



The Role of Invariant Natural Killer T Cells in Cancer

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Authors' contributions

This work was carried out in collaboration between all authors. Authors HN and EM designed and wrote the manuscript. Authors EDB, EVV and KV revised the manuscript. All authors read and approved the final manuscript.

Review Article

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ABSTRACT

Invariant natural killer T (iNKT) cells are a unique subset of T lymphocytes that recognize glycolipid antigens presented by the class I-like non-polymorphic histocompatibility complex (MHC) molecule, CD1d. They express both innate and adaptive immune cells' surface receptors, but act more like cells of the innate immune system. Although iNKT cells represent a relatively small population of T lymphocytes, they can rapidly produce copious amounts of cytokines after activation which can polarize different axes of the immune response. Many glycolipid agonists have been discovered of which the marine sponge-derivative called α -Galactosylceramide (α -GalCer) is a potent ligand for iNKT cells. iNKT cells have been described by many researchers as a critical immunotherapeutic target characterized by having tumor-suppressive potential. However, their actual role in immune responses is still unclear. In addition, the need for appropriate preclinical models that mimic human diseases is important for better understanding the iNKT cell biology. This review describes the characteristics of iNKT cells and their role in immunotherapy in cancers such as multiple myeloma and how they can interact with the components of the neighbouring environment.

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1. NATURAL KILLER T CELLS: GENERAL OVERVIEW

Natural killer T (NKT) cells are T lymphocytes expressing the receptors of the adaptive immune system, but in reality reacting as cells of the innate immune system. Therefore, they can easily harmonize the functions of both innate and adaptive immune systems [1,2]. These innate T lymphocytes express both T cell surface receptors (CD3 and $\alpha\beta$ -TCRs) and NK cell surface receptors (NK1.1 in mouse or CD161 in human, NKG2D, CD44, CD56, CD69, CD94, CD122 and the Ly49 family) and recognize self and foreign [3,4] lipid cognate antigens in the context of the non-polymorphic CD1d of the class I-like major histocompatibility complex (MHC) molecules [5]. The innate-adaptive potential of iNKT cells can profoundly modulate different pathological statuses such as cancer, autoimmunity, allergy and microbial infections [3,4,5,6]. The highest frequency of murine iNKT cells is found in the liver [7]; they have more anti-tumor protective capacity than NKT cells from other tissues [8]. iNKT cells can be found in spleen, bone marrow, thymus, lymph nodes and blood but in lower frequency compared to liver and with variable functionality [8,9]. In humans, it is reported that the highest prevalence of iNKT cells is found in the omentum [10], where it represents 10% of the T cell population, while in human peripheral blood it represents between 0.1-0.5% of the T cell population [1,9] taking into consideration the high variability between individuals [3].

1.1 NKT Phenotypic Classification

Different populations of NKT cells are well-known [11], the most interesting classical population is the invariant NKT (iNKT), also called type I NKT cells. iNKT cells are a thymus-dependent population differentiated from conventional T cells at the double positive (DP; CD4⁺CD8⁺) thymocyte stage, late during T cell ontogeny [1,3,12]. They interact with CD1d-glycolipid complexes on DP cells in the thymic cortex during thymic development to express their canonical TCR. Self glycolipid antigens were identified such as the lysosomal glycosphingolipids Isoglobotrihexosylceramide (iGb3) [13,14] and β -D-glucopyranosylceramide (β -GlcCer) [15] and recently the peroxisomal derived lipid plasmalogen lysophosphatidylethanolamine (Plasmalogen lysoPE) [4,16]. However, a study by Pei et al. suggested that endogenous antigens for mouse NKT cells may not be glycosphingolipids [17]. The iNKT cells achieve their innate-like characteristics during thymic development under the direction of the transcription factor promyelocytic leukaemia zinc finger protein (PLZF) [18,19]. PLZF can direct the iNKT phenotype through chromatin modification in association with culin 3, an ubiquitin ligase [3,4,20]. Furthermore, homotypic interactions between the receptors of the signalling lymphocyte activation molecule (SLAM) family members are involved in part in iNKT maturation [3,4,21]. Other transcriptional factors are also involved such as T-bet, retinoic acid receptor-related orphan receptor- γ t (ROR γ t) and GATA-binding protein 3 (GATA3) [4]. iNKT cells migrate to the peripheral tissues where they can continue their maturation [3]. In addition to the recognition of endogenous antigens, iNKT cells can recognize exogenous synthetic glycolipid antigens such as α -Galactosylceramide (α -GalCer) by presentation through CD1d molecules [22,23]. This leads to the activation of their unique semi-invariant TCR α -chain encoded by V α 14.J α 18 paired with V β 8.2/ β 7/ β 2 in mice and V α 24.J α 18 paired with V β 11 in humans [3,24]. The development of α -GalCer loaded CD1d tetramers allowed specific detection, isolation and characterization of iNKT cells [25,26]. It is now well known that α -GalCer is the prototype

