Review paper

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A brief review of clinical trials involving manipulation of invariant NKT cells as a promising approach in future cancer therapies

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Abstract

In the recent years researchers have put a lot of emphasis on the possible immunotherapeutic strategies able to target tumors. Many studies have proven that the key role in recognition and eradication of cancer cells, both for mice and humans, is being conducted by the invariant natural killer T-cells (NKT). This small subpopulation of lymphocytes can kill other cells, either directly or indirectly, through the natural killer cells' (NK) activation. They can also swiftly release cytokines, causing the involvement of elements of the innate and acquired immune system. With the discovery of α-galactosylceramide (α-GalCer) – the first known agonist for iNKT cells – and its later subsequent analogs, it became possible to effectively stimulate iNKT cells, hence to keep control over the tumor progression. This article refers to the current knowledge concerning iNKT cells and the most important aspects of their antitumor activity. It also highlights the clinical trials that aim at increasing the amount of iNKT cells in general and in the microenvironment of the tumor. For sure, the iNKT-based immunotherapeutic approach holds a great potential and is highly probable to become a part of the cancer immunotherapy in the future.

Key words: *immunotherapy, iNKT, invariant NKT, CD1d, tumor immunology.*

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Introduction

Among the broad and heterogenic population of lymphocytes, recently the natural killer T-cells (NKT) have generated a lot of interest. These cells are rare and generally represent only from 0.001% up to less than 1% of the human peripheral blood lymphocytes [1, 2], although for some individuals this amount may be higher, reaching as much as 3% of peripheral blood lymphocytes [3, 4]. The basis for such variability in the range of NKT frequencies is not clear, but is unquestionably connected with the influence of genetic factors [2]. Despite their small amount in human blood they significantly affect the immune system [5, 6]. Ongoing studies have revealed their involvement in immune responses, especially during infections, allergies, autoimmune disease, or even cancer. Most of the available information on NKT cells comes from research conducted on mice. However, both phenotypic and functional features of the mouse and human NKT cells appear to be similar [7, 8]. NKT gained their name after a unique cell surface feature: they express receptor characteristic to T lymphocytes (αβ-TCR) and lineage markers of Natural Killer cells (NK) (e.g. human CD161, mouse NK1.1) [7, 5, 9-11]. In light of current data, the above definition seems to be insufficient and incomplete. The expression of NK-markers is not necessarily needed to identify a cell as an NKT cell. Additionally, the level of NK cell markers expressed on NKT cells seems to vary in accordance with their maturation, activation state, and presumably their genetic background [12, 13]. NK1.1 together with other NK-typical molecules may be acquired by CD8+ T lymphocytes upon appropriate stimulation or during a viral infection [14]. NKT cells nowadays are defined by the restriction of their TCR receptors. While the TCR receptors on T cells react with peptide antigens in the context of major histocompatibility complex (MHC) class I or II molecules, the NKT cells, with use of their TCR receptors, recognise the lipid and glycolipid antigens presented by non-polymorphic MHC class I-like glycoprotein, known as CD1d (Fig. 1) [15]. The NKT cells can react with both endogenous and exogenous antigens, regulating a variety of autoimmune diseases, but also providing protection against different pathogens like Sphingomonas spp. or Borrelia burgdorferi [11, 16, 17]. In order to be recognised, and to stimulate the

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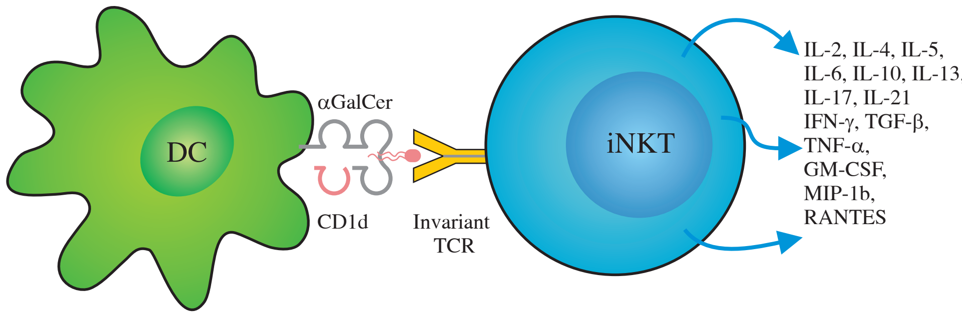

Fig. 1. Mode of glycolipid presentation by CD1d to NKT cells. An antigen-presenting cell (APC) – here dendritic cell (DC) – with the help of CD1d molecules present on its surface, presents antigen (α-GalCer) to an NKT cell. The latter recognizes proper ligand through iTCR. As a result NKT rapidly secretes Th1 and Th2 cytokines [11, 123]

NKT cells, the aforementioned antigens have to be at first internalised, processed, and finally presented through the cells containing CD1d. These molecules are constitutively expressed on antigen-presenting cells (APC): dendritic cells (DCs), B lymphocytes, and macrophages [18].

Some tumour cells also highly express CD1d molecules and are able to present endogenous tumorous glycolipids. By recognising them, the NKT cells can directly conduct lysis on the sensitive CD1d$^+$ tumour cells. This observation indicates the NKT cells as improvers of antitumor response, thus encouraging investigators to further examine ways to strengthen the immune response against cancer in future therapies. The CD1d restriction, relative to the NKT cell reagents, allows the detection, enumeration, and characterisation of functions, as well as examination of the responsiveness of these cells [12, 17, 19]. In this review, an outline of anti-tumour properties of the NKT cells has been presented. It also presents the clinical approaches concerning this unique population of cells as possible enhancers in future anti-cancer strategies.

