression of CD28 [42].

The interaction between ICOS and its ligand ICOSL is important for normal *i*NKT cell homeostasis. A lack of ICOS signaling in both C57BL/6 and Balb/c mouse strains results in reduced *i*NKT cell numbers in the spleen and liver [39,47,48]. Signaling through ICOS but not CD28 is required for *i*NKT cell survival in the periphery [47]. Whether ICOS costimulation is needed for the intrathymic development of *i*NKT cells is not completely clear. The absence of either ICOS or ICOSL dramatically decreases *i*NKT cell numbers in the thymus of C57BL/6 mice [39] but not in Balb/c mice [47]. Moreover, C57BL/6 radiation chimeras harboring bone marrow cells from $ICOS^{+/+}$ and $ICOS^{-/-}$ mice exhibit lower percentages of $ICOS^{-/-}$ *i*NKT cells but normal proportions of T cells, NK cells and other major immunocyte populations [48]. These findings also highlight the role of genetic factors in *i*NKT cell costimulation.

Recent studies using stimulatory or blocking mAbs and gene knockout approaches have established the importance of the ICOS–ICOSL pathway in the effector responses of *i*NKT cells. In the presence of a suboptimal dose of anti-CD3 mAb, bead-coated anti-ICOS costimulated IL-4 secretion by hepatic *i*NKT cells [48]. Anti-ICOSL and anti-B7.1/B7.2 mAbs could each partially inhibit IL-4, IL-10, IL-13 and IFN-γ production by α-GalCer-stimulated *i*NKT cells [42]. In combination, these mAbs abolished cytokine production in an additive fashion, suggesting that ICOS and CD28 costimulate *i*NKT cells independently of each other. This was also true for α-GalCer-induced *in vivo* responses, including cytokine release, bystander cytotoxic activity and the prevention of metastasis. In a separate study, interfering with the ICOS–ICOSL pathway decreased the production of IL-4, IL-5, IL-10, IL-13 and IFN-γ by *i*NKT cells, whereas IL-2 production remained intact [47]. Both the $CD4^+$ and DN subsets of *i*NKT cells seem to require ICOS signaling for optimal cytokine production.

Signaling through ICOS might also play a role in *i*NKT cell-induced pathology. In a mouse model of asthma, airway hyperreactivity (AHR) mediated by $CD4^+$ *i*NKT cells depended on ICOS–ICOSL interactions [47]. These interactions also contribute to liver injury associated with concanavalin A (ConA)-induced hepatitis, a commonly used mouse model of human autoimmune hepatitis with documented involvement of IL-4 produced by *i*NKT cells [48].

Collectively, the above observations indicate that intact costimulation through ICOS plays an important role in normal homeostasis and the peripheral survival of *i*NKT cells and might influence their cytokine responses under physiological and pathological conditions.

PD-1/PD-L pathway—PD-1 is a monomeric receptor with a coinhibitory function in T cells [49]. The selective upregulation of PD-1 is associated with the functional exhaustion of virus-specific $CD8^+$ T cells in mice and humans with chronic viral infections [50,51].

PD-1 binds to two separate ligands PD-L1 and PD-L2 and has a higher affinity for the latter [49]. B7.1 was recently discovered to serve as an additional binding partner for PD-L1 in both mouse and human cells [52,53]. *i*NKT cell numbers in the thymus, spleen and liver of $PD\text{-}L1^{-/-}$, $PD\text{-}L2^{-/-}$ and $PD\text{-}L1^{-/-}$ $PD\text{-}L2^{-/-}$ mice are similar to those in wild-type animals [54], suggesting that PD-1–PD-L interactions are dispensable for *i*NKT cell development.

PD-1 expression is low in resting *i*NKT cells, rapidly upregulated upon treatment with a single dose of α-GalCer and maintained throughout the anergic phase of α-GalCer-experienced *i*NKT cells [46,55]. The same treatment can transiently increase the expression of PD-L1 and PD-L2 on APCs [46] and that of PD-L1 on *i*NKT cells[55]. Importantly, α-GalCer-induced anergy is not observed in $PD\text{-}1^{-/-}$ mice and can be prevented in wild-type animals receiving a combination of anti-PD-L1 and anti-PD-L2 mAbs [46]. The blockade of PD-L1 but not PD-L2 reversed α-GalCer-induced anergy *ex vivo* [55]. Although a potential

role for PD-L1–B7.1 interactions in *i*NKT cell anergy has not been ruled out, these observations collectively suggest that the PD-1/PD-L1 axis is a major contributor to this phenomenon. It is noteworthy that the PD-1–PD-L pathway seems to be selectively required for α-GalCer-mediated anergy because interfering with this pathway does not prevent *i*NKT cell anergy induced by heat-inactivated *Escherichia coli* or the *v*NKT cell glycolipid agonist sulfatide [46].

In a mouse melanoma model, the antimetastatic activity of α-GalCer, which was otherwise hampered by a prior injection of the same glycolipid, could be rescued by blocking PD-1–PD-L interactions during the initial glycolipid priming [46]. Coadministration of anti-PD-L mAbs and α-GalCer starting a few days after the melanoma challenge also reduced metastatic burden. This mimics an effective treatment regimen for established cancer.