ligand that causes proliferation, cytokine secretion and apoptosis of iNKT cells [27,28,29]. iNKT cells can be divided into different sub-populations according to their CD4/CD8 expression; in mice and humans CD4⁺ and double negative (DN;CD4⁻CD8⁻) populations are present. A further small CD8⁺ (CD8 α and CD8 $\alpha\beta$) population is found in humans [9,24,30,31]. It is described that CD8 α ⁺ iNKT cells secrete more Interferon gamma (IFN- γ) than the other sub-populations [32]. In mice, most of the liver and spleen iNKT cells express NK1.1 and interleukin (IL)-12 receptors and can be either CD4⁺ or CD4⁻, this sub-population of iNKT is called Th1-like iNKT cells [4,33,34]. Th1-like iNKT cells produce IFN- γ and small amounts of IL-4 and require IL-15 for their development [33]. Other sub-populations of iNKT cells called Th2-like and Th17-like iNKT cells have been described [34,35,36]. A novel subpopulation of iNKT cells are the iNKT_{FH} cells which produce IL-21 and who help B cell responses against lipid-containing antigens, similar to T_{FH} cells [37,38,39]. Type II NKT cells are a non-classical CD1d-restricted subset that express more diverse $\alpha\beta$ -TCRs and are non-reactive to α -GalCer, but respond to other lipids such as sulfatide, β -Glucosylceramide (β -GlcCer)(C24:0) and β -Galactosylceramide (β -GalCer) [40,41,42,43]. Of this group, two sub-populations have been identified in mice; namely CD4⁺ and CD4⁻, whereas CD4⁺ is the most predominant [41,44]. Many express NK1.1, as they represent 25-28% of the sulfatide/CD1d tetramer⁺ cells in the liver and spleen of mice, while in the thymus most of the sulfatide/CD1d tetramer⁺ cells do not express NK1.1 [41]. Some studies indicated the regulatory role of these cells in tumor immunity as they can functionally oppose iNKT cells [45]. Another subset of NKT cells is termed type III NKT cells or NKT-like cells and although they express NK1.1 receptors, they are CD1d-independent and are thereby not reactive to glycolipid antigens [11]. The characteristics of the different (sub) types of NKT cells are summarized in Table 1.

1.2 NKT activation pathways

It has been described that iNKT cells can be either activated directly or indirectly [3,46]. The direct pathway occurs through microbial or viral-derived cognate glycolipids that are processed by CD1d on antigen presenting cells (APCs) such as the regular dendritic cells (DCs), macrophages and B-cells. These cognate antigens can be recognized directly by the invariant TCR of the iNKT cells; thus, inducing a strong TCR signal that leads to the production of both IFN- γ and IL-4 [3,47] (Fig. 1A). In addition, the direct pathway can occur through exogenous antigens such as α -GalCer.

The indirect activation of iNKT cells involves cytokine secretion by APCs such as IL-12, IL-18 and type I IFNs (α/β) in response to the activation of pattern-recognition receptors (PRRs) of the toll-like receptors (TLRs) on the APCs together with presentation of self-antigens through CD1d [3,47]. This activation occurs through the engagement of some microbial products to the TLRs, which are considered as non-cognate antigens to the iNKT cells and lead to IFN- γ secretion [47]. In some cases, indirect activation strengthens weak interactions of endogenous or self glycolipid antigens with iNKT cells (Fig. 1B) [47,48].

Table 1. Characteristics of type I and II NKT cells and their sub-populations

Characteristics	Type I NKT (also called iNKT, Classical NKT)			Type II NKT (also called Non-classical NKT)
CD1d-restricted	Yes			Yes
TCR-chain	V α 14.J α 18 paired with β -chains of V β 8.2, 7 or 2 (mice) V α 24.J α 18 paired with V β 11 (humans)			Diverse (e.g. V α 3.2J α 9 and V α 8 (mice))
Antigens	α -GalCer and its analogues Microorganism-derived glycolipids Self glycolipids (e.g. iGb3)			Sulfatide β -GlcCer β -GalCer
Frequency in PB	0.2-0.5% (mice) 0.1-0.5% (humans)			low, but higher in humans than mice
Sub-populations	Th-1 like iNKT (CD4 ⁺ , DN) In humans, a small portion of DN expresses CD8 ⁺ (referred as CD4 ⁻ CD8 ⁺)	Th-2 like iNKT (CD4 ⁺)	Th-17 like iNKT (DN)	(CD4 ⁺ , CD4 ⁻ (mice)) CD4 ⁻ was reported as CD4 ⁻ CD8 ⁺ , but no CD4 ⁻ CD8 ⁻ was reported
NK1.1 (CD161) expression	+	-	-	+/-
Cytokine receptor	IL-12R	IL-25R	IL-23R	-
Major cytokine secretion	IFN- γ , IL-4	IL-4, IL-10, IL-13, GM-CSF	IL-17, IL-21, IL-22	IFN- γ , IL-4
Major tissue distribution (mice)	Liver, spleen	Lung	Lymph nodes, lung	Liver, spleen

iNKT cell, invariant natural killer T cell; TCR, T cell receptor; α -GalCer, alpha-Galactosylceramide; β -GlcCer, beta-Glucosylceramide; β -GalCer, beta-Galactosylceramide; iGb3, Isoglobotrihexosylceramide; PB, Peripheral blood; Th, T helper; DN, double negative; IL-R, Interleukin receptor; IFN- γ , Interferon gamma; IL, Interleukin; GM-CSF, Granulocyte/macrophage colony stimulating factor. (Ref. 1, 3, 4, 11, 39, 40, 82).