Subsets of natural killer T-cells

Invariant natural killer T-cells

Depending on the composition of T-cell receptors and the type of molecule presenting the identified antigens to which they respond, the NKT cells are currently classified into three main groups: type I, type II, and NKT-like cells. The TCRs of most NKT cells in human bodies are formed by a canonical α chain, comprised of Vα24 and Jα18 gene segments, and a β chain of limited variability – usually Vβ11. Such NKT cells are known as type I or invariant NKT (iNKT). Homologous Vα14-Jα18 and Vβ8.2, Vβ7, Vβ2 create TCRs of the iNKT in mice bodies [15, 20-22]. Such TCRs restrict the gamut of recognised antigens and facilitate reactivity to CD1d. Despite iNKT cell populations using conserved TCRs, they differ essentially. The differences may concern the phenotype, localisation, or functions of both murine and human iNKT cells. The Vα24$^+$ NKT cells can be further classified as: CD4$^+$CD8$^-$, CD4$^-$CD8$^+$, and double negative (DN) for CD4 and CD8 expression [6, 23]; murine iNKT are either CD4$^+$CD8$^-$ or DN [8, 24]. Particular subpopulations differ potentially from each other in terms of cytokine production. The human iNKT cells with CD4 molecule produce a mixture of Th1 and Th2 cytokines, whereas the DN produces only Th1-type cytokines [6]. Such differences are less evident for mice [6, 9]. The discrepancies in cytokine release seem to depend on the tissue localisation, which can at least partially explain why the iNKT cells obtained from different organs present different functions [25]. The iNKT cells occur mainly in the blood and various tissues including the thymus, spleen, liver, and bone marrow. Studies on mice have shown that the liver-resident iNKT cells are the most prevalent [10]. Their protection against tumours is also stronger than in thymus or spleen [25]. Strangely enough, the number of the iNKT cells in most human organs is lower than for mice, and their appearance seems to vary substantially among individuals [26].

Recent studies have revealed the existence of the iNKT subpopulations that differ in terms of production of particular cytokines and expression of specific transcription factors. This connotes with the classification of conventional T lymphocytes. Mature iNKT cells may be divided into three dominant groups: NKT1, NKT2, and NKT17. These cells express transcription factors that typically regulate the cell lines of Th1, Th2, and Th17 (T-bet, GATA3, and RORγt, respectively); they also produce similar cytokines to the foregoing subsets of T lymphocytes [27-29]. The T-bet NKT1 release large amounts of IFN-γ – a cytokine involved in antiviral and antitumor response. The NKT2 seem to take part in the course of diseases with Th2 dominance through the secretion of IL-4, IL-9, IL-10 or IL-13 [30]. At present, a lot of focus has been placed on the NKT17, which release IL-17A upon stimulation and promote the inflammation state [29]. Researchers have also identified a group of iNKT cells with FoxP3$^+$ expression, which act similarly to regulatory T lymphocytes by inhibiting the proliferation of other cells. A novel subpopulation of iNKT cells named NKT10 was discovered in 2014. They were observed to produce IL-10 under steady-state conditions in mice and human bodies, in this way impairing anti-tumour response [31].

Variant natural killer T-cells

The second identified subset of NKT cells is called *variant* NKT (vNKT), or more commonly type II NKT. They are more heterogeneous than type I in both α and β chain usage [7]. Their TCRs form diverse combinations on the cells' surface. Table 1 shows their main features in comparison to the iNKT. This subset often plays an opposite or cross-regulating role with the iNKT, especially under conditions of immune dysregulation such as cancer. Like the iNKT, they are present in human and mice organisms [32]. Since their discovery in 1995 [33], only a limited number of studies have reported their physiological functions and our knowledge about them is still quite limited [34].

The natural killer T-like cells

All of the other other NKT cells form the group of the NKT-like cells – the most heterogeneous compartment of NKT cells. They constitute a subset of T lymphocytes that express some natural killer cell receptors. However, they are not restricted by CD1d molecules, so they are also called CD1d *independent* NKT (CD1d^{ind} NKT) [35]. Scientists have confirmed a highly specialised effector-memory phenotype of these lymphocytes, thus their percentage in peripheral blood increases with age. In comparison, the amount of human iNKT in peripheral blood decreases with age [36]. The majority of NKT-like cells are CD16^{-}, and CD8 dominates over the expression of CD4 [37]. The functionally mature CD3^{+}CD56^{+} NKT-like cells have been observed to show high tumour-killing abilities against many tumour cell targets [38-40]. They hold high levels of granzyme and can produce substantial amounts of proinflammatory cytokines like IFN-γ and TNFα [41, 42]. The frequency of CD3^{+}CD56^{+} NKT-like cells has been reported to decrease significantly among patients with progressive chronic lymphocytic leukaemia [43], which suggests their protective role against cancer. These cells can be generated when cultured *in vitro* as one of the cytokine-induced killer (CIK) cells [44].

Given that far more is known about the iNKT cells and their antitumor activity, this review will focus predominantly on these cells and recent immunological approaches based on implementing them into cancer treatment.

The means of tumour cell recognition by invariant natural killer T-cells

The progress in the characterisation of iNKT that has occurred in recent years has allowed us to form a belief about how they recognise tumour cells and disallow them to evade an immune response [45]. Research indicates participation of CD1d in this process. These molecules are expressed on cells of the monocytic lineage like monocytes, macrophages, and dendritic cells [46-50], as well as on B lymphocytes. They are also present on malignant human haematopoietic cells, originating from the corresponding tissues, e.g. a few types of leukaemia cells of patients with acute myeloid leukaemia (M4 or M5 AML and juvenile myelomonocytic leukaemia) [51], malignancies originating from Langerhans cells, or interdigitating dendritic cells [46]. Tumour cells of patients with B-cell malignancies are also CD1d-positive, like B-precursor acute lymphoblastic leukaemia with MLL/AF4 gene rearrangement and chronic lymphocytic leukaemia (CLL) [51]. Studies conducted by Metelitsa *et al.* [46] confirmed that CD1d^{+} myelomonocytic leukaemia cells trigger a cytotoxic feedback via the NKT cells. The above-mentioned path of activating iNKT cells is called a "direct activation" (Fig. 2A); it constitutes one of the possibilities of recognising and eliminating tumour cells. Similarly, Renukaradhya *et al.*

Table 1. Classification of NKT cells into two types of cells [2, 34, 122]

	Type I	Type II
Other names	Classical NKT, invariant NKT, Vα14iNKT	Non-classical NKT
Percentage	> 95% NKT	< 5% NKT
Surface markers	CD4+CD8–, CD4–CD8+, CD4–CD8– (H) CD4+CD8–, CD4–CD8– (M)	CD4+CD8–, CD4–CD8– (M)
CD1d	Dependent	Dependent
TCRs	Invariant αβTCR (Vα14Jα18 M, Vα24Jα18 H)	Diverse TCRs
Reactivity with α-GalCer	Yes	No
Cytokine production	IFN-γ, IL-4, IL-3	IL-4, IL-13, IL-10, IFN-γ