A recent investigation has uncovered important but opposing roles for PD-L1 and PD-L2 in two mouse models of *i*NKT cell-mediated asthma [54]. PD-L1$^{-/-}$ mice developed mild AHR in response to model allergenic challenges and their *i*NKT cells secreted high concentrations of IFN-γ in response to α-GalCer. By contrast, the development of AHR and airway inflammation was exacerbated in PDL2$^{-/-}$ mice, and *i*NKT cells from these animals produced elevated levels of IL-4 compared with *i*NKT cells obtained from wild-type or PD-L1$^{-/-}$ animals. Consistently, while the adoptive transfer of *i*NKT cells purified from wild-type or PD-L2$^{-/-}$ mice into *i*NKT cell-deficient Jα18$^{-/-}$ mice restored allergen-induced AHR in these animals, Jα18$^{-/-}$ recipients reconstituted with PD-L1$^{-/-}$ *i*NKT cells did not develop severe AHR.

Altogether, the PD-1–PD-L pathway does not seem to be required for the intrathymic development and peripheral homeostasis of *i*NKT cells but mediates α-GalCer-induced anergy. Furthermore, PD-L1 and PD-L2 might differentially regulate *i*NKT cell responses at least in allergic asthma.

BTLA/HVEM pathway—BTLA is a polymorphic molecule exhibiting allelic variation across mouse strains [49]. Herpes virus entry mediator (HVEM), which binds to herpes simplex virus glycoprotein D and mediates viral entry into host cells, was recently identified as the ligand for BTLA [56]. HVEM is a member of the TNFR superfamily, and the BTLA–HVEM interaction constitutes the first example of crosstalk between costimulatory members of the Ig and TNFR superfamilies.

BTLA signaling seems to be dispensable for *i*NKT cell development and maintenance [57]. Thymic, splenic and hepatic *i*NKT cells express BTLA at levels similar to those found in conventional T cells. BTLA$^{-/-}$ mice secrete heightened levels of cytokines including IL-4 and IFN-γ following α-GalCer injection [58], and NKT cells obtained from these animals hyperproliferate and produce more cytokines than wild-type NKT cells in response to α-GalCer. Two independent studies have reported that BTLA inhibits *i*NKT cell-induced pathology associated with ConA hepatitis [57,58]. Compared with wild-type controls, BTLA$^{-/-}$ mice mounted a more vigorous cytokine response and showed higher mortality following ConA injection. In addition, Jα18$^{-/-}$ mice reconstituted with hepatic BTLA$^{-/-}$ *i*NKT cells were moderately more susceptible to ConA-inflicted liver damage than those receiving wild-type *i*NKT cells. Therefore, signaling through BTLA may negatively regulate *i*NKT cell responses in health and disease.

Costimulatory molecules of the TNFR/TNF superfamily—TNFR and TNF family members are type I and type II transmembrane proteins, respectively and participate in various biological processes including T and *i*NKT cell costimulation. Ligation and trimerization of TNFR family members can recruit adapter proteins called TNFR-associated

factors (TRAFs) that activate several signaling cascades, notably those involving nuclear factor (NF)-κB.

CD40L/CD40 pathway—CD40L–CD40 interactions result in bidirectional signaling with important consequences not only for T or *i*NKT cells but also for their engagement partners.

Activated *i*NKT cells express functional CD40L [41,59] and provide “innate help” to B cells [60]. They also engage in productive crosstalk with DCs [61] (Figure 1). In fact, reciprocal *i*NKT cell–DC interaction is a prerequisite for α-GalCer-induced effector responses. Following glycolipid presentation by DCs, *i*NKT cells, predominantly the $CD4^+$ subset in mice, express CD40L, which then cross-links CD40 on DCs [59,61]. This results in the production of IL-12 by DCs, which in turn stimulates IFN-γ secretion by *i*NKT cells. CD40 costimulation and IL-12 are apparently not essential for α-GalCer-mediated IL-4 production. It is therefore not surprising that CD40L–CD40 interactions skew *i*NKT cell responses towards a T_H1 phenotype. In mice, antibody blockade or genetic disruption of this pathway inhibits the production of IFN-γ but not IL-4 in response to α-GalCer [41,61,62]. T_H1-type antimetastatic properties of α-GalCer and its enhancement of cytotoxic activities of splenic and hepatic mononuclear cells are also missing in $CD40^{-/-}$ mice [41]. In addition, the presentation of α-GalCer by APCs pretreated with an agonistic anti-CD40 mAb induced T_H1-biased *i*NKT cell responses *in vitro* and aggravated EAE *in vivo* [43]. Therefore, the CD40–CD40L pathway plays an important role in the generation of T_H1-type *i*NKT cell responses.

OX40/OX40L pathway—OX40 serves as a “second-wave” costimulatory receptor supporting the continued survival, effector function and memory responses of conventional T cells, notably $CD4^+$ T cells. Although a TCR-mediated signal 1 is sufficient for OX40 induction, CD28–B7 interactions augment and sustain the subsequent expression of OX40. The sequential expression/function of CD28 and OX40 in T cells reiterates the concept that effector and memory T cells are reliant on inducible costimulatory molecules such as OX40, and clearly disputes the previously popular view that these cells are completely costimulation-independent.