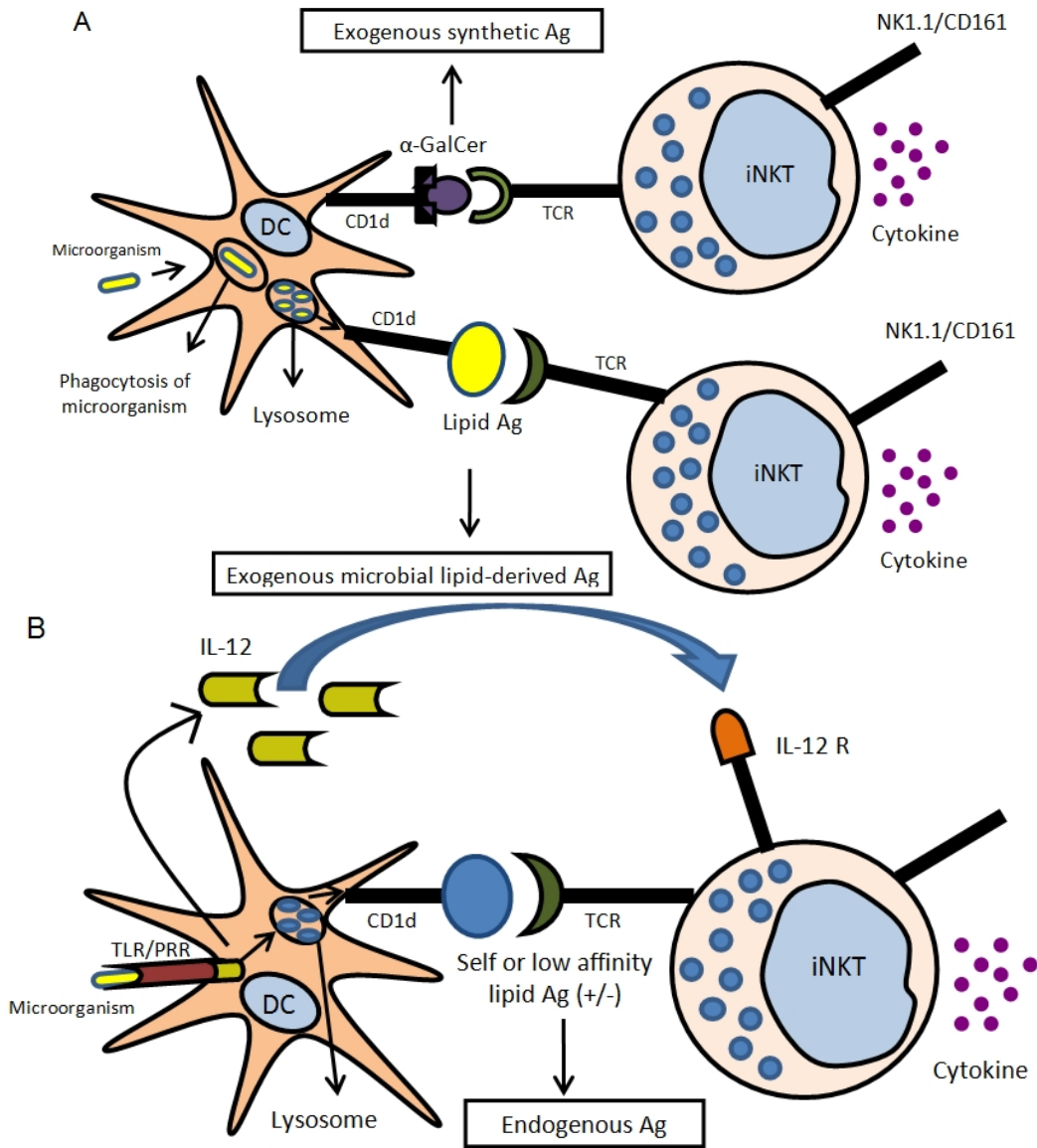


Fig. 1. Major pathways of iNKT cell activation

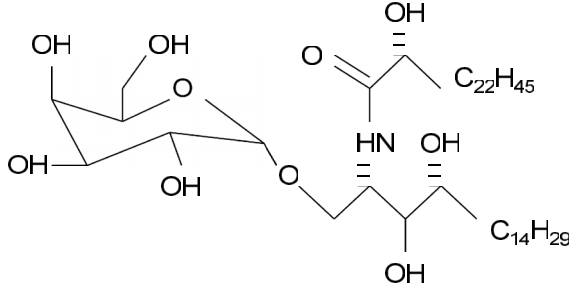
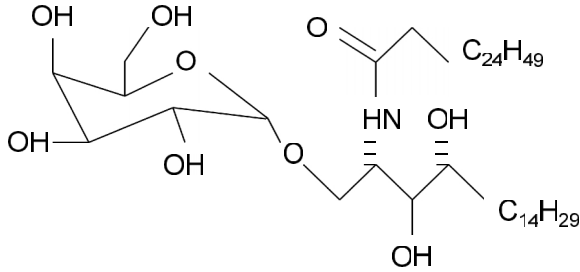
(A) Direct activation of iNKT cells by conjugation of the TCR of the iNKT with the CD1d molecule of DCs. In this case, the DC presents exogenous glycolipids such as the synthetic α -GalCer or the microbial-derived Ag and activates iNKT in the absence of co-stimulation. This activation is CD1d dependent and can lead to IFN- γ and IL-4 cytokine secretion. (B) Indirect activation of iNKT cells by cytokine secretion of DCs such as IL-12. Engagement of microbial Ag to pattern-recognition receptor (PRR) of the toll-like receptor (TLR) on the APC triggers IL-12 co-stimulation. IL-12 secreted by DCs binds to its receptor on iNKT cells (IL-12R) and activates iNKT. The activation that occurs in the presence or absence of self or low affinity lipid Ag can lead to IFN- γ secretion. Besides IL-12, other cytokines may be secreted by APC such as IL-18 and type I-IFN (α & β) and can activate iNKT in a CD1d independent manner. iNKT, Invariant natural killer T; DC, Dendritic cell; TCR, T cell receptor; α -GalCer, Alpha-Galactosylceramide; Ag, Antigen; IL-12, Interleukin 12, IL-12R; Interleukin 12 receptor; TLR, Toll-like receptor; PRR, Pattern-recognition receptor; IFN, Interferon.