M – mouse; H – human

Table 2. A summary of clinical trials using iNKT-related therapies

Clinical trial	Number of patients	Tumor-type	Applied treatment	Clinical outcome	Time of observation	References
Giaccone *et al.*	24	Solid tumors	α-GalCer (*i.v.*)	SD (7)	2002	[99]
Nieda *et al.*	12	Solid tumors	α-GalCer-immature DCs (*i.v.*)	Reduction of tumor markers (2)	2004	[103]
Nicol *et al.*	12	Solid tumors	α-GalCer-immature DCs (*i.v.* and *i.d.*)	SD (3) PR (3)	2011	[107]
Chang *et al.*	6 enrolled 5 completed	Solid tumors, myeloma	α-GalCer-mature DCs (*i.v.*)	Reduction of serum or urine M protein (3) SD (1)	2005	[105]
Richter *et al.*	6	Asymptomatic myeloma (AAM)	α-GalCer-mature DCs + lenalidomide (*i.v.*)	Increase of iNKT, NK, monocytes, eosinophils	2013	[110]
Ishikawa *et al.*	11 enrolled 9 completed	Lung cancer	α-GalCer-immature DCs- rich APCs (*i.v.*)	SD (5)	2005	[104]
Motohashi *et al.*	23 enrolled 17 completed	Lung cancer	α-GalCer-APCs (*i.v.*)	SD (5)	2009	[113]
Uchida *et al.*	9	HNSCC	α-GalCer-APCs (via nasal submucosa)	SD (5) PR (1)	2008	[106]
Kurosaki *et al.*	17	HNSCC	α-GalCer-APCs (via nasal and oral submucosa)	Increase of iNKT and IFNγ	2011	[115]
Nagato *et al.*	4	Lung cancer	α-GalCer-APCs (*i.v.*)	Infiltration and activation of iNKT	2012	[116]
Motohashi *et al.*	6	Lung cancer	α-GalCer-activated iNKT (*i.v.*)	SD (4) PR (2) Level 2: SD (2)	2006	[117]
Kunii *et al.*	8	HNSCC	α-GalCer-APCs (via nasal submucosa) + α-GalCer-activated iNKT (intra-arterial infusion)	SD (4) PR (3)	2009	[119]
Yamasaki *et al.*	10	HNSCC	α-GalCer-APCs (via nasal submucosa) + α-GalCer-activated iNKT (intra-arterial infusion)	SD (5) PR (5)	2011	[120]

SD – stable disease; PR – partial regression; HNSCC – head and neck squamous cell carcinoma; AMM – asymptomatic myeloma

[52] revealed that the expression of CD1d molecules on B cell lymphoma NSO cells for mice correlates with iNKT cell-mediated lysis. Still, the role played by CD1d and their part in triggering cytotoxic mechanisms has not been fully established yet. Further investigations need to be conducted to determine how the CD1d of malignant cells can be distinguished from the other ones. The fact that tumours try to overcome iNKT-mediated immunity by losing the expression of CD1d on their surface or by repressing a synthesis of ligands for the TCR of iNKTs, should also be remembered [53].

Although direct recognition of CD1d-positive malignant haematopoietic cells is effective, most solid tumours and cell line models do not express CD1d, or its expression is very low [14, 51]. Still, iNKT cells may become activated indirectly (Fig. 2B). This mechanism involves APCs that are able to present CD1d-restricted antigen derived from tumour cells, and in this way activate the iNKT. In turn, the iNKT cells promote activation of NK cells. Thereby, the tumour cell elimination is conducted indirectly by activating the NK cells [54]. Wu *et al.* [55] conducted the first analysis of a ganglioside precursor disialohematoside (GD3) – a natural compound that is recognised with the above-described mechanism by our immune system. GD3 is a ganglioside highly expressed by many types of tumour, such as melanoma, sarcoma, or neuroblastoma, but very poorly by normal human or mice cells. Using mice immunised with human melanoma SK-MEL-28 cell line, the authors observed a response from the NKT cells. Since this tumour cell line is CD1d$^-$, the GD3 must be cross-presented by murine APC to the NKT cells. The cross-presentation of tumour-derived glycolipids seems to play an especially important role in the recognition of CD1d$^-$ tumours [52, 55].

Another suggested way of reducing the growth of tumours is connected with tumour-associated macrophages (TAMs). Perhaps, the activated iNKT and NK cells could suppress the production of pro-angiogenic factors produced by these macrophages (Fig. 2C). This thesis, however, requires further research for confirmation [54].

Invariant natural killer T-cells-mediated anti-tumour response

Since the discovery of iNKT cells, many studies have tried to describe their activity with the help of potent exogenous stimulators such as IL-12 or α-GalCer. Soon, they were followed by reports concerning the role of iNKT in tumour surveillance in physiological conditions (without stimulation) [56]. Smyth *et al.* [57] performed a study involving the use of knockout mice lacking the Jα18 gene. Such a change in the DNA resulted in a deficiency of murine iNKT cells, which correlated with an increased tendency for spontaneous development of methylcholanthrene-induced cancers like sarcomas and B16F10 melanoma tumours [39, 40]. They are characterised by early appearance and high frequency [57]. Subsequently, Crowe *et al.* [58] proved that this effect could be reversed after administrating the liver-derived iNKT cells in the early phase of tumour growth. However, the transfer of thymic or splenic iNKT was not as potent, which suggested functional discrepancies between subsets of iNKT cells [58]. Swan *et al.* [59] performed a study on mice deficient in p53