Steady-state *i*NKT cells homing to certain peripheral organs (e.g. liver and pancreas) express substantial levels of OX40 [63]. $OX40^{-/-}$ mice show diminished serum concentrations of IFN-γ following α-GalCer injection, and the disruption of the OX40–OX40L pathway partially blunts IFN-γ response to α-GalCer *in vitro* [64].

OX40–OX40L interactions might mediate the crosstalk between *i*NKT cells and DCs during antitumor and antiviral immune responses. In a melanoma model, the intratumoral administration of DCs genetically modified to express OX40L recruited *i*NKT cells and slowed tumor growth [64]. The success of this vaccination strategy was dependent on CD1d expressed by DCs and the engagement of OX40 on tumor-infiltrating *i*NKT cells that produce IFN-γ. The protective role of *i*NKT cells in this setting was confirmed by the observation that $OX40L^+$ DCs suppress tumor growth in *i*NKT-deficient mice reconstituted with wild-type but not $OX40^{-/-}$ *i*NKT cells.

The OX40–OX40L pathway also promotes the crosstalk between *i*NKT cells and plasmacytoid DCs (pDCs), which are specialized in type I IFN production [65]. CpG-containing oligodeoxynucleotides, which mimic microbial DNA binding to TLR-9 in pDCs, synergized with α-GalCer to induce IFN-γ production by human *i*NKT cells [66]. This effect required the CD1d presentation of α-GalCer by myeloid DCs, the secretion of IFN-α by CpG-stimulated pDCs and the engagement of OX40 on *i*NKT cells by pDC-expressed OX40L. Reverse signaling through OX40L can also promote pDC function in the context of

*i*NKT–pDC cooperation. Pancreatic and hepatic *i*NKT cells upregulated OX40 following the infection of mice with lymphocytic choriomeningitis virus (LCMV) and controlled early viral replication in these organs [63]. This effect was mediated by IFN-α secreted by pDCs after they interacted with *i*NKT cells via the OX40–OX40L pathway.

Signaling through OX40 has also been implicated in pathogenic T_H2-type responses. In a mouse model of house dust mite (HDM)-induced allergy, eosinophilic airway inflammation, T_H2-type cytokine responses (IL-4, IL-5 and IL-13) and elevated HDM-specific IgE levels were dependent on OX40 costimulation [67]. These responses required OX40 engagement on $CD4^+$ and *i*NKT cells during the sensitization and re-exposure phases, respectively. Allergic inflammation in this model was attenuated in *i*NKT cell- and OX40-deficient mice or by the instillation of a blocking anti-OX40L mAb during intranasal challenge with HDM. Moreover, adoptive transfer of $OX40^{+/+}$ but not $OX40^{-/-}$ *i*NKT cells into HDM-sensitized, *i*NKT-deficient animals restored allergic responses following a subsequent challenge with HDM. Therefore, interfering with OX40–OX40L interactions might be an attractive approach in the treatment of respiratory allergy.

4-1BB/4-1BBL pathway—4-1BB has emerged as a crucial costimulator of survival signaling, predominantly in activated and memory $CD8^+$ T cells [68]. The 4-1BB–4-1BBL pathway seems to be dispensable at early stages of T cell activation when other costimulatory signals are abundant but plays an important part in augmenting TCR signals and sustaining effector functions when other costimulatory signals are limiting. 4-1BB can also be induced in memory $CD8^+$ T cells by IL-2 and IL-15, thus potentially contributing to their survival in the absence of overt antigenic stimulation.

NKT cell development and/or peripheral maintenance depend on 4-1BB because $4\text{-}1BB^{-/-}$ mice have decreased frequencies and absolute numbers of $CD3^+DX5^+$ NKT cells in their thymuses, spleens and livers [69]. It is noteworthy that this cell population contains but is not necessarily equal to CD1d $tetramer^+$ *i*NKT cells. 4-1BB expression is induced on splenic and hepatic *i*NKT cells following TCR stimulation [70]. $4\text{-}1BB^{-/-}$ mice produce less IL-4 soon after injection with anti-CD3 mAb, a response mediated by NKT cells [69]. In addition, an agonistic mAb to 4-1BB could augment α-GalCer-induced cytokine production by *i*NKT cells *in vitro* and *in vivo* [70].

In a lipopolysaccharide model of toxic shock where NKT cells contribute to the pathogenesis of fulminant hepatitis, a blocking anti-4-1BB mAb was protective and curtailed cytokine upregulation in NKT cells [69]. In a mouse model of pulmonary inflammation, an agonistic anti-4-1BB mAb enhanced the detrimental effect of α-GalCer and worsened AHR and inflammatory cell accumulation in an IL-4 receptor-dependent fashion [70]. Interestingly, administering a therapeutic cocktail containing the same mAb and α-GalCer eradicates established mammary and renal carcinomas in mice in an IFN-γ-dependent manner [71]. Therefore, the outcomes of 4-1BB triggering might vary depending on the experimental model used.

GITR/GITRL pathway—Glucocorticoid-induced TNFR family-related gene (GITR) is a relatively new member of the TNFR superfamily with low expression on resting conventional T cells and upregulated levels on activated T cells [72]. The constitutively high expression of GITR is detectable on nTreg cells. GITR ligation promotes the proliferative and cytokine production capacities of effector T cells and regulates the suppressor function of nTreg cells [72].