Recently, additional mechanisms for iNKT cell activation have been described during inflammation (reviewed in [47]). Namely, iNKT activation can be promoted by diverse stimulatory and co-stimulatory signals such as the induction of CD1d expression on APCs by engagement of peroxisome proliferator activated receptor (PPAR) γ through bacterial products such as lipopolysaccharide (LPS) or by cross linking class II MHC complexes with TCR V β chains e.g. V β 8.2 on iNKT cells through superantigens such as staphylococcal enterotoxin B (SEB). Other signals may involve activation of iNKT Fc γ receptors by antigen-IgG complexes, interaction of NK1.1 receptors with their counter-parts on APCs, activation of TLRs of previously TCR-activated iNKT cells and engagement of T cell immunoglobulin-like mucin-like (TIM)-1 expressed on iNKT cells to phosphatidylserine on apoptotic cells [47].

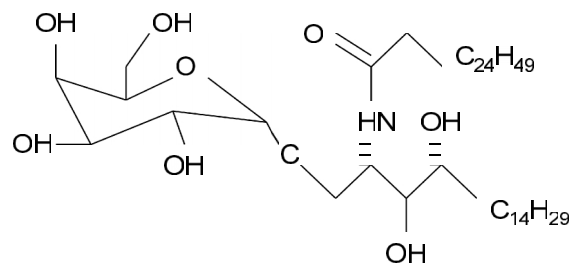
1.3 Polarization toward Th1 or Th2, iNKT Cell Ligands: α -GalCer and its Derivatives

The discovery of α -GalCer allowed better understanding of the TCR of iNKT cells. α -GalCer, also known as KRN7000, is a synthetic glycolipid analogue to the natural compound Agelasphin-9b which was originally extracted from the marine sponge *Agelas mauritianus* or from microorganisms symbiotic with the sponge [22,23,49,50]. KRN7000 or (2S,3S,4R)-1-O-(α -D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol is a glycosphingolipid with an α -anomeric linked galactose molecule which has a molecular formula of C₅₀H₉₉NO₉ [6,22]. α -GalCer which acts as a potent agonist for TCR was first examined in a B16 melanoma model in C57BL/6 mice and showed potent anti-tumor activities [23]. It can elicit both Th1 (IFN- γ) and Th2 (IL-4) cytokine secretion [27,28,29]. Since then α -GalCer has been described as a useful agent for cancer therapy [23] as it can react with both human and murine iNKT cells [27]. In general, naturally occurring iNKT lipids are classified into two groups: α -linked ceramide-containing glycolipids derived from the cell wall of gram-negative *Sphingomonas* species (recently called *Novosphingobium*) and glycerol-containing glycolipids derived from *Borrelia burgdorferi* and *Streptococcus* species [3,4,47]. The α -linked glycosidic bonds between the carbohydrate head and the lipid backbone of these compounds provide an antigen that can be recognized by iNKT cells [4]. Using α -GalCer as an immunomodulatory therapeutic agent is useful but caution is needed. Firstly, its high affinity to TCR makes it highly recognisable by iNKT cells which causes a cytokine storm followed by a strong anergy phase [51]. This cytokine storm can lead to unpredictable and variable patterns of immune responses after administration [51,52]; Secondly, as a long-term therapeutic agent, it has its flaws as it can be metabolized by glycosidase enzymes [51,52]. Several new derivatives of the α -GalCer glycolipid have been synthesized by modifying the sugar moiety and/or truncating the aliphatic tails [29] such as α -C-GalCer, naphthylurea 6"-derived α -GalCer (NU- α -GalCer), C20:2 and OCH [3,29,53,54,55,56,57,58,59]. Some can strongly elicit Th1 responses, while others elicit a Th2 response. The different analogues of α -GalCer are summarized in Table 2.