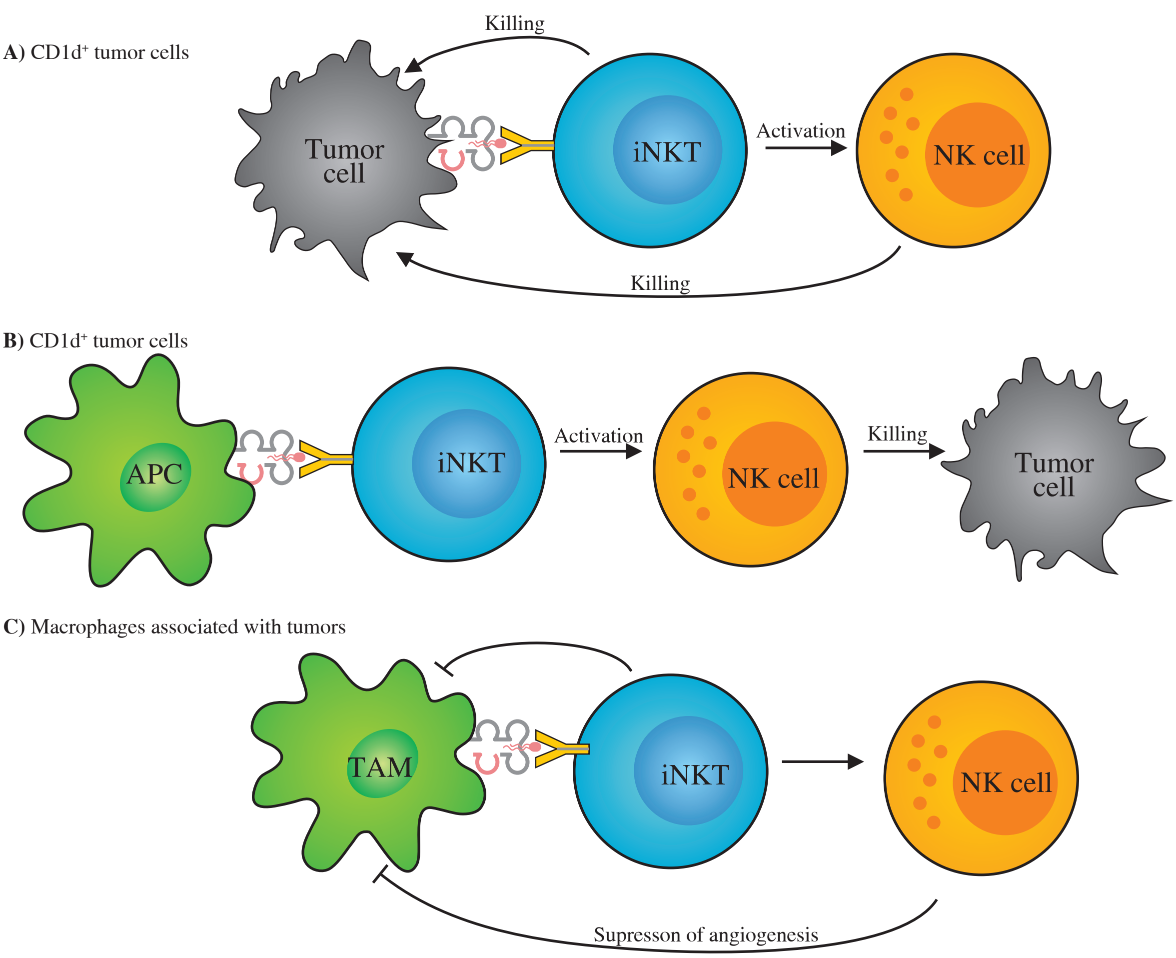

Fig. 2. Methods for suppressing tumor cells by invariant natural killer (iNKT) T cells. A) Tumor cell that possess CD1d molecules on its surface, can present antigens to iNKT cell what results in the activation of the latter. Activated lymphocyte in turn prompt tumor cells death in either direct way or indirectly via activity of natural killer (NK) cell. B) iNKT cell participate in the process of eliminating tumor cell without CD1d expression as well. They are activated by antigen presenting cells (APC) and kill tumor cell via activating NK cell. C) iNKT cell by suppressing tumor-associated macrophage can also influence on tumor cell restricting secretion of macrophages proangiogenic factors [54]

– an important suppressor of tumorigenesis in mice and human organisms. The lack of iNKT cells resulted in increased tumour occurrence for these animals. The above-mentioned studies confirmed the undeniable role of classic NKT cells in antitumor immunity and suggested endogenous ligands as effective activators of these cells. The antitumor properties questioned seem to derive from combining a number of different lines of action. After the antigen recognition, the activated iNKT cells rapidly release cytokines and chemokines, through which they affect many immune cells, or may present a direct cytolytic effect.

Invariant natural killer T-cells-cytotoxic activity

Classic NKT cells have their own lytic activity. They produce cytotoxic molecules able to eradicate tumour cells. Up to 85% of these compounds consist of perforin and granzyme B, accompanied by Fas ligand (FASL), tumour necrosis factor α (TNF-α), and TNF-related apoptosis-inducing ligand (TRAIL). Metelitsa *et al.* [46] examined the NKT cells for cytotoxicity against CD1d-positive myelomonocytic leukaemia cells. The cultured NKT cells demonstrated constitutive expression of high levels of mRNA for perforin and granzyme B. The stimulation with use of α-GalCer led to a further increase in their level. In turn, the concentration of TNF-α ligands was very low in the resting state. The iNKT cells transcribed them later, after α-GalCer stimulation. The authors concluded that perforin together with granzyme B are responsible for the early cytolytic activity of iNKT cells, while ligands belonging to the TNF-α family provide additional cytotoxicity.

This study also pointed to the presence of CD56 molecules in iNKT cells. The CD56-positive cells were noted

