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The regulatory role of invariant NKT cells in tumor immunity

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Abstract

Invariant natural killer T (iNKT) cells are a unique population of T lymphocytes, which lie at the interface between the innate and adaptive immune systems, and are important mediators of immune responses and tumor-surveillance. iNKT cells recognize lipid antigens in a CD1d-dependent manner; their subsequent activation results in a rapid and specific downstream response, which enhances both innate and adaptive immunity. The capacity of iNKT cells to modify the immune-microenvironment influences the ability of the host to control tumor growth, making them an important population to be harnessed in the clinic for the development of anti-cancer therapeutics. Indeed, the identification of strong iNKT cell agonists, such as α-galactosylceramide (α-GalCer) and its analogues, has led to the development of synthetic lipids which have shown potential in vaccination and treatment against cancers. In this Masters of Immunology article we discuss these latest findings, and summarise the major discoveries in iNKT cell biology, which have enabled the design of potent strategies for immune-mediated tumor destruction.

Introduction

Invariant Natural Killer T cells (iNKT) cells represent a distinct population of T lymphocytes, which have features of both conventional T cells as well as natural killer (NK) cells [1]. As a result of their unique ability to recognize CD1d-bound endogenous lipid antigens, iNKT cells have a constitutive memory phenotype and are capable of rapidly responding to stimulation, producing a broad range of cytokines. In addition, through direct interactions, in particular via CD1d and CD40L-CD40 signalling, as well as indirect interactions with other immune cells, iNKT cells are capable of maturing dendritic cells (DC) and activating B cells, and thus are crucial in enhancing antigen-specific B- and T-cell responses [2]. The use of iNKT-cell deficient mice and iNKT cell-specific adjuvants has provided compelling evidence demonstrating that iNKT cells play an important role in mounting an antitumor response. Indeed, the importance of iNKT cells in tumor immunosurveillance is further emphasised with the observation that reduced iNKT cell numbers and function have been documented in a large number of cancer patients, including in patients with progressive malignant multiple myeloma [3], prostate cancer [4] and a broad range of other solid malignancies [5]. In this Master of Immunology article, we will discuss

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the role of iNKT cells in enhancing tumor immunity and introduce clinical strategies that are currently being considered to harness iNKT cells in cancer patients to encourage stronger anti-cancer immune responses.

NKT cells: classification and subsets

In contrast to conventional T cells, which recognize protein-derived antigens presented by major histocompatibility complex (MHC) class I and class II molecules, the T-cell receptors (TCR) on NKT cell recognize both exogenous and endogenous lipids presented in the context of the non-polymorphic, MHC class I-like CD1d molecules [6, 7]. NKT cell development requires thymic selection, similarly to that of conventional T cells, which results in the release and expansion of a population of cells with the ability for specific antigen recognition, but also with a range of innate immune functions [2]. Analysis of the phenotype and cytokine profile of NKT cells has led to the identification of two main NKT-cell subsets: invariant NKT (iNKT) cells, otherwise known as type I NKT cells, and diverse NKT cells, which are more commonly called type II NKT cells [8]. iNKT cells express an antigen-specific TCR composed of a semi-invariant α-chain (Vα14-Jα18 in mice and Vα24-Jα18 in humans) paired with a restricted repertoire of β-chains (Vβ2, Vβ7 and Vβ8.2 in mice, or Vβ11 in humans) [9]. Similarly, type II NKT cells are CD1d-restricted, but in contrast to iNKT cells, they express a polyclonal TCR repertoire, and are more comparable to the highly diverse TCRs of conventional $CD4^+$ and $CD8^+$ T cells [10-12]. The importance of antigen presentation by CD1d molecules in NKT-cell activation and development was highlighted by the observation that *Cd1d*$^{-/-}$ mice lack both iNKT cells and type II NKT cells [13-15]. Indeed, to distinguish the roles of the two NKT populations, researchers commonly compare the phenotype of *Cd1d*$^{-/-}$ mice [13-15] with that of *Jα18*$^{-/-}$ mice [16], which lack only iNKT cells. Notably, recent studies have highlighted that *Jα18*$^{-/-}$ mice exhibit additional defects in the T-cell repertoire [17], therefore, the iNKT cell relevance of results obtained using *Jα18*$^{-/-}$ mice should be considered in the context of these findings. The heterogeneity of $V\alpha14^+$ iNKT cells has been further appreciated with the identification of several subsets of iNKT cells with distinct developmental and functional properties [18-21]. Indeed, a distinct Vα50-Jα10 iNKT-cell subset was identified, which although absent in *Cd1d*$^{-/-}$ mice, was found to be present in *Jα18*$^{-/-}$ mice [22]; it is clear that considering these subsets will be critical in order to accurately interpret forthcoming data.