Table 2. Summary of different α -GalCer derivatives and their modification

Name	Structure	Modification	Biased Immune Response	Ref.
Agelasphin-9b		Parent molecule isolated from an extract of okinawan marine sponge <i>Agelas mauritianus</i>		[21] [45] [46]
α - GalCer		A prototype ligand for iNKT cells synthesized from Agelasphin-9b	Th1-Th2	[21] [22] [26]

α -C-GalCer



O-Glycosidic bond is replaced by CH₂-based glycosidic bond

Th1

[3]

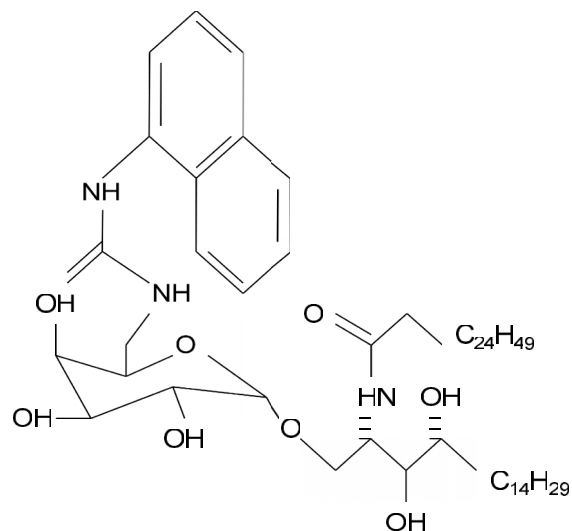
[49]

[50]

[51]

[54]

Nu- α -GalCer



6'-derivatized galactose modified α -GalCer analogue

Th1

[52]

C20:2		C20 fatty acid with cis-unsaturation at position 11 & 14	Th2	[3] [53] [54]
OCH		Truncated analogue of α -GalCer, truncation of sphingosine	Th2	[3] [28] [53] [54]
Plakoside A		Naturally occurring glycosphingolipid similar to α -GalCer isolated from the marine sponge <i>Plakortis simplex</i> , different analogues are synthesized from it	Th1	[55]

ThrCer		<p>Non-glycosidic lipid comprising ceramide. The absence of alcohol moieties at C(5) and C(6) results in an ether linked to sugar alcohols with four carbons</p>	Th1	<p>[3] [54] [56]</p>
HS44		<p>Non-glycosidic aminocyclitolic ceramide analogue. Sugar moiety is replaced by carba- cyclitol and O-glycosidic bond is replaced by amino bond</p>	Th1- Th2	[48]

Many newly discovered pharmacological compounds can lead to the expansion and activation of iNKT cells. A novel non-glycosidic iNKT agonist called HS44 was recently generated. It is an aminocyclitol ceramide analogue in which the O glycosidic bond is exchanged by an amino bond [52]. Although it has a weaker TCR affinity compared to α -GalCer; it is able to induce a robust IFN- γ secretion with less Th2 response, as well as a specific efficient iNKT immune response in a B16 melanoma lung metastases model [52]. The other non-glycosidic compound threitolceramide (ThrCer) was shown to activate both human and murine iNKT cells and prime Ag-specific T and B cells [60]. To search other new agonists, Inafuku et al. examined the anti-metastatic effect of the naturally occurring glucosphingolipid β -GlcCer on murine liver tumor cells [61]. They found that intraperitoneal administration of β -GlcCer (C16:0) enhanced NKT activation and IFN- γ production from intrahepatic NKT cells. Loading of irradiated E μ -myc tumor cells with the α -GalCer analogue β -mannosylceramide (β -ManCer) induced prolonged anti-E μ -myc lymphoma protection. The cytokines IFN- γ , IL-12 and TNF- α were not detected 24 hours after vaccination compared to α -GalCer, indicating that different anti-tumor mechanisms may be involved [62].

2. HARNESSING INKT CELLS IN CANCER IMMUNOTHERAPY

2.1 NKT Immunosurveillance

Many trials have shown the possibility of recruiting the innate phenotypic characteristics of iNKT cells as an immunotherapeutic tool in malignant, infectious, inflammatory and autoimmune diseases [5,6,63,64] despite their small numbers and their semi-invariant nearly monospecific TCR repertoire [4]. The function of iNKT cells in tumor suppression has been reported in many murine models and in some human studies, and is mainly executed through their invariant canonical TCR receptors [65]. Studies using J α 18 and CD1d knockout mice that lack iNKT cells and CD1d-dependent NKT cells respectively have proven this hypothesis [65,66,67]. Upon activation of iNKT cells through APCs, different cytokines (including IFN- γ , tumor necrosis factor alpha (TNF- α), transforming growth factor beta (TGF- β), IL-2, IL-4, IL-6, IL-13 and some production of IL-17), chemokines (including macrophage inflammatory protein (MIP-1 α) and RANTES) and growth factors (including GM-CSF) are intensively produced [3,6]. These cytokines can activate other mediators of the immune system as shown in Fig. 2.

This would suggest the immunoregulatory role of iNKT cells in various immune responses. For example, the proinflammatory Th1 cytokines such as IFN- γ increase anti-tumor and anti-microbial immunity, while the anti-inflammatory Th2 cytokines such as IL-2 decrease autoimmunity [6]. Furthermore, iNKT cells can exert a direct cytolytic activity against tumor cells through granzyme B, perforin, FasL and others [3,6]. Many hypotheses have been offered to explain the paradoxical activities of these cells [57]. The presence of different functional iNKT subsets that respond to different stimuli could lead to this differential outcome [9,57]. A specific iNKT subset may also respond differently to a specific stimulus based on the antigenicity of the ligand or the context in which it is presented. For example, DCs provide a strong CD1d/TCR signal and if this is combined with a strong agonist ligand, it increases the potential of a Th1 over a Th2 immune response. Other non-professional APCs are more likely to elicit a Th2 response [57]. The antitumor activity of iNKT cells was demonstrated in different models of cancer [68]. However, the paradoxical role of iNKT cells as immunosuppressive cells can be observed depending not only on the strength of the antigenic signal, but also by the cytokine profile secreted by the presenting cells [57,68]. For instance, the pro-inflammatory IL-12 which is produced by myeloid DCs positively triggers a