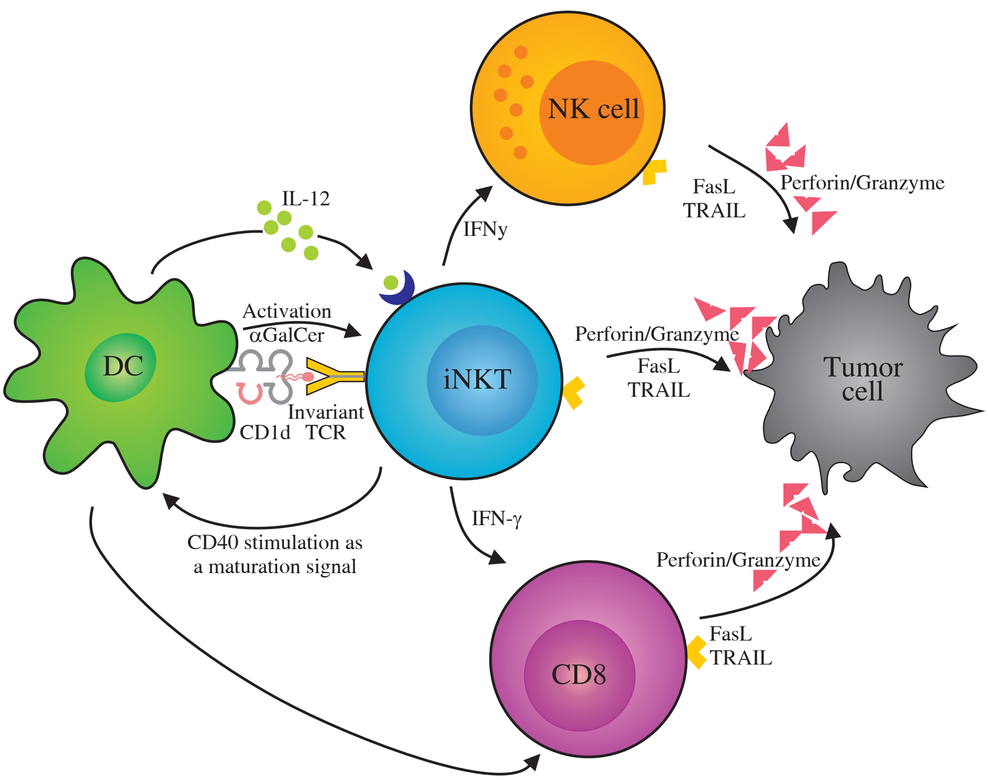

Fig. 3. An influence of iNKT cells on tumor activity by causing adjuvant effect [60]. After recognizing a lipid antigen, here α-galactosylceramide (α-GalCer), presented by CD1d of dendritic cells (DCs), iNKT cells increase the expression of CD40L. It touches off the higher expression of costimulatory molecules (like CD40 or CD80) on DCs and induces their maturation. Activated DCs start to secrete IL-12, which binds with an appropriate receptor on iNKT cells, activating them. In this way, iNKT cells start to release cytotoxic molecules: perforin and granzyme B, accompanied by the expression of FasL and TRAIL. Such action effect a direct death of tumor cells. Activated iNKT cells also start to secrete different cytokines, led by IFN-γ. Through IFN-γ, they can stimulate NK cells and CD8+ T lymphocytes to express their own cytotoxicity against tumor cells. Apart from the expression of perforin, granzyme B or FasL, NK cells start to release their own IFN-γ, providing an 'adjuvant effect' of iNKT cells' action

to be more cytotoxic, more frequently expressing perforin without stimulation. Such dissimilarity was not observed under the influence of α-GalCer [46]. The engagement of the direct-killing mechanism seems to be variable and depends on the employed tumours model [60].

Multimodal activity of released cytokines

Above and beyond the described cell-death-inducing mechanism, the reaction of Vα24 NKT with antigens (e.g. α-GalCer) results also in the release of cytokines [61]. Initially, only secretion of IL-4 and INF-γ was detected [62], arising within hours of the activation. This observation was later partially explained after the finding of constitutive expression of mRNA for these cytokines in iNKT [13, 63, 64]. Subsequent studies have revealed that the pool of produced cytokines varies considerably as it also includes IL-2, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, IL-22, TNF-α, transforming growth factor β (TGF-β), and granulocyte-macrophage colony-stimulating factor (GM-CSF) [29, 62, 65, 66]. Thereby, the iNKT cells can have an impact on several other types of cells of an innate and adaptive immune system [13] including DC, macrophages, neutrophils, NK cells, conventional T and B lymphocytes, lymphocytes Tγδ, or type II NKT cells. A secretion of both Th1 and Th2 cytokines makes iNKT cells multimodal in their action, hence it is more difficult to predict the consequences of their actions *in vivo* [57]. Referring to cancer, the iNKT cells can launch an antitumor response through proinflammatory Th1 cytokine cascade, triggering "adjuvant effects" (activation of other antitumor cytolytic cells), and through revealing direct cytotoxicity. However, the role played by the NKT is far more complex because they may act on the contrary through IL-13 or the mentioned type II NKT [52]. This functional heterogeneity should be further explored in order to create future strategies that promote anti-tumour effects.

Taking a closer look into the process leading to the cytokine release, it all starts from the recognition of an appropriate antigen (e.g. α-GalCer). Activated iNKT cells up-regulate CD40L molecules on their surface, to which DCs respond by remodelling their markers (enhancement of costimulatory molecules: CD40, CD80, and CD86). The described interaction between iNKT and DCs induces the maturation of the latter. DCs activated in this way start to secrete IL-12 [67], while production of IL-23 is inhibited [68]. The IL-12 acts on cells that possess corresponding receptors on their surface. The iNKT have substantial amounts of the mature form of these receptors (IL-12R), becoming the main recipient of a released cytokine. By binding it, it activates the iNKT. The activation signal can also be transmitted by the reaction between CXCR6 receptor on the iNKT and CXCL16 ligand on APCs [69]. Fully activated iNKT cells secrete large amounts of IFN-γ and IL-2, through which they influence e.g. NK and $CD8^+$ T cells to express cytotoxic functions [5, 70, 71]. Activated NK cells start to secrete their own IFN-γ. Thus, the IFN-γ is first produced by the NKT cells, and later by the NK cells. The adjuvant effect of iNKT cells is emerging this way [72]. Furthermore, the DCs matured in a response to iNKT cells cross-present the tumour-derived antigens to $CD4^+$ and $CD8^+$ T lymphocytes and exert a significant influence on the efficacy of immune responses [73, 74]. A study by Hermans *et al.* [73] implies that the enhanced T-cell responses that they observed were caused by conditioning of DC by the iNKT cells. This observation additionally developed the 'adjuvant effect' of the iNKT cells. The described interactions are illustrated in Figure 3. IFN-γ seems to play a critical role in the tumour rejection. It mediates the emergence of the 'adjuvant effect'. The loss of this activity has been observed in a study ran by Gonzales *et al.* [75], who used mice lacking the receptor of IFN-γ. Other studies conducted on patients with different cancer types (colon cancer, head and neck cancer, breast cancer, renal cancer, and melanoma) have shown, besides a decrease in the iNKT, a maintained ability to produce the IFN-γ. However, in patients with lung cancer, advanced prostate cancer, or other undefined advanced cancers except from glioma, the iNKT responded poorly, even under stimulation with α-GalCer [76].

The influence of iNKT involves also B lymphocytes, which respond by an increase in secreted levels of IgG, thereby participating in the fight against tumour cells by antibody-dependent cellular cytotoxicity (ADCC) mechanism [76].