Although a lack of reagents to monitor type II NKT cells has slowed down functional and phenotypic analysis of these cells, access to CD1d tetramers loaded with iNKT-cell agonists has allowed characterisation of the frequency and phenotype of iNKT cells both in mice and humans [23-25]. In mice, iNKT cells comprise approximately 1–3% of the lymphocytes in the circulation and lymphoid organs, and are unusually enriched in the liver where they can comprise up to 30% of resident lymphocytes [26]. Conversely, although found to be enriched in the adipose tissue and omentum [27], the frequency of iNKT cells in the human periphery is lower and more variable than in mice [28].

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iNKT cells recognize a diverse range of antigens

Despite their semi-invariant TCRs, iNKT cells are able to recognize a diverse range of antigens [29]. Structural and functional studies have been fundamental in determining which features of lipid recognition modulate the potency and activation of iNKT cells, and importantly, have been crucial in optimising the design of iNKT-cell agonists suitable for use in the clinic [30-36]. α-galactosylceramide (α-GalCer), derived from the glycosphingolipid extract of the marine sponge *Agelas mauritianus*, was the first lipid identified which potently activates iNKT cells [37]; the α-linked glycan in α-GalCer has since been shown to be a structural motif common to many of the identified α-linked bacterial pathogens, which can directly and potently activate iNKT cells [38-41]. Recently a β-linked lipid, Asperamide B, was identified as the first example of a fungal-derived iNKT-cell agonist [42], although in other models of fungal infection iNKT-cell reactivity was shown to be driven through Dectin-1- and MyD88-mediated upregulation of IL12 by antigen-presenting cells (APC) [43]. In addition to recognising synthetic and microbial-derived antigens, iNKT cells react against $CD1d^+$ APCs in the absence of exogenous antigens, a feature defined as autoreactivity. iNKT-cell autoreactivity underpins the constitutive memory phenotype of iNKT cells and their ability to be activated during a wide variety of immune responses including infections, cancer and autoimmunity [44, 45]. Although complete elucidation of endogenous and exogenous lipids mediating iNKT-cell activation has been challenging due to poor sensitivity of assays, which are often unable to detect low lipid concentrations purified from cellular extracts and pathogens, seminal studies in the last year identified the gut mucosa [46-48] and alternative enzymatic pathways in mammals [49, 50] as potential sources of exogenous and endogenous iNKT-cell lipid agonists. Further investigations are warranted to fully characterise these lipids, which will be highly valuable for understanding the role of iNKT cells in cancers, where endogenous lipids undoubtedly play a key role in triggering the immune response.

iNKT-cell activation and down-stream signalling

Activation of iNKT cells can occur directly or indirectly

i) Direct activation of iNKT cells involves the endocytosis of glycolipid antigens by APCs and their presentation to iNKT cells via CD1d-antigen complexes. In addition to direct iNKT-cell activation by exogenous lipid agonists, we and others have shown that signalling events downstream of Toll-like receptors (TLR) [44, 45, 51], inflammasome components NOD1 and NOD2 [52] and the Formyl Peptide Receptor 2 (FPR2), which recognizes Serum Amyloid A-1 [53], results in the loading of CD1d molecules expressed on APCs with endogenous lipid antigens, and subsequent iNKT-cell activation. Additionally, since a number of tumor cells express CD1d [3, 54-57], it is hypothesised that tumor cells may also present endogenous lipids to iNKT cells directly, although to date the identity of such tumor cell-derived endogenous iNKT-cell agonists remains contentious. Importantly, CD1d-dependent activation of iNKT cells triggers release of IFNγ and interleukin (IL)4, as well as of a diverse range of other cytokines including IL2, IL5, IL6, IL10, IL17, IL21, TNFα, TGFβ and