Th1 response, while the anti-inflammatory IL-10 secreted by myeloid DCs in case of weak antigenic signalling triggers a Th2 response [57,68]. The suppressive effect of NKT cells on CD8+ T cells has also been reported [6,69]. This is mediated by reducing CD8+ T cell cytotoxic activity through the production of IL-13, which signals through the IL-4R-STAT6 pathway [69]. Besides APCs, certain tumor cells also express CD1d, as well as some non-hematopoietic cells [57]. However, not all tumor cells have CD1d molecules; it can be poorly expressed or downregulated during iNKT immunosurveillance [67].

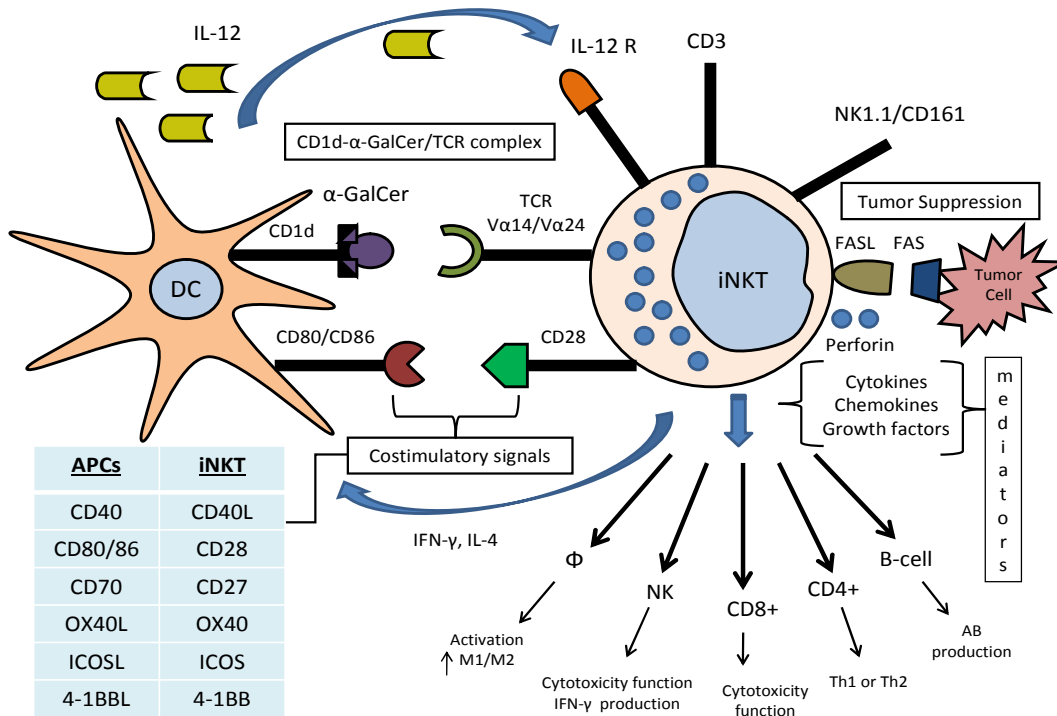


Fig. 2. Interaction of iNKT cells with other cells of the innate and acquired immune system

DCs stimulate iNKT cell activation through TCR engagement, co-stimulatory molecule ligation and soluble cytokine secretion. Activated iNKT cells can exert a cytolytic function through FasL, perforin and granzyme B. They release a storm of soluble mediators such as cytokines, chemokines and growth factors and activate other immune cells. Collectively, these events can modulate the immune system and accelerate tumor rejection. iNKT, Invariant natural killer T; DC, Dendritic cell; APCs, Antigen presenting cells; TCR, T cell receptor; α -GalCer, Alpha-Galactosylceramide; Ag, Antigen; IL-12, Interleukin 12; IL-12R, Interleukin 12 receptor; TLR; Toll-like receptor; PRR, Pattern-recognition receptor; IFN- γ , Interferon-gamma; IL-4, Interleukin 4; FASL, FAS ligand; Φ , Macrophage; NK, Natural killer; Th, T helper; AB, Antibody.

Interestingly, hematological malignancies such as multiple myeloma (MM) were found to express high CD1d levels but most of the solid tumors are hidden to iNKT cytotoxicity [65]. It has also been reported that iNKT cells can mediate tumor killing by downstream activation of other effector bystanders such as NK cells, CD4+ T cells, CD8+ T cells and B cells (marginal zone of B cell) and vice versa [58,67,70]. NKT cells exert indirect effects when activating these cells [6] by inducing the secretion of different cytokines such as IL-2, IL-12 and IFN- γ [70,71,72,73]. iNKT cells were found to regulate the secondary antigen-specific CD8+ and

CD4⁺ T cell immune responses [74,75]. Activated CD8⁺/CD4⁺ T cells were adoptively transferred into J α 18 and CD1d knockout mice and no antitumor immune responses were observed compared to their recipient mice, indicating the regulatory antitumoral role of NKT cells [74,75]. Furthermore, iNKT cells can be stimulated not only in a CD1d-dependent manner but also by co-stimulatory receptors. Also, activated iNKT cells can induce DC activation and maturation by upregulation of surface co-stimulatory molecules such as CD40, CD27, CD80, CD86, CD70, ICOSL and OX40L [57,58,76,77,78,79] and can induce them to secrete soluble mediators such as the Th1 cytokine IL-12 [58,80]. iNKT cells themselves obtain an overexpression of CD40L and IL-12 receptor on their surfaces, which leads to large IFN- γ production [65,76,81]. The role of IFN- γ in anti-tumor activity was demonstrated in studies using immunodeficient IFN- γ knockout mice, which showed no immune response upon α -GalCer activation [82].