Invariant natural killer T-cells in relation to TAMs

As mentioned earlier, iNKT cells cannot kill CD1d-negative tumour cells directly [77]. Although the iNKT activate NK cells and cause augmentation of their cytotoxicity [78], the NK cell migration to the established tumours in human bodies is inferior [79]. For example, their level in neuroblastoma (tumour without CD1d expression) is significantly low [80]. This finding suggests that the iNKT may act by another mechanism, much more sophisticated than NK mediation, involving the control of tumour initiation and metastatic process. The iNKT cells infiltrate primary and metastatic human tumours, where they interact with neoplastic cells or other immune cells present in the tumour microenvironment. According to Song *et al.* [53], who conducted research on neuroblastoma, the accumulation of the iNKT cells occurs due to the expression of high levels of CCL2 by the tumour cells. The same cytokine also attracts monocytes and other immature myelomonocytic precursors of TAMs. TAMs are precisely the crucial attachment point of a proposed novel antitumor mechanism. They develop under the influence of IL-6 – a tumour growth-promoting cytokine secreted by neuroblastoma cells [53]. TAM cells promote angiogenesis [81], protect tumour cells from chemotherapy-induced apoptosis [82], facilitate the metastasis of tumour [83], and cause T-cell death through the expression of programmed-death ligand-1 [53]. As TAMs are the dominant CD1d-positive cells in the examined tumour microenvironment, by cross-presenting neuroblastoma-derived antigens, they enable the iNKT cells to destroy the tumour in a CD1d-dependent way. Killing the TAMs would terminate their growth support for tumour cells [51, 53, 84]. The presented mechanism may partially explain the affiliation of the iNKT on CD1d-negative tumours with better disease outcome in neuroblastoma and other types of cancer [53].

Invariant natural killer T-cells in relation to myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) incorporate immature macrophages, granulocytes, DCs, monocytes, and other myeloid cells at early stages of differentiation [85]. A low incidence of these occur for healthy individuals [86]. Growing tumours release factors (e.g. IL-6, IL-10) that disrupt the normal differentiation of myeloid-line cells and lead to their accumulation in blood, spleen, or undifferentiated bone marrow cells [87]. MDSCs enhance the tumour progression in those tissues. Their main functions are immunosuppression and constitution of a tumour suppressive environment. They have been observed to inhibit CD4+ and CD8+ T lymphocytes, B lymphocytes, and NK cells and induce CD4+CD25+FoxP3+ regulatory T lymphocytes [86]. There have been reports suggesting iNKT as key cells in overcoming immune suppression mediated by the MDSC [88, 89]. Studies performed on mice prove that iNKT cells convert CD11b+ Gr1+ MDSC into stimulatory APCs or suppress them in association with a CD1d molecule [88]. The NKT cells resisted the MDSCs effects and were observed to reverse their effect on T-cell proliferation [90]. The interaction of iNKT cells with MDSCs in human organisms has been incompletely understood. In one model, the iNKT cells interact with CD1d on the surface of MDSCs, which results in an increase in CD80/86, CD70 and ICAM-1 expression, and transformation of the MDSCs into the DC phenotype. The MDSCs converted through CD80 and CD70 molecules react with those on the T cells (CD28 and CD27), supporting their response against cancer [91]. Cancer immunotherapy designed to strengthen the anti-tumour activity of iNKT cells may not only remain effective in the presence of MDSCs, but also erase the suppression of MDSCs on immune responses.

Application in clinical trials

Strategies to combat tumours are among the most rapidly evolving areas of contemporary medicine. Even the development of chemotherapy, radiotherapy, and drug design have not sufficiently reduced the human morbidity and mortality caused by cancer. Tumours learn to develop different mechanisms that allow them to avoid the immune system. For this reason, over the last years, researchers have made efforts to strengthen human immune response and help the healthy cells to control cancer. Probably, manipulating the iNKT cells can act as such an immunotherapeutic tool because they recognise and destroy the tumour cells.

Researchers have observed that a decline in the number of iNKTs is often accompanied by their functional alternation in patients with many types of tumours, like prostate cancer or head and neck cancer [18, 53, 60]. A low level of iNKT cells in blood circulation has been found to correlate with poor prognosis in patients with acute myeloid leukaemia and head and neck cancer [18, 92, 93]. The impairment of the iNKT cell functions correlated with the clinical stage of tumours. iNKT cells have been observed to accumulate in the tumours (e.g. colorectal cancer), in the mechanism of a tumour tissue infiltration, which related to their improved survival [94, 95]. Therefore, actual therapeutic strategies focus on restoring the proper amount and functional condition of the iNKT cells, aiming especially at potentiating a Th1-type response.

Administration of soluble α-linked galactosylceramide

The discovery of α-linked galactosylceramide (α-GalCer) has been extremely helpful in extending our knowledge on iNKT biology. α-GalCer had been obtained from marine sponges as one of the compounds having anti-tumour properties. Researchers have received encouraging data in terms of effectiveness and safety from *in vitro* studies and murine models [57, 96-98]. To verify if the activity of α-GalCer for humans is relevant to the preclinical outcomes, the first in-man administration was performed. It was an open-label, non-randomised phase I study that included 24 patients with refractory solid tumours [99]. They were intravenously injected with a soluble α-GalCer at a varying range of doses in four weekly cycles. Giaccone *et al.* [99] provided basic information about the pharmacokinetics of α-GalCer in the human organism, indicating that it does not accumulate in the body and is not detectable in urine. It is safe when applied at a wide range of doses, and generally has been well tolerated. Its toxicity, assessed using the National Cancer Institute Common Toxicity Criteria, was minimal. Only one of the patients developed grade 3 symptoms: fever with vomiting and shivering. No serious adverse events were observed. Although previous studies conducted on mice have reported that α-GalCer induced liver damage, such toxicity was not observed for humans [99]. Concerning the number of iNKT cells, their rapid (within 24 hours) decrease after α-GalCer administration was noted, just like in the murine models [100]. Only later it occurred that their disappearance was connected with a TCR down-regulation at that time. Within ten days, most of the iNKT cells died by apoptosis, while the remaining part went into an anergy state. In this condition, the iNKT cells had become unresponsive to another administration of ligand for at least thirty days. It occurred that this mechanism depends on the interaction between programmed cell death-1 and its ligand [101]. The conducted study met with little success and did not show any clinical improvement, but the malignant disease of seven patients maintained at a stable level. Thus, other schemes should be taken into consideration in order to effectively increase the amount of iNKT cells for cancer patients [99].

Administration of α-linked galactosylceramide-pulsed immature dendritic cell

Murine models were also tested in terms of the iNKT reaction on α-GalCer-pulsed dendritic cells instead of the α-GalCer alone [14, 97, 102]. Based on the favourable results of these studies, many clinical trials have been undertaken using α-GalCer combined with the variations in APC platform [103-106] (Table 2).