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granulocyte-macrophage colony stimulating (GM-CSF) [1, 58-60], in addition to chemokines, such as RANTES, Eotaxin, MIP-1α and MIP-1β [61]. IFNγ and IL4 transcription is activated during iNKT-cell thymic development, and preformed IL4 mRNA in the cytoplasm allows for rapid responses upon antigen stimulation [62, 63]. In concert with cytokine release, activation of iNKT cells through TCR stimulation augments the bi-directional cross-talk with DCs in a CD40/CD40L and CD1d-dependent manner; this interaction promotes the maturation, activation and the upregulation of co-stimulatory receptors such as CD80 and CD86 on DCs, as well as the release of IL12. Interestingly, depending on the lipid antigen presented, iNKT cells may also modulate up-regulation of inhibitory molecules (such as PD-L1 and PD-L2) on $CD8\alpha^+$ DCs, which may be the mechanism behind the Th2-polarizing effect of some iNKT-cell agonists [64]. As a result of direct interaction with iNKT cells, DCs can prime antigen-specific $CD4^+$ and $CD8^+$ T cells [65-67]. Licensing by iNKT cells of $CD8\alpha^+$ DCs results in the secretion of the chemokine CCL17, which attracts naive $CD8^+$ T cells expressing the chemokine receptor CCR4 [68]. iNKT cells can also directly provide B-cell help through CD1d expression on B cells [69, 70]. This ability to prime the adaptive immune response indicates that iNKT-cell agonists could be used in the clinic to harness iNKT cells, where they have previously been shown to have adjuvant effects in combination with a number of vaccines [71].

ii) iNKT cells can be activated via soluble factors (indirect NKT-cell activation) released by TLR-activated DCs, such as type I IFN, IL12, and IL18 [44, 45, 51, 72-75], or by co-stimulatory molecules like OX40/OX40L [76].

Structural and functional analyses of the interaction between the iNKT TCRs and CD1d molecules loaded with endogenous and exogenous iNKT-cell agonists are of importance to characterize further how the quality of iNKT-cell activation can be modulated by the binding affinity, concentration, hydrophobicity and stability of glycolipid-CD1d complexes [31, 32, 77, 78]. Indeed, low antigen concentration or weak binding affinity of CD1d/lipid complexes to the iNKT TCRs induce GM-CSF and IL13, whereas a higher antigen concentration or higher binding affinity of CD1d/lipid complexes induce IL4 and IFNγ, along with increased expression of GM-CSF and IL13 [79]. In line with this, the lipid C-glycoside, an analog of α-GalCer, has a weak binding affinity to the iNKT-cell TCR, but as a result of the formation of a stable complex with CD1d, and thus its extended survival *in vivo*, is still able to induce IFNγ production from iNKT cells [80]. These mechanisms demonstrate how antigenic activation of iNKT cells can enhance both cell-mediated and humoral immunity through direct or indirect interaction with other immune cells.

iNKT cells in tumor immunity

The initial observation that α-GalCer injected into mice could protect against tumor progression [81, 82], led to the subsequent discovery that α-GalCer specifically activated iNKT cells in a CD1d-resticted manner [37]. In addition to exerting a protective role in a range of different tumor models when *in vivo* activated with α-GalCer [83] or IL12 [16], iNKT cells also play a critical role during tumor immunosurveillance. Indeed, following

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adoptive transfer of iNKT cells into *Jα18*$^{-/-}$ mice Crowe and colleagues demonstrated their ability to protect mice from methylcholanthrene (MCA)-induced sarcomas via direct interaction of the iNKT TCR with CD1d molecules [84], confirming and extending previous observations by the same group using MCA tumor models [83]. The role of iNKT cells in tumor immunosurveillance has been confirmed in other murine studies including a p53 deficiency model [85] and a TRAMP model [86], all of which showed enhanced tumor growth in iNKT cell-deficient mice (*Jα18*$^{-/-}$ mice or *Cd1d*$^{-/-}$ mice), as compared with wild-type animals. Notably, not all iNKT-cell subsets are equally protective, as rejection of MCA-1 sarcomas and B16F10 melanomas was mediated exclusively by the liver-derived CD4^{-} iNKT-cell subset [87].

Activation of iNKT cells during immunosurveillance can occur either directly, through presentation of self-lipids by CD1d positive tumors, or indirectly, by cross-presentation of tumor lipids by APCs [88]. Evidence for direct presentation stems from the observation that overexpression of CD1d by the B-cell lymphoma NS0 induces cytokine production by iNKT cells and iNKT cell-dependent lysis [89]. Consistent with these findings, in a mouse model of breast cancer metastases, tumor down-regulation of CD1d molecules inhibits iNKT-mediated antitumor immunity and promotes metastatic breast cancer progression [57]. Furthermore, human iNKT cells were found to recognize and kill CD1d^{+} osteosarcoma cells, but not CD1d^{-} osteoblasts, confirming the CD1d restriction of iNKT cell-dependent cytotoxicity [90]. Notably, these studies and others [91, 92] have confirmed iNKT cell-dependent cytotoxicity against CD1d^{+} tumor cell lines without pulsing with α-GalCer, underscoring the notion that the iNKT cell TCR can interact with endogenous antigenic lipids expressed by human and mouse tumor cells, which can lead to direct iNKT-cell activation [90].