2.2 iNKT in Malignancies

2.2.1 Preclinical studies

Injection of α -GalCer has been shown to induce a powerful activation of iNKT cells in mice that leads to a robust secretion of cytokines such as IFN- γ and IL-12 and consequently a rapid downstream activation of NK cells, DCs and the cells of the adaptive immune system which results in tumor inhibition [67,74,75]. Nevertheless, a defect in iNKT number and/or function has been observed in tumor models. Furthermore, iNKTs failed to respond to additional doses of α -GalCer which is pointed out as anergy and is associated with poor prognosis in patients with solid and hematological malignancies [83]. The majority of iNKTs undergo activation-induced cell death after stimulation with α -GalCer [26]. The role of programmed cell death (PD)-1 in induction of anergy of iNKT in α -GalCer treated mice was clear in PD-1 deficient mice compared to wild type [84]. It was found that activated iNKT cells rapidly overexpress the inhibitory costimulatory receptor PD-1 on their surfaces which can last for approximately one to two months [84,85]. The nature of this anergy is unclear and it may be due to the lack of co-stimulatory signals expressed on DC surfaces due to the presentation of α -GalCer by non-professional APC [58,67,86,87]. Other evidence indicated the possibility of iNKT migration from blood to other surrounding tissues or their ability to down-regulate their TCR-receptor post-activation [58,88]. In contrast to preclinical studies, studies in patients reported that iNKT cell number was higher in certain types of cancer such as primary colorectal carcinomas and intrahepatic tumors which may indicate the role of iNKT cells in immunosurveillance [89,90]. However, the loss of IFN- γ production from iNKT cells was still evident in patients with melanoma and renal cell cancer [83]. Their circulating myeloid DCs reduced the activity and changed the Th1 cytokine profile of iNKT cells [83].

The anti-metastatic effect of 3 subsequent intraperitoneal injections of α -GalCer in murine lung and liver metastasis models was mainly due to consecutive secretion of IFN- γ by iNKT and NK cells and not due to their cytotoxic activity through perforin, Fas-ligand or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). The antimetastatic activity disappeared in immunodeficient mice lacking NKT cells; however, to identify the mechanisms involved in this process more investigation is needed [91]. Other studies compared the effect of the route of α -GalCer administration on iNKT cell activation in mice and found that repeated intradermal injections were more potent than intravenous delivery in terms of preventing iNKT anergy and priming specific immune responses. The DCs present in the skin may namely conjugate with α -GalCer and migrate to the lymph nodes, leading to the activation of iNKT cells residing in the skin-draining lymph nodes (DLN). Free α -GalCer may also travel directly to the skin DLN, conjugate with proper APCs and induce iNKT cell

proliferation [87]. Several approaches have been done to overcome iNKT anergy. For example, α -GalCer was introduced in the context of DCs to enhance iNKT activity against different tumors [92,93,94,95]. Other groups showed that using antibodies to block the PD-1/PD ligand 1 (PD-L1) pathway after α -GalCer iNKT activation was able not only to prevent anergy but also to reactivate non-responding iNKT cells [85] and enhance the anti-metastatic activity of α -GalCer [84]. In this case the combined therapy of α -GalCer and PD-1/PD-L blocking antibodies was sufficient to enhance IFN- γ production and to reduce the number of lung nodules in a B16 melanoma lung metastases model [84,85].

2.2.2 Clinical trials

As preclinical studies have established the background for iNKT cell-based immunotherapy, clinical trials are still under investigation with some initial success in the treatment of certain cancer types. Treatment with free α -GalCer, α -GalCer pulsed mature or immature DCs, transfer of ex-vivo expanded iNKT cells or a combination has been performed in several clinical trials with cancer patients such as squamous cell carcinoma, renal cell cancer, head and neck cancer, MM and other metastatic tumors with mixed results [79,96,97,98]. For example, manipulation of DCs can lead to the activation and expansion of human iNKT cells as observed with iNKTs of melanoma patients. In this study, CD1d/IL-12 transduced allogenic DCs derived from an acute-myeloid leukemia cell line were loaded with specific Ag and α -GalCer. The stimulated iNKT cells produced a large spectrum of IFN- γ which was effective in activating Ag-specific CD8+ CTL in vitro [99]. The impact of these methods on the expansion of IFN- γ -producing iNKT cells from cancer patients despite their low number and functional defect could be of benefit to fight cancer clinically [100]. The same was also observed in 4 patients at stage IIB or IIIA of advanced non-small cell lung cancer (NSCLC), who received a single intravenous injection of α -GalCer loaded autologous APCs 7 days prior to operation [98]. This pre-operative administration significantly induced the infiltration of tumor infiltrating lymphocytes and V α 24 iNKT cells into the primary tumor site and augmented IFN- γ production by these cells. This adjuvant immunotherapy and increased IFN- γ production had a positive prognostic value and positive impact on the tumor microenvironment [98]. In another study, intravenous administration of α -GalCer loaded peripheral blood mononuclear cells (PBMNCs) cultured with IL-2/GM-CSF was administered 4 times to phase I/II advanced or recurrent NSCLC patients [101]. NKT cell number was highly increased during the first two injections, indicating that the endogenous expansion of NKT cells occurred once during the treatment course, while PBMNCs were able to produce IFN- γ in response to α -GalCer immunotherapy even during later injections. Therefore, the study suggested that the increase in IFN- γ producing cells, detected in the peripheral blood was correlated to the prolonged median survival time in well responding patients (31.9 months) compared to poor-responding patients (9.7 months) [101]. In advanced cancers such as anal cancer and renal cell cancer, intravenous injection of α -GalCer loaded mature DCs led to sustained expansion of iNKT cells in the blood of the patients that could be detected 6 months after vaccination [94]. These results open the door to use iNKT cells in combined immunotherapy.