Nieda *et al.* [103] used autologous, immature, monocyte-derived DC pulsed with α-GalCer. The study included a group of 12 patients with metastatic tumours. Injections were well tolerated, without any serious side effects. Most of the patients experienced only minor adverse events. Soon after the injection, a transient decrease in peripheral blood of the iNKT, but also NK, T, and B cells was observed, followed by a small increase of the iNKT cells within a few days. Consequently, a larger number of NK and T cells were activated, leading to an increase in the IFN-γ level. Just like in the previously described trial on mice model, Nieda *et al*. did not detect any reductions of tumour. However, in two of the patients tumour markers decreased, which indicated an antitumor effect of the α-GalCer-pulsed, immature, monocyte-derived DCs [103].

Nicol *et al.* [107] conducted a phase I clinical trial using the same APCs as in Nieda's study. In order to estimate the safety and tolerability, they used dose-escalation and different routes of administration: intravenous and intradermal. Twelve patients with different metastatic diseases, who could not be treated with standard therapy, took part in the study. Immature DCs (obtained from monocytes cultured with GM-CSF and IL-4) were pulsed with α-GalCer one day before the administration. The surface of immature APCs proved to be richer in CD1d molecules than for the mature forms. Subsequently, contact with iNKT would lead to their maturation. The iNKT cells stimulated in the above-described way showed an adjuvant effect by activating other peripheral blood lymphoid cells, e.g. NK cells and T lymphocytes, and by causing an increase of the IFN-γ detected in the serum. Overall, the therapy was well tolerated. Most of the patients experienced temporary intensification of inflammation symptoms in the place where metastasis had been diagnosed (in connection with iNKT activation). These changes were more common after intravenous, rather than intradermal injections. The cause lies in the accuracy with which most of the α-GalCer-pulsed, immature DCs administered intradermally stay in the skin, with only a small part moving into the lungs, liver, or spleen. The same cells injected intravenously encounter the iNKT cells without obstacles. The progression of the disease, as observed in six out of 12 cases, evolved into a stabilisation or minor objective improvement, and in three cases lasted up to one year [107].

Administration of α-linked galactosylceramide-pulsed mature dendritic cell

Another phase I clinical study, run by Chang *et al.* [105], evaluated the efficacy of α-GalCer-loaded mature DCs administered to patients with advanced cancer. Some of the earlier findings indicated that DCs were preferable to monocytes in presenting the α-GalCer. The maturation process of DCs correlated with an increase in their ability to activate iNKT cells [108], mainly because of more effective co-stimulation and cytokine production (IL-2, IL-7, IL-12, IL-15) [110]. Chang *et al.* [105] obtained a reliable intensification of the iNKT cells' *in vivo* expansion for humans. After injections with α-GalCer-loaded mature DCs, all patients experienced a substantial growth in the number of iNKT cells (more than 100-fold). In some cases, this state persisted for more than six months after injection. In comparison, after using α-GalCer-loaded immature DCs or α-GalCer alone, the increase was barely transient. The authors also detected higher amounts of cytokines associated with maturation of the DCs. Three patients experienced clinically relevant decreases in urine and serum M protein, and one patient showed disease stabilisation [105].

The α-GalCer-loaded, monocyte-derived mature DCs (obtained in a similar way as in the previous study) have also been used in a trial conducted by Richter *et al.* [110]. However, in this case the investigators decided to apply those cells accompanied with an immunomodulatory drug, lenalidomide. They assumed that such a means of treatment might enhance the anti-tumour response, as lenalidomide co-stimulates human T lymphocytes, including the iNKT cells [111]. An important factor in choosing the patients for the trial was a CD1d+ character of myeloma cells, thus sensitive to the iNKT cell lysis. The effects of the administered therapy differed from those observed earlier with α-GalCer monocyte-derived DCs alone. A short-term exposure of six patients with asymptomatic myeloma provided clear data on a decline in the amount of iNKT cells, and a broad immune activation involving iNKT cells, NK cells, monocytes, and eosinophils. The NK cells up-regulated CD56 and NKG2D and increased their number. No toxicity was observed, nor intolerance. The study pointed out promising possibilities of using medicines in future therapies to help with harnessing the immunological response against tumours [110].

Administration of α-linked galactosylceramide-pulsed antigen-presenting cells

Other researches have modified the administration approach by choosing APC cells, obtained from peripheral blood mononuclear cells in the presence of GM-CSF and IL-2, and pulsing them with the α-GalCer. Ishikawa *et al.* [104] applied this strategy for 11 patients with advanced and recurrent non-small-cell lung cancer. The intravenously injected α-GalCer APCs seemed to migrate to the lung and activate the iNKT cells *in situ* [112]. These results are very promising because the above approach could be implicated especially for non-small-cell lung cancer patients after radical surgery. More than half of such patients experience local or distant micro-metastases that are impossible to remove during surgery. Applying such post-surgical immunotherapy could suppress the growth of the micro-metastases. Authors of the study have not observed any major toxicity or severe side effects. In five cases the disease has remained stable [104].

In 2009, Motohashi *et al.* [113] published the results of a phase I-II study with administration of similar APCs. The study included 23 patients with advanced non-small cell lung cancer, but only 17 of them completed the treatment. For most patients (in 10 out of 17 cases), the injection induced a noticeable increase of the amounts of NK and NKT cells in peripheral blood, as well as the produced IFN-γ level. Those for whom the IFN-γ growth was more than two-fold compared to the baseline had an estimated median survival time of almost 32 months. The median survival time of seven patients who were non- or poor responders was only 10 months. The authors concluded that iNKT secretion elevated the IFN-γ level, which may be associated with enhancement of the bearing patients' cancer survival [113, 114].

Uchida *et al.* [106] also picked the α-GalCer-loaded APCs, but injected them into the nasal submucosa of nine patients with HNSCC. The use of this novel route of administration resulted in rapid migration of the APCs into ipsilateral regional lymph nodes and started a regional anti-tumour response. The number of circulating iNKT cells increased in four patients and slightly decreased among others. The quantity of NK cells also increased in most cases. For the first time, an obvious regression of tumour could be observed. Its diameter decreased for one patient from 22 to 7 mm. Another positive aspect is that in five patients the disease has remained stable [60, 106].

In continuation of this study [115], the peripheral blood mononuclear cells collected from patients with head and neck squamous cell carcinoma (here 17) were cultivated with GM-CSF and IL-2, followed by the load of α-GalCer on the day before administration. The injection, however, was applied into the nasal and oral submucosa. Depending on the route of administration, the difference in migration sites could had been observed. The APCs from the nasal route migrated to the lateral neck lymph nodes, whereas those from oral route migrated to the submandibular lymph nodes. A greater increase of the amount of iNKT cells and IFN-γ was detected in patients after the nasal administration. The unique microenvironment of submandibular lymph nodes is rich in Langerhans-like DCs, which tend to induce suppressive cells such as regulatory T cells. This is the probable background of the observed immunological suppression in patients after oral injection [114, 115].