$GITR^{-/-}$ mice have intact thymic, splenic and hepatic *i*NKT cell compartments [73]. GITR expression on *i*NKT cells is constitutive and further enhanced upon TCR ligation [73,74].

The role of GITR in the regulation of *i*NKT cell responses is controversial because both costimulatory and coinhibitory properties have been reported. Although an agonistic anti-GITR mAb has been shown to augment the cytokine responses of an *i*NKT hybridoma and primary $TCRb^{+}NK1.1^{+}$ cells to α-GalCer and anti-CD3, respectively [74], a later study found that the same mAb inhibits α-GalCer-induced proliferation and cytokine production by CD1d tetramer^{+} NKT cells [73]. The $TCR\beta^{+}NK1.1^{+}$ fraction contains both *i*NKT and *v*NKT cells with potentially opposing characteristics [75], whereas CD1d tetramer-positive cells more accurately represent *i*NKT cells [5,6]. Moreover, $GITR^{-/-}$ mice used in the latter study exhibited boosted cytokine responses to α-GalCer and prolonged survival following glycolipid therapy in a metastatic tumor model in comparison with wild-type animals. Therefore, we favor a coinhibitory role for GITR in the context of *i*NKT cell activation.

The TIM domain family

TIM family members are type I transmembrane glycoproteins with a wide range of ligands and diverse roles in immunity [76]. They contain a single IgV domain and a glycosylated mucin domain that distinguishes them from other costimulatory molecules. Eight predicted *tim* genes exist in the mouse genome, four of which encode functional proteins (TIM-1, TIM-2, TIM-3 and TIM-4), whereas the human TIM gene family has only three members encoding TIM-1, TIM-3 and TIM-4. We will discuss TIM-1 [kidney injury molecule-1 (KIM-1)] because it is relevant to *i*NKT cell immunobiology.

Both TIM-1 and TIM-4 are constitutively expressed by *i*NKT cells [77]. Several ligands have been identified for TIM-1, including TIM-4, TIM-1 itself and phosphatidylserine. In the presence of a TCR signal, several agonistic anti-TIM-1 mAbs have been shown to suppress the IFN-γ secretion capacity of *i*NKT cells while increasing or not altering their IL-4 responses *in vitro*. Coinjection of mice with α-GalCer and an anti-TIM-1 mAb also lowers IFN-γ and increases IL-4, IL-10 and IL-13 secretion in comparison with animals receiving α-GalCer only, which also correlates with the intracellular cytokine content of *i*NKT cells. Although the potential effects of anti-TIM-1 mAbs in the absence of glycolipid treatment have not been investigated, these results implicate TIM-1 signaling in the induction of T_H2-skewed *i*NKT cell responses.

In summary, the thymic ontogeny, peripheral maintenance and effector functions of *i*NKT cells, including their cytokine responses in health and disease, can be regulated by costimulatory members of the Ig superfamily, the TNFR/TNF superfamily and the TIM domain family. However, because the costimulation requirements of *i*NKT and conventional T cells are not identical, one cannot extrapolate the experimental results obtained from conventional T cell costimulation studies to *i*NKT cells.

Clinical implications

KRN7000, the prototype ligand for *i*NKT cells with a unique α-GalCer structure, was initially discovered in a screen for novel anticancer agents [78] and was demonstrated to trigger the antitumor and antimetastatic activities of *i*NKT cells in mouse models [79]. These activities are mainly attributed to the ability of *i*NKT cells to mature DCs and stimulate their IL-12 production, secrete IFN-γ and boost NK cell- and cytotoxic T lymphocyte-mediated cytotoxicity, and counteract or eliminate immunosuppressive or tolerogenic leukocytes within the tumor microenvironment.

The recognition mode of *i*NKT cells is evolutionarily conserved to the extent that human *i*NKT cells recognize mouse CD1d and vice versa [80]. In addition, *i*NKT cells from both species are responsive to α-GalCer. These observations prompted several clinical trials of α-

GalCer or *ex vivo*-expanded *i*NKT cells in patients with cancer or viral diseases [81–89], which are summarized in Table 2.

The goal of *i*NKT cell-based cancer immunotherapy is to expand *i*NKT cells, overcome their functional inadequacies, especially in terms of IFN-γ production, and potentiate T_H1-type responses [79]. An ideal protocol should minimize toxicity and adverse immunological reactions without compromising the clinical response. Costimulatory manipulations might offer novel opportunities to achieve these objectives. Moreover, pAPCs from cancer patients are often dysfunctional [79], and this can be potentially reversed through optimized costimulation.

If a treatment regimen involves systemic α-GalCer administration, coinjection of agonistic mAbs to costimulatory molecules such as CD40, CD28 and 4-1BB might prove effective. This might not only strengthen *i*NKT cells and their downstream effects but also lower the therapeutic dose of α-GalCer or mAbs, thereby avoiding the potential toxicity associated with high-dose monotherapy. Such strategies might also benefit from the concomitant disruption of T_H2-promoting costimulatory pathways or the blockade of coinhibitory molecules. The PD-1–PD-L pathway is of particular interest given its role in $CD8^+$ T cell exhaustion resulting from persistent viral infections [50,51] and chronic antigenic stimulation in cancer [90] in addition to its involvement in α-GalCer-induced *i*NKT cell anergy [46,55]. Combining α-GalCer administration with a blockade of PD-1–PD-L interactions may improve treatment outcomes in chronic viral diseases or when repeated α-GalCer injections might be needed, for instance during disease relapse in cancer.