CD1d is preferentially expressed in hematopoietic cells [93], especially those of myelomonocytic and B-cell lineages, and accordingly, malignancies originating from such tissues have also been found to be CD1d-positive [3, 54, 55, 89, 94, 95]. Interestingly, CD1d expression has also been found on select solid tumors, such as prostate cancer [4, 56], breast cancer [57], renal cell carcinoma [96] and specific nervous system tumors including malignant glioma [97] and paediatric medulloblastoma [98]; however many other human and murine solid tumors are generally thought to be CD1d-negative, or to down-regulate CD1d molecules. Lack of CD1d expression in tumors results in their lack of recognition by iNKT cells, and has, in some models, been correlated with tumor progression. It remains to be determined, however, whether the lack of detection of CD1d molecules on the surface of such tumors could stem from sub-optimal antibody staining or the local down-regulation of CD1d, and thus whether these tumors are able to present endogenous lipid is not yet defined. Given that CD1d molecules are widely expressed by normal cells, it remains unclear as to whether a different set of unidentified self-iNKT-cell agonists can be presented by CD1d molecules expressed by transformed cells, as compared to normal cells. Furthermore, although it is commonly accepted that endogenous lipids are likely to be responsible for activating iNKT cells in the inflammatory tumor microenvironment, the mechanisms by which iNKT cells are activated during tumour growth remain elusive. Further investigations are warranted to elucidate these findings.

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A hypothesis: The role of the endoplasmic reticulum (ER)-stress response in modulating iNKT-mediated tumor immunity

In non-sterile disease models, pathogen-associated molecular patterns (PAMP) act as TLR agonists, and through the up-regulation of endogenous ligand presentation and the release of soluble factors by APCs, have been shown to enhance the activation of iNKT cells [44, 45, 51]. In light of this, we put forward the hypothesis that a similar mechanism may be involved in iNKT-mediated tumor surveillance. Indeed in recent years a new concept of 'immunogenic cell death' [99] has emerged, which links ER stress with the release of damage-associated molecular patterns (DAMP) during anticancer therapy, and through recognition by pattern recognition receptors (PRR), such as TLR4, the release of DAMPs by dying cancer cells results in the activation of a cancer-specific immune response [100]. Although it remains unclear whether these DAMPs can influence iNKT cell antitumor responses, in support of this idea, we and others have shown that stimulation of TLR4 on APCs can enhance presentation of iNKT-cell agonists and stimulate iNKT-cell activation [44, 45, 101]. In line with this, the Unfolded Protein Response (UPR), which is also triggered by ER stress, increases the activity of the ER lipid transfer protein microsomal triglyceride transfer protein (MTP) [102], which is involved in CD1d loading [103, 104]. Lastly, an additional UPR component, XBP-1, which modulates phospholipid synthesis and is required for ER membrane expansion under ER stress [105], has been shown to positively control hepatic lipogenesis at basal levels [106]. Disruption of XBP-1 led to decreased fatty acids and sterols in primary hepatocytes, possibly by directly trans-activating key genes in this metabolic pathway [106].

As well as tumor-intrinsic ER-stress signalling, which promotes tumor survival and proliferation, the tumor-cell UPR can function in a cell-extrinsic manner, transmitting ER stress to tumor-infiltrating myeloid cells, in a mechanism termed transmissible ER stress (TERS) [107]. Although not yet assessed in the context of cancer, ER stress was correlated with abnormalities in the function and frequency of NKT cells in hepatic steatosis, where it was suggested that ER disruption might lead to dysregulation of iNKT-mediated innate immunity through decreased expression of membrane CD1d resulting in reduced iNKT-cell activation [108]. While in this model ER stress had a negative effect on iNKT-cell activation, in light of the reported effects of ER stress on lipid metabolism and CD1d loading discussed above, further experimentation needs to be performed to dissect whether changes in lipid metabolism due to ER stress in cancer cells may modulate iNKT-cell activity.