In a subsequent study conducted by Nagato *et al.* [116], the researchers focused on closer examination of tumour infiltration by the iNKT under the influence of α-GalCer-loaded APCs. Four patients with operable advanced lung cancer formed a treatment group, whose outcomes were compared with those from a control group of six patients. α-GalCer-loaded GM-CSF and IL-2-cultured APCs were administered intravenously a week before the surgery, after which tumour infiltrating cells (TILs) were collected from the resected lung tissue. Further analysis revealed a considerable increase of the iNKT cells, especially among TILs, as well as the IFN-γ production in comparison to the control group. The induced iNKT cells' activation and infiltration to the tumour microenvironment contributed to a higher anti-tumour response. Proposed administered post-operative therapy could help to decrease severe toxic effects of chemotherapeutical agents, normally applied after a non-small cell cancer surgery [116].

Administration of activated invariant natural killer T-cells

Patients suffering from cancer have been reported to have not only low levels of iNKT cells, but also a reduced ability of those cells to develop properly. Since iNKT cells can expand after proper *in vivo* stimulation, an idea of creating a clinical trial based on these reports emerged. Motohashi *et al.* [117] published the results of such a study in 2006. In order to obtain the activated iNKT cells, peripheral blood mononuclear cells were stimulated with α-GalCer and IL-2 for up to three weeks. *In vitro* expanded cell fractions enriched with the activated iNKT cells were injected intravenously in two doses. The number of iNKT cells in peripheral blood increased in two out of three cases after the second dose, such as the IFN-γ production. The safety of this therapy was estimated as high; no severe adverse events developed. All six patients completed the study; however, none of them met the criteria of either complete or partial response. After applying this therapy, four of the patients were classified with stable, and two of them with progressive state of a disease. The authors have been following these two patients for nine and 12 months beginning from the end of the trial and confirm that their stable state has been maintained.

It is possible that studies performed on a larger scale or for a longer period of time could have provided more precise and conclusive information. As iNKT cells have a potent antitumor activity both *in vivo* and *in vitro*, the application of the above method of treatment in cancer patients seems to be promising [60, 117, 118].

Combination strategy

On the basis of encouraging results of previous clinical trials, the idea of combination immunotherapy appeared. Such phase I study was performed on eight participants who suffered from HNSCC. The protocol assumed an infusion of α-GalCer-pulsed APCs into the nasal submucosa (twice), followed by the administration of *in vitro* expanded iNKT cells into the tumour-feeding artery. In seven out of eight cases the results showed a significant increase in the number of iNKT cells and IFN-γ-producing cells [119]. Similar positive changes in the level of iNKTs have previously been reported after α-GalCer-loaded APC monotherapy [106]. The benefits of extension by adding the activated iNKT cells included a higher level of IFN-γ producing cells, and hence significant antitumor activity. This approach was well tolerated by all the participants, three of whom revealed strong partial response, and four were classified with stable disease [114, 119].

The combination therapy was also applied in a designed phase II study with participation of 10 patients suffering from HNSCC. The observed changes in the amount of the iNKT cells, especially within the tumour-infiltrating lymphocytes, led to measurable clinical effects: five out of 10 patients demonstrated tumour regression. Such good results encourage further research in this direction [120].

Conclusions

Studies conducted in recent years have revealed the existence of a certain regularity among many patients suffering from different cancer types: a low amount of iNKT cells. Moreover, surgical tumour removal or radiotherapeutic approach did not restore the iNKT cell levels [121]. Research has often linked this observation with poor clinical outcomes in patients with malignancies [18], noted for instance in cases of HNSCC [92]. A low level of iNKT cells infiltrating the tumour tissue of colorectal cancer or neuroblastoma has also been associated with worse survival capabilities [94]. Therefore, the current approaches focus much attention on deepening our knowledge of the iNKT biology and methods of rejuvenating the proper amount and functional condition of the iNKT cells in tumour-bearing patients. Identification of the best possible agonists that could successfully activate iNKT cells without inducing anergy (a state when cells do not respond on activation) has also become a very important issue [54]. For now, only α-GalCer is used in clinical trials. Under its influence, the activated iNKT cells mediate in the anti-tumour response with other elements of the immune system, e.g. NK cells. In a paper published in *Clinical Cancer Research*, Giaccone *et al*. [99], for the first time in history, have applied α-GalCer in humans. The intravenous injection temporarily reduced the number of circulating iNKT cells for at least one week and increased the GM-CSF and TNF-α levels, but only in patients with a relatively high pre-treatment iNKT level [99]. Although this method did not result in any significant clinical effect, it has laid the foundation for others to emerge and follow in this direction. Researchers have cultured different types of APCs, loaded them with α-GalCer, administered, and examined during clinical trials. DCs transpired to have a superior ability to present antigens and express costimulatory molecules on their surface, which enables antitumor properties of the used ligand [54]. The matured forms of DCs provided higher expansion of IL-12 and IFN-γ levels in the serum [105]. Other undertaken courses of action have been focused on combination therapy or different routes of administration: intra-arterial, submucosal, and sub-nasal [106, 115, 117, 119, 120]. The outcomes indicate an increased level of the iNKT, as well as IFN-γ-producing cells in blood, compared to the α-GalCer APCs level in monotherapy [106]. However, there remains much to be determined: the optimal internal route of administration and the interval between the doses or the appropriate agonists, which would induce iNKT activation without driving them into an anergy state. Efforts should also be made to approach the iNKTs with type II NKTs and learn more about their possible interactions during immunosurveillance.

So far, all immunotherapeutic approaches based on α-GalCer usage in patients suffering from different tumours have appeared to be safe and well tolerated by the patients. No severe adverse events have been reported. It is now clear that iNKT cells possess valuable anti-tumour activity against many types of cancer and are able to induce a total or at least partial clinical response, even for aggressive tumour types. This is a substantial promise of changes in tumour-fighting therapies that might be implicated in the near future. Immunotherapy affecting the iNKT cells could have been used after radical surgery in non-small cell lung cancer patients in order to suppress growth of irremovable micro-metastases [104]. Unfortunately, the clinical responses observed at present in many trials have not been consistent and predictable. Further studies are required for the realisation of a widespread clinical administration dream.

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