Direct injection of α-GalCer exerts a plethora of effects through transactivation of various cell types, some of which might not be desirable. Systemic administration of mAbs might target both *i*NKT and non-*i*NKT cells, and injection of some mAbs might even trigger detrimental responses. One classic example was the case of excessive toxicity and a severe systemic inflammatory response experienced by healthy volunteers receiving an agonistic anti-CD28 mAb [91], which had not been observed in animal models. Such adverse complications might be eliminated or minimized if “costimulation-optimized” α-GalCer-coated autologous pAPCs or *i*NKT cells expanded *ex vivo* using such pAPCs are infused back into patients.

*i*NKT cells infiltrate some tumors, and positive associations exist between the presence of *i*NKT cells within certain tumors and long-term survival in patients [92]. When injected intratumorally, DCs modified to express high OX40L levels recruit *i*NKT cells, provoke tumor-specific CTL responses and suppress tumor growth in a mouse model [64]. Therefore, in circumstances when tumors are readily accessible and have not yet metasta-sized, costimulation-optimized pAPCs that simultaneously display α-GalCer might harness intratumoral *i*NKT cell populations for cancer immunotherapy.

Other *i*NKT cell-based immunotherapies can be envisaged and pursued in future investigations. One option is to coadminister costimulatory mAbs and CD1d-transfected tumor cells coated with α-GalCer. Costimulation-optimized pAPCs copulsed with tumor lysate (or tumor-derived peptides) and α-GalCer might also be an attractive vaccine candidate for cancer.

In mouse models where tumor rejection is mediated by *i*NKT cells, hepatic DN cells are reportedly superior to their $CD4^+$ counterparts and thymus-derived *i*NKT cells [12]. It is not currently understood whether these findings mimic anticancer *i*NKT cell responses in humans and whether/how costimulatory requirements might differ across the various *i*NKT subsets.

Recently, there has been increasing interest in synthesizing α-GalCer analogs that polarize immune responses towards either a T_H1 or T_H2 phenotype. One such compound is a C-glycoside analogue of α-GalCer (α-C-GalCer), which is a potent inducer of IFN-γ and IL-12 production in mice [93] and a candidate therapeutic for cancer and infectious diseases. A combination immunotherapy regimen utilizing *i*NKT cell glycolipid agonists, an anti-4-1BB mAb and a mAb against a TRAIL receptor was successful in rejecting mouse mammary and renal carcinomas [71]. Importantly, α-C-GalCer was more potent and less hepatotoxic than α-GalCer in "NKTMab therapy".

T_H2-favoring glycolipids are potential therapeutic options for autoimmune disorders and transplant rejections resulting from pathogenic T_H1-type responses. OCH, a sphingosine-truncated derivative of α-GalCer with T_H2-promoting properties, prevented collagen-induced arthritis in a mouse model [94] as well as insulitis and type 1 diabetes in nonobese diabetic (NOD) mice [95]. When combined with rapamycin, OCH delayed T_H1-mediated graft rejection in two mouse models of cardiac allotrans-plantation [96]. C20:2, an α-GalCer variant featuring a truncated fatty acyl side chain with two unsaturation sites at carbons 11 and 14, can also prevent autoimmune manifestations in NOD mice [97]. Importantly, this glycolipid is presumed to be superior to OCH in deviating human *i*NKT cell responses towards a T_H2 phenotype [97]. Future investigations will address the efficiency of these glycolipids in combination therapies, including those targeting costimulatory molecules.

Concluding remarks and future directions

Recent years have witnessed increasing interest in *i*NKT cells and their immunomodulatory properties. The impressive adjuvanticity of *i*NKT cell ligands has led to many preclinical studies with promising results and perceived potential for benchtop-to-bedside translation. Despite recent advances in the field, many important questions remain regarding *i*NKT cell activation, costimulation and effector functions (Box 1). Addressing these questions will improve our understanding of *i*NKT cell biology and pave the way for effective *i*NKT-based therapies in a wide range of diseases. Several factors need to be considered for the rational design of such therapies. The choice of an α-GalCer derivative will have to be tailored to the specific disease. Combining glycolipid therapy with other agents (e.g. costimulatory or coinhibitory mAbs) or strategies (e.g. cancer chemotherapy) could induce fruitful clinical responses while minimizing the toxicity and adverse effects otherwise caused by each treatment alone. Manipulation of more than one costimulatory/coinhibitory pathway might further optimize these therapies; the timing of each intervention will be critical because the initial priming and effector responses of *i*NKT cells are regulated by first- and second-wave costimulatory molecules, respectively.

When glycolipid-pulsed pAPCs are employed instead of directly injected α-GalCer, the expansion protocol, maturation status and costimulatory capacities of these pAPCs need to be optimized first. For *ex vivo i*NKT cell expansion, it is imperative to use costimulation-optimized APCs and to determine which *i*NKT cell subsets from which tissues yield the most desirable response.