NKT cell-mediated adjuvant effects on innate and adaptive immunity against cancer in mice

The ability of iNKT cells to activate antitumor immune responses can be jump started by using exogenous iNKT-cells agonists, such as the prototypic ligand α-GalCer [109-112]. Injection of α-GalCer was found to inhibit tumor metastases and increase survival in a range of murine cancer models, including models of B16 tumor challenge [109], spontaneous sarcomas in $p53^{-/-}$ mice [113] and the colon carcinoma model C26GM [114]. In line with this, injection of α-GalCer-pulsed DCs [115], or intravenous administration of either live or irradiated B16 tumor cells loaded with α-GalCer [116] was shown to elicit an innate iNKT

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and NK cell response that rejects the tumor. The α-GalCer-mediated antitumor activity of iNKT cells has since been shown to be dependent on IFNγ production and NK cells [110, 117, 118], dendritic cell maturation, activation and IL12 release, and ultimately the activation of $CD8^+$ cytotoxic T cells, $CD4^+$ Th1 cells, and gamma-delta (γδ) T cells that further target and kill tumor cells [65, 116, 119]. Indeed, administration of α-GalCer into mice injected with a T-cell lymphoma enhanced the generation of tumor-specific cytotoxic T cells in an IFNγ- and NK-cell-dependent manner [120]. This pathway was further emphasised in murine models of lung and liver metastasis, where the anti-metastatic activity of α-GalCer was dependent on IL12- and IL18-mediated enhancement of IFNγ production by iNKT and NK cells [118].

Upon activation, both murine and human iNKT cells can exhibit potent cytotoxic functions to promote the killing of tumor cells, such as acute myeloid leukaemia, through the expression of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) [121]. This observation was also confirmed with iNKT cells from patients with malignant melanoma, whereby upon α-GalCer/DC activation, the patient-derived iNKT cells displayed potent perforin-dependent cytotoxic activity against a range of tumor cell lines [122]. Interestingly, the transfer of perforin-deficient iNKT cells into $Ja18^{-/-}$ mice with MCA-induced tumors restored tumor resistance, suggesting that in this model direct perforin-dependent tumor lysis by iNKT cells is not critical [84]. Taken together, these observations imply that both direct and indirect mechanisms of iNKT-cells activation play a key critical role in iNKT cell-mediated tumor immunosurveillance [88, 116].

Studies aimed at enhancing iNKT cell-mediated antitumor immunity have shown that the use of soluble α-GalCer leads to potent stimulation of iNKT-cell subsets and may result in iNKT-cell over-activation and anergy [123, 124]. Given these considerations, the search for efficient iNKT agonists with functional differences compared with α-GalCer is an ongoing goal in the field, which attracts the work of many laboratories. Indeed, in recent years, many α-GalCer analogs have been formulated that exhibit different properties, including optimised cytokine induction profiles, which are aimed at targeting specific subsets of iNKT cells in a number of different clinical settings [125-133].

Harnessing iNKT cells to optimize vaccination strategies in cancer patients

Activity of iNKT cells in cancer patients

A large number of pre-clinical and clinical trials have been performed to investigate whether activation of iNKT cells could be a therapeutically beneficial approach in human patients suffering from cancer and other infectious diseases. Reduced iNKT-cell frequency and function has been observed in patients with haematologic cancers [3, 134] and a range of solid tumors [4, 135], as compared with that of healthy volunteers, independent of tumor type and tumor load. In line with these observations, reduced iNKT-cell frequency was shown to correlate with poor overall survival in acute myeloid leukaemia [136], and head and neck squamous cell carcinoma [137], while increased numbers of intra-tumor or circulating iNKT cells have been associated with improved prognosis in colon cancer, prostate cancer, haematologic malignancies, and neuroblastoma [138-140]. Whether immune-cell subsets found in peripheral blood are accurate representative of systemic

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cancer immunity in humans remains to be established in all cancer models [141]; relative NKT-cell deficiencies have, however, also been observed locally in solid tumors and the surrounding tissues, such as in neuroblastoma [142] and colorectal cancer [27]. Interestingly, other investigators have reported elevated iNKT-cell frequency in some tumors [143, 144]; and increased iNKT-cell frequency in the microenvironment of colorectal cancers is thought to be a positive prognostic indicator [145, 146]. The high variability in iNKT-cell frequencies in humans, in addition to the defective numbers shown in cancer and other diseases, reduces the effectiveness of targeting iNKT cells in these individuals. Indeed, studies have reported that NKT cell-based treatments may only be beneficial for patients with high iNKT-cell frequency [147]. To overcome these limitations, universal efforts have been directed at optimising the development of synthetic iNKT-cell agonists to enhance iNKT-cell activation and antitumor function.

iNKT cell-based cancer immunotherapy

Three main iNKT cells-directed therapeutics have been exploited thus far; these include, but are not limited to: administration of iNKT cell-activating ligands (all human studies described to date have used α-GalCer), administration of APCs pulsed with α-GalCer, transfer of *ex vivo*-expanded and/or activated iNKT cells, and finally a combination of these methods.