3. CELLULAR COMMUNICATIONS OF INKT IN THE TUMOR MICROENVIRONMENT

The interaction between tumor cells and the microenvironment promotes the progression of tumor development. The contribution of iNKT cells in organizing the tumor microenvironment is still unclear. In recent years, a type of innate immune cell called tumor-associated

macrophages (TAMs) was shown to play a role in inducing angiogenesis in certain types of cancer [102] such as MM [103]. TAMs, when activated by MM cells and mesenchymal stromal cells, secrete cytokines, growth factors, proteolytic enzymes and inflammatory mediators to enhance tumor survival and progression [104]. iNKT cells on the other hand, together with NK cells, can suppress the production of pro-angiogenic factors produced by TAMs such as vascular endothelial growth factor (VEGF) and basic-fibroblast growth factor (bFGF) [67,104]. Song et al. demonstrated a novel mechanism through which human V α 24 iNKT cells mediate antitumor activity by killing TAMs in the tumor microenvironment. TAMs and their myelomonocytic precursors namely secrete IL-6 which induces tumor growth of primary and metastatic CD1d⁻ neuroblastoma cells in patients [105]. iNKT cells infiltrate these tumor sites where they can recognize and kill CD1d⁺ TAMs, the only CD1d expressing cell in the tumor tissue [105]. Moreover, TAMs can represent endogenous CD1d ligands and are therefore primed to be destroyed by iNKT cells [105]. In contrast, another study in neuroblastoma showed a novel mechanism explaining how tumor cells can escape from V α 24 iNKT cell immunosurveillance. TAMs are recruited by tumor cells mostly under hypoxic conditions and as such are used as traps for iNKT cells. The activation of the NF- κ B signalling pathway induces TAMs to secrete the chemokine CCL20 at the tumor site, which in turn, attracts iNKT cells to the hypoxic niches. Hypoxia causes a defect in iNKT function and viability [106]. It was however found that IL-15 protects iNKT cells from hypoxia and enhances their anti-tumor characteristics, therefore, this defect can be reversed by adoptive transfer of IL-15 over-expressing iNKT cells [106].

Recently, another type of immune cell termed myeloid-derived suppressor cell (MDSC) has been investigated. It can induce tumor cell growth by suppressing the immune response in different types of cancers such as MM and lung cancers [107,108,109,110]. An increase in the MDSCs was reported in the blood and BM of patients with active MM compared to healthy donors [111,112] and they specifically suppress T cell responses [103,112]. It has been shown that iNKT cells play a role in reducing the number of MDSCs in melanoma; this strategy of tumor immunosurveillance could be promising in controlling cancer cell proliferation [107]. MDSCs can be controlled by iNKT cells in several ways such as the reduction of nitric oxide synthase 2 (NOS2) and arginase 1 production as well as the decrease in the production of NO and reactive oxygen species (ROS) [58,113]. Activation of these pathways by MDSCs leads to an inhibition in T cell proliferation and function [113].

iNKT cells have also been shown to interact with CD4⁺CD25⁺ T regulatory (Treg) cells. Treg cells are found to exert an inhibitory effect on iNKT cells in mice and humans [114,115]. Activation of NKT cells by α -GalCer-loaded DCs was diminished when Treg cells were added to the co-culture [114]. Moreover, combining α -GalCer injection with Treg depletion in a murine mammary breast cancer model increased the survival rate from 44% (α -GalCer treatment alone) to 85% (combined treatment) [114]. In cancer patients (such as ovarian carcinoma), the number of Treg cells is often raised and correlated with poor prognosis [116]. This is consistent to what has been observed in MM patients at diagnosis, where the number of Treg cells is elevated, together with impairments of antigen-specific immune responses [111]. In contrast, another study showed that α -GalCer stimulated CD4⁺ NKT cells were able to enhance Treg cell proliferation via IL-2 secretion [117].

Another cross-talk exists between NKT and NK cells and this has been demonstrated in cytotoxicity studies where α -GalCer-activated NKT cells were able to indirectly provoke the cytotoxic activity of NK cells by the secretion of IFN- γ and IL-2 [118].

Angiogenesis refers to the formation of new blood vessels from pre-existing vasculature either in healthy conditions or disease [119,120]. Angiogenesis in solid and hematological tumors has been described as a crucial step for