In treating cancer and infectious diseases, robust T_H1-type immune responses induced by *i*NKT-based strategies might be associated with autoimmune sequelae requiring careful monitoring and timely management. Finally, although we depend heavily and inevitably on animal models for testing novel therapeutic approaches, mouse models or even nonhuman primate preclinical studies might not necessarily predict treatment outcomes in humans. Nevertheless, the promise of *i*NKT cell-based therapies, once optimized, is likely to outweigh the potential complications, and exciting scientific and clinical achievements are anticipated from future investigations in this active area of research.

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Box 1. Outstanding questions

- Are $CD4^+$, $CD8^+$ and DN *i*NKT cell subsets regulated by similar or different costimulatory/coinhibitory signals provided by distinct tissue microenvironments?
- How do costimulatory signals affect the activation threshold of *i*NKT cells and lipid raft-aggregation, as well as the stability and sustenance of the *i*NKT:APC immunological synapse [17]?
- What are the costimulatory requirements for *i*NKT cells responding to T_H1- and T_H2-skewing α-GalCer analogs including α-C-GalCer, OCH and C20:2?
- Is costimulation needed for the cytolytic functions of *i*NKT cells? *i*NKT cells are "naturally" cytotoxic, may detect endogenous glycolipids expressed by malignant cells [79] and lyse tumor cells pulsed with α-GalCer *in vitro*. They also express Fcγ receptors [98] and might thus be involved in antibody-dependent cell-mediated cytotoxicity.
- Do "atypical" costimulatory molecules modulate *i*NKT cell responses? Cell surface proteins other than classical costimulatory molecules might regulate *i*NKT cell functions, with a good example being the human NK cell marker CD161 [99]. Whether/how other proteins shared by NK and NKT cells fulfill a similar costimulatory role for *i*NKT cells is not fully understood. Several glycosylphosphatidylinositol-anchored proteins (e.g. mouse Thy-1 and human CD55) costimulate conventional T cells [100,101] and might also regulate *i*NKT cell responses to glycolipid antigens.
- When used in *i*NKT-based combination immunotherapies, how do mAbs to costimulatory molecules affect immunosuppression mediated by nTreg cells expressing the same targeted molecule(s)? This is an important question in light of the reported crosstalk between *i*NKT and nTreg cells [102].
- How are *i*NKT cell functions influenced by promiscuous costimulatory molecules interacting with multiple ligands? For instance, the relative contributions of the PD-L1–PD-1 and PD-L1–B7.1 interactions to *i*NKT cell responses need to be explored. This will be facilitated by using 9G2 and 2H11 mAbs in parallel. The former blocks the interaction of PD-L1 with both PD-1 and B7-1, whereas the latter inhibits the PD-L1–B7-1 interaction only [54].
- Are *v*NKT cells costimulation-dependent? *i*NKT and *v*NKT cells might counter-regulate each other in various diseases [75]. The identification of sulfatide as a *v*NKT cell ligand and its employment in CD1d tetramer reagents detecting *v*NKT cells [103] will help address this question. Mice solely deficient in *v*NKT cells do not yet exist. However, using $CD1d^{-/-}$ mice lacking both *i*NKT and *v*NKT cells in parallel with $J\alpha18^{-/-}$ mice selectively deficient in *i*NKT cells will be a useful approach to this question [79].
- How do costimulatory/coinhibitory signals affect the responsiveness of lipid-reactive human T cells restricted by CD1 molecules other than CD1d?

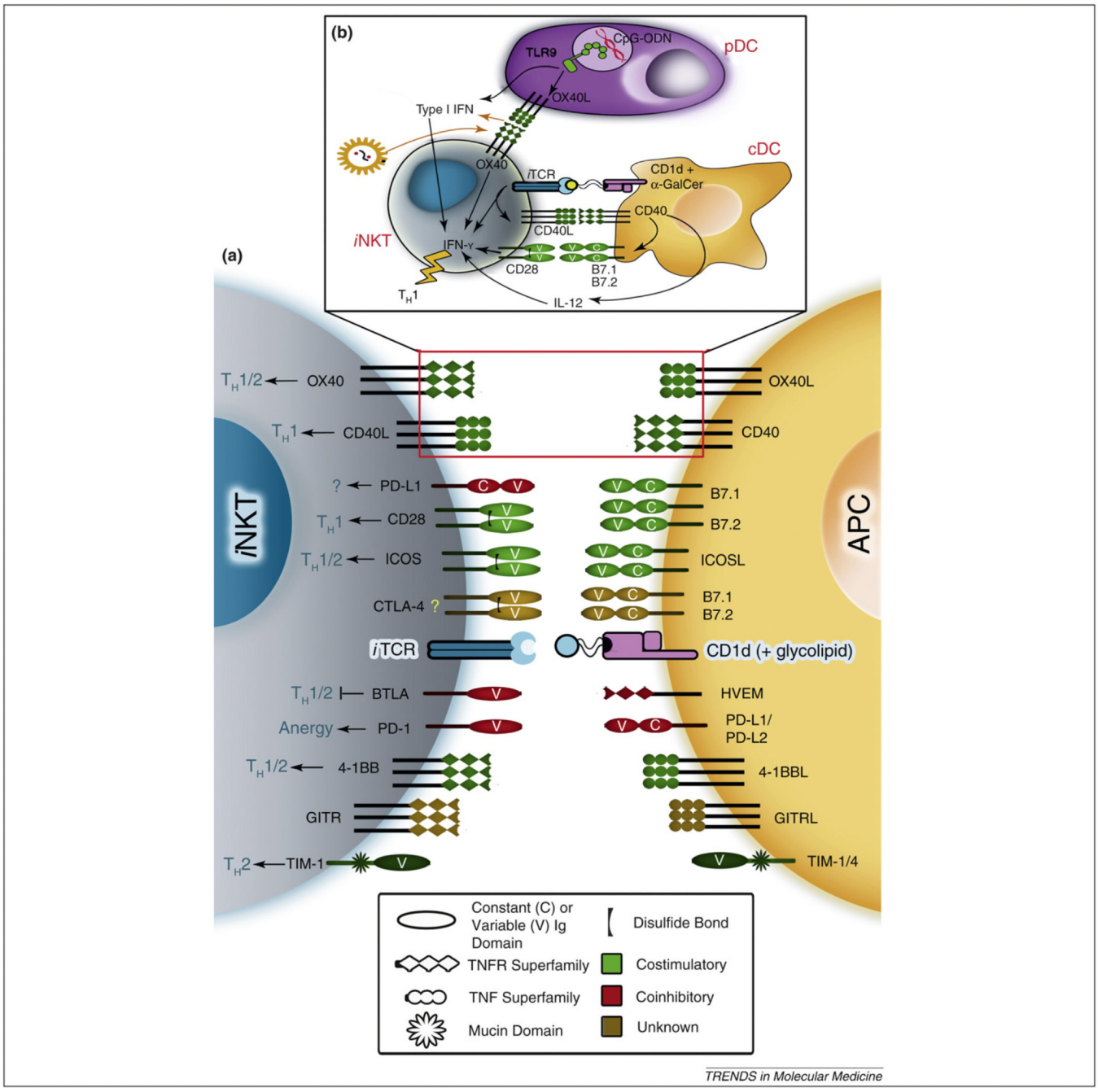

Figure 1.

Costimulatory and coinhibitory interactions between *i*NKT cells and APCs. *i*NKT cells recognize and respond to glycolipid antigens (e.g. α-GalCer) presented in the context of CD1d expressed by APCs. These responses are regulated by several costimulatory (green) and coinhibitory (red) molecules belonging to the Ig superfamily, the TNFR/TNF superfamily or the TIM family (**a**). Cell surface molecules represented in brown have yet to be firmly classified as costimulatory or coinhibitory in *i*NKT cell responses. For instance, the expression and function of CTLA-4 in *i*NKT cells have not been established. Costimulatory interactions affect T_H1- and/or T_H2-type cytokine production by *i*NKT cells. These interactions might also mediate crosstalk between *i*NKT cells and DC subsets (**b**).

TLR-9 triggering by CpG-ODNs stimulates pDCs to produce type I IFN. This effect together with the OX40–OX40L interaction contributed by pDCs synergizes with an *i*TCR signal to promote IFN-γ production by *i*NKT cells [66]. The *i*TCR signal is dependent on α-GalCer presentation by conventional DCs (cDCs) to *i*NKT cells. This signal also leads to CD40L upregulation in *i*NKT cells, which engages with CD40 in cDCs and stimulates their IL-12 secretion and enhanced B7 expression [59,61]. IL-12 and CD28–B7 interactions in turn act on *i*NKT cells and modulate their cytokine responses. Infection with viral pathogens such as LCMV might result in the enhanced expression of OX40 in *i*NKT cells [63]. This represents yet another mechanism by which the OX40–OX40L-mediated interaction between *i*NKT cells and pDCs can stimulate type I IFN secretion by pDCs. Ultimately, this three-way communication results in a T_H1-type *i*NKT cell response.

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Table 1

Expression of costimulatory and coinhibitory molecules by conventional T cells and iNKT cells

Family	Type	Molecule	Cell Surface Expression		Ligand(s)	Ligand Expression	References
			*i*NKT cells	Conventional T cells			
IgSF	**Costimulatory**	CD28	+	+	B7.1 (CD80) B7.2 (CD86)	APCs (↑) Activated conventional T cells (↑) APCs (+,↑)	[25,38,41,43]
		ICOS (CD278)	+, ↑ ($CD4^+$>DN)	↑	ICOSL (LICOS, B7h, B7RP-1, B7-H2, GL50, CD275)	B cells (+) Macrophages (+) DCs (+) Non-lymphoid cells (↑)	[25,39,42,47,48]
	Coinhibitory	CTLA-4 (CD152)	?	↑	B7.1 (CD80) B7.2 (CD86)	APCs (↑) Activated T cells (↑) APCs (+, ↑)	[25,45]
		PD-1 (CD279)	+, ↑	↑	PD-L1 (CD274, B7-H1)	Resting and activated conventional T cells (+, ↑) B cells (+, ↑) NK cells (+, ↑) Macrophages (+, ↑) DCs (+, ↑) Non-hematopoietic cells (+, ↑)	[40,49,54,55]
					PD-L2 (CD273, B7-DC)	Activated DCs (↑) Macrophages (↑)	
		BTLA (CD272)	+	↑	HVEM (CD270)	Most immune cells and in all internal organs (+)	[49,57,58]
TNFSF	**Costimulatory**	CD40L (CD154)	↑ ($CD4^+$>DN)	↑ ($CD4^+$>$CD8^+$)	CD40	B cells (+) DCs (+) Macrophages (+) Non-hematopoietic cells (fibroblast, endothelial, epithelial) (↑)	[59–61]
TNFRSF	**Costimulatory**	OX40 (CD134)	+	↑ ($CD4^+$>$CD8^+$)	OX40L (CD252)	B cells (↑) DCs (↑) Macrophages (↑) Conventional T cells (↑) Endothelial cells (↑)	[63–66]
		4-1BB (CD137)	↑	↑ (including memory $CD8^+$)	4-1BBL (CD137L)	DCs (↑) B cells (↑) Macrophages (↑)	[68–71]
	Coinhibitory	GITR (CD357)	+, ↑	+, ↑	GITRL	B cells (+, ↑) Macrophages (+, ↑) BMDCs (+, ↑) Endothelial cells (+, ↑)	[72–74]
TIM Family	**Costimulatory**	TIM-1 (KIM-1)	+	↑ (T_H2>T_H1)	TIM-1, TIM-4, Ptd-L-Ser	APCs (+, ↑)	[77]