1. Intravenous injection of α-GalCer—α-GalCer remains the best-characterised iNKT agonist in tumor immunity to date. Although promising data utilising this agonist have been generated in murine models and *in vitro*, the fundamental question remains whether iNKT-cell activation by select agonists is relevant in the clinic. The first clinical study of α-GalCer used repeated intravenous (IV) injection of α-GalCer at varying doses in patients with solid tumors [148]. No dose-limiting toxicity was observed, suggesting that activation of iNKT cells through IV injection of α-GalCer is a safe, well-tolerated treatment in humans. Although iNKT cell numbers appeared to decrease in the periphery, likely resulting from down-regulation of the TCR following iNKT-cell activation [149], Giaccone and colleagues observed elevated serum levels of iNKT cell-associated cytokines, including TNFα and GM-CSF [148], and disease stabilisation for a median of 123 days in 7 of 24 patients. Similar to murine studies in which injection of soluble, but not cell-associated α-GalCer leads to iNKT-cell anergy [123] in a PD-1/PDL-1-dependent manner [150], follow up studies in humans identified α-GalCer-induced iNKT-cell anergy using this administration method [151].

2. Adoptive transfer of α-GalCer-pulsed APCs—Studies with murine tumor models demonstrated that co-injection of α-GalCer and tumor antigens [65], or alternatively administration of α-GalCer-pulsed DCs [152], induced prolonged cytokine responses as compared with injection of soluble α-GalCer. Although the reasoning behind the differing immune responses is unclear, it has been hypothesised that the type of APC and method of administration could play an important role. Indeed, whereas IV injection of pulsed DCs induced a strong cytokine response, α-GalCer-pulsed DCs injected subcutaneously in mice did not stimulate a particularly effective iNKT-cell response [152]. In addition, DCs were found to stimulate a stronger iNKT-cell response in comparison to B cells [153]. A large

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number of clinical trials have since utilised *ex-vivo*-generated, or isolated APCs pulsed with α-GalCer, which has thus far been shown to be safe and well tolerated.

The first phase-I trial reported utilised IV administration of α-GalCer-pulsed monocyte-derived DCs, which were given at two weekly intervals to patients with metastatic tumors [154]. Although activation of iNKT cells increased serum levels of cytokines including IFNγ and IL12 and the trans-activation of both T and NK cells, only 2 of the 12 patients enrolled exhibited a decrease in serum tumor markers, indicating minimal efficacy of this treatment [154]. Two later studies using α-GalCer-pulsed, monocyte-derived DCs were published; the first, using weekly IV injections of IL2-cultured DCs in patients with advanced or recurrent non-small-cell lung cancer, demonstrated an expansion of iNKT-cell frequency and elevated IFNγ levels by PCR analysis [151]. IFNγ ceased to be detected onwards of the second injection, possibly consistent with the onset of iNKT-cell anergy [151]. Comparably, Chang and colleagues reported that the injection of α-GalCer-pulsed monocyte-derived DCs also induced elevation of iNKT-cell frequency to greater than 100-fold, as well as higher serum concentrations of IFNγ and IL12 [155]. iNKT-cell activation could be seen for up to 6 months in some patients and was consistent with an increase in the levels of IL12p40, IP-10, and MIP-1β, and an increase in cytomegalovirus-specific $CD8^+$ memory T cells [155]. Uchida and colleagues modified the administration approach by utilising injection of α-GalCer-pulsed peripheral blood APCs directly into the nasal sub-mucosa of patients with head and neck cancer [156]. Elevation in iNKT cell numbers and NK activation was observed in approximately half of the patients, and a reduction or stabilisation of tumor growth was seen in 6 of 9 patients [156]. A follow up study demonstrated that administration via the nasal sub-mucosa was optimal over administration via the oral sub-mucosa [157]; notably, authors also reported that oral-administration was linked to the expansion of $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells [157].

More recently, four additional studies were published in which cancer patients were injected with APCs pulsed with α-GalCer either IV or intradermally (ID) [158-161]. Injection of APCs generated in the presence of GM-CSF and IL2 into patients with non-small-cell lung cancer demonstrated expansion of iNKT cells, and in patients with elevated level of IFNγ, a possible prolongation in survival was observed, although no partial or complete clinical responses were detected [161]. Elevated IFNγ production, as well as expansion and infiltration of iNKT cells were also observed following injection of GM-CSF/IL2-generated α-GalCer-pulsed APCs prior to surgery [159]. For patients with cancers of differing origin and metastatic potential, Nicol and colleagues reported that IV injection of pulsed APCs stimulated antitumor activity both at the main tumor site, and in sites of metastasis [158]; more than half of the patients showed disease stabilisation or a reduction in tumor mass [158]. Finally treatment of patients with multiple myeloma using the combined regimen of α-GalCer-pulsed APCs and the immune-modulatory drug lenalidomide elicited elevated IL2 in the serum, as well as a decrease in tumor-associated monoclonal immunoglobin levels (M spike) [160, 162]. Taken together, these findings demonstrate that α-GalCer-pulsed APCs represent a possible therapeutic strategy to enhance antitumor immunity. While further optimisation of loading and delivery and a more detailed understanding of the mechanisms

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of action are required, α-GalCer-pulsed APCs show promise for reducing tumor growth and metastasis.

3. Adoptive transfer of *ex-vivo*-activated iNKT cells—An alternative strategy to compensate for the decreased iNKT-cell frequency observed in patients with cancer involves expanding autologous iNKT-cell populations *in vitro*. Firstly, adoptive transfer of *in vitro*-activated iNKT cells into patients with non-small-cell lung cancer resulted in *in vivo* iNKT-cell expansion, downstream activation of NK cells and IFNγ release [163]. Interestingly, the combined transfer of iNKT cells and α-GalCer-pulsed DCs has been reported to induce substantial antitumor immunity in patients with head and neck squamous cell carcinomas [164, 165]. In these studies, patients demonstrated a partial response or stabilisation of the disease, and in some cases, tumor regression [164, 165]. Optimisation of the current protocols holds high potential in tumor immunotherapy. Indeed, functionally competent iNKT cells have recently been differentiated from induced pluripotent stem cells (iPSC) in mice, which may represent a novel approach to expand iNKT cells for cancer therapy in humans [166].

Conclusion & future perspectives

Murine studies and clinical trials performed to date have demonstrated that therapies involving the manipulation of iNKT cells are not only feasible but also appear to be generally well tolerated by mice and human patients alike, and in some cases induce significant tumor regression, disease stabilization, or possible prolongation of survival. Many of the approaches used thus far induce iNKT-cell activation; however it remains to be determined which route of administration, APC type, and dosing interval are the most efficacious. Although pre-clinical studies in animal models may help answer these questions, ultimately, appropriately designed clinical trials in humans will guide protocol optimization. Our ability to manipulate these cells in antitumor therapeutics is critically dependent on our understanding of iNKT cell biology and of the factors which activate and regulate these cells; the identification and optimisation of iNKT-cell agonists which can promote Th1 immune responses without inducing iNKT-cell anergy is of high priority. Notably, despite the clear ability of exogenously-activated iNKT cells to initiate potent antitumor activity in response to immunotherapeutic stimuli, whether this represents a physiologic role for NKT cells in tumor rejection, and if so, which signalling cascades are required, remains unclear. Additionally, in light of the identification of developmentally and functionally distinct subsets of iNKT cells and type II NKT cells, emphasis should be put on characterising the roles and interactions of these cells during immunosurveillance therefore improving the specificity of NKT-targeted agonists.