+: Constitutive; ↑: Inducible.

Abbreviations: **BMDC**: bone marrow-derived dendritic cell; **BTLA**: B and T lymphocyte attenuator; **CD**: cluster designation; **cDC**: conventional dendritic cell; **CTLA-4**: cytotoxic T lymphocyte-associated antigen-4; **DN**: double negative; **GITR(L)**: glucocorticoid-induced TNFR family-related (ligand); **HVEM**: herpes virus entry mediator; **ICOS(L)**: inducible costimulator (ligand); **IgSF**: immunoglobulin superfamily; **iNKT cell**: invariant natural killer T cell; **KIM**: kidney injury molecule; **NK**: natural killer; **nTreg cell**: naturally occurring regulatory T cell; **(p)APC**: (professional) antigen-presenting cell; **PD-1**: programmed death-1; **pDC**: plasmacytoid dendritic cell; **Ptd-L-Ser**: phosphatidylserine; **TH**: T helper; **TIM**: transmembrane (or T cell) immunoglobulin and mucin; **TNFSF**: tumor necrosis factor **(TNF)** superfamily; **TNFRSF**: tumor necrosis factor receptor superfamily.

Table 2

A summary of human clinical trials using *i*NKT-based therapeutic approaches in cancer and viral diseases

Tumor/virus type(s)	No. of patients	Therapeutic agent/cells	Adverse events (one or more patients)	Clinical outcomes	References
Various refractory solid tumors	24	KRN7000 (*i.v.*)	Minor[a]	7 cases of stabilization 15 cases of tumor progression	[81]
Various metastatic tumors	12	KRN7000-pulsed, immature MoDCs (*i.v.*)	Transient tumor-associated flares Minor systemic side effects	↓ Serum tumor markers, Tumor necrosis ↓Serum levels of hepatic enzymes	[82]
Advanced or recurrent non-small cell lung cancer	11 enrolled 9 completed	KRN7000-pulsed, immature MoDC-rich APCs (*i.v.*)	Minor[a] ↑ serum K^+, creatinine, or total bilirubin	No changes in 5 cases Progression in 4 cases	[83]
Myeloma, anal squamous cancer, renal cell cancer	6 enrolled 5 completed	KRN7000-pulsed, mature MoDCs (*i.v.*)	Positive rheumatoid factor Transient positive antinuclear antibodies	Not evaluated	[84]
Advanced or recurrent non-small cell lung cancer	6	*i*NKT cells stimulated *ex vivo* with KRN7000-pulsed autologous PBMCs (*i.v.*)	Minor[a] Transient arrhythmia ↑ LDH, γ-GTP, or total bilirubin	6 cases of stabilization 2 cases of progression	[85]
Unresectable or recurrent head and neck cancer	9	Activated autologous KRN7000-pulsed APCs (via nasal submucosa)	Temporary anemia	1 partial tumor regression No changes in 5 cases Progressions in 2 cases	[86]
Head and neck squamous cell carcinoma	8	*In vitro* expanded *i*NKT (intra-arterial infusion) and KRN7000-pulsed APCs (via nasal submucosa)	Minor[a] Pharyngocutaneous fistula	Partial response in 3 cases Stable disease in 4 cases Progressive disease in 1 case	[87]
Hepatitis C virus	40 enrolled; 38 completed	KRN7000 (*i.v.*)	Minor[a]	1 case of ↓ viral RNA	[88]
Hepatitis B virus	27 enrolled; 22 completed	KRN7000 (*i.v.*)	Minor[a] ALT flare Severe chills	Transient ↓ viral DNA (3 cases) Sustained ↓ viral DNA (1 case)	[89]

Abbreviations: **ALT**: alanine aminotransferase; **APCs**: antigen-presenting cells; γ-GTP: γ-glutamic-pyruvic transaminase; ***i*NKT cells**: invariant natural killer T cells; **i.v.**: intravenous; **KRN7000**: α-galactosylceramide; **LDH**: lactate dehydrogenase; **MoDCs**: monocyte-derived dendritic cells; **PBMCs**: peripheral blood mononuclear cells.

[a]Minor includes one or more of the following: fever, lethargy, malaise, headache, vomiting, chills, transient flush, fatigue, myalgia, rhinitis.