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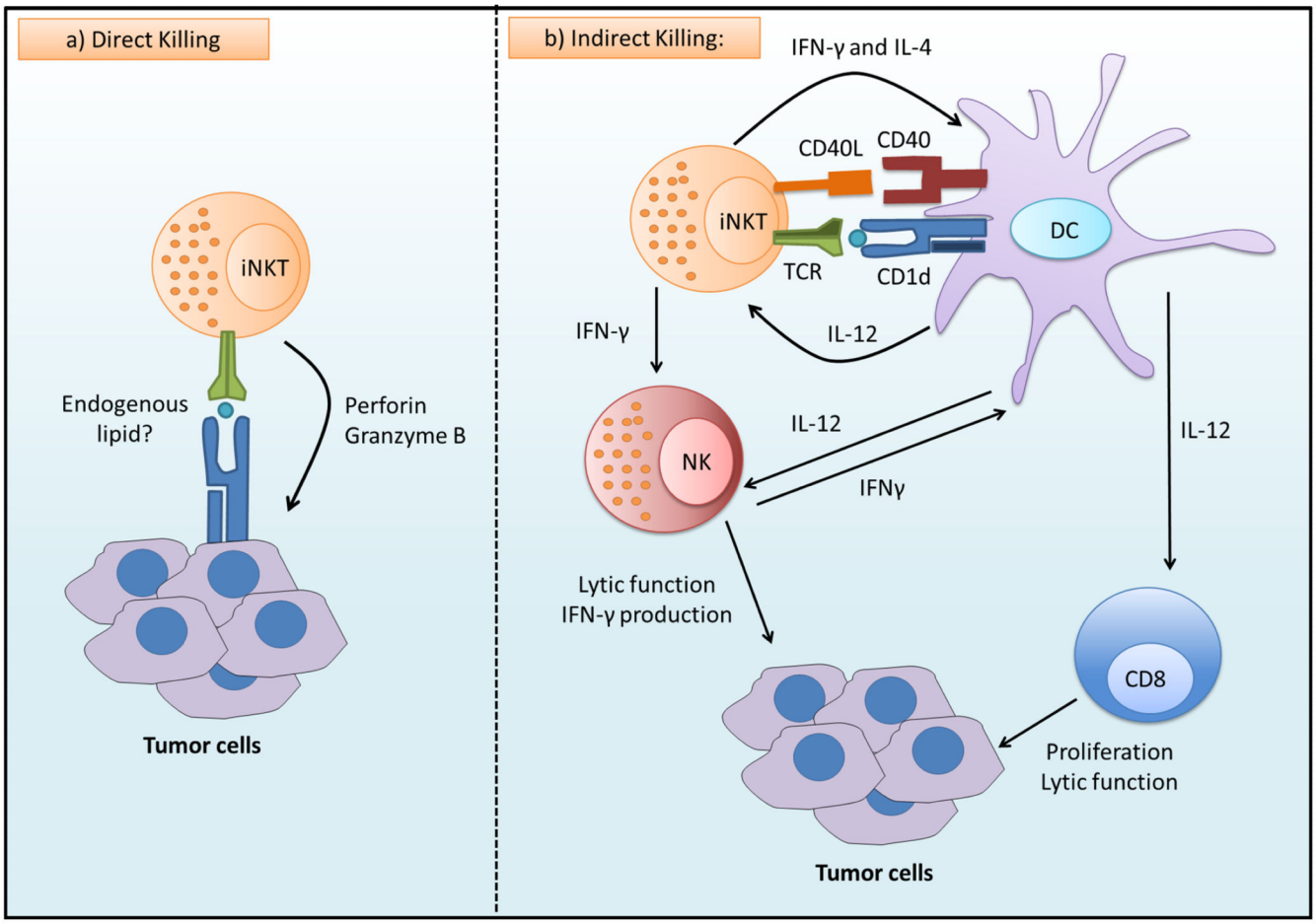

Figure 1. Antitumor activities of iNKT cells

a) Invariant natural killer T (iNKT) cells can recognize endogenous lipids presented by CD1d molecules on tumor cells and subsequently eliminate tumor cells directly through iNKT cell-mediated lysis. **b)** In the absence of CD1d expression on tumor cells, iNKT cells may become activated in response to CD1d-expressing or Toll-like receptor (TLR)-activated antigen-presenting cells (APC). Bi-directional activation of iNKT cells and APCs promotes NK-cell activation and the activation of the tumor-specific T-cell response, thereby indirectly mediating tumor-cell killing. (This figure is created by Hemza Ghadbane of the Weatherall Institute of Molecular Medicine and the University of Oxford.)

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Figure 2. Structure and interactions of the prototypic iNKT-cell agonist α-GalCer with CD1d molecule and TCR

a) The biochemical structure of the prototypic iNKT-cell agonist, α-GalCer. **b**) The crystal structure of α-GalCer (red) loaded onto human CD1d molecules (grey) and binding to the iNKT-cell TCR (yellow/orange). Figure was generated using PyMOL and the Protein Data Bank using accession number 2PO6 from [78], and adapted by permission from Macmillan Publishers Ltd: Nature [78], copyright 2007. The head group of the lipid is exposed and allows for interaction with the iNKT-cell TCR. Modifications to the head-group, tail length or saturation affect the ability of iNKT-cell agonists to activate iNKT cells [31], a property

which has been utilised to optimise anti-cancer therapeutics. (Panel **a** of this figure is generated by Hemza Ghadbane of the Weatherall Institute of Molecular Medicine and the University of Oxford.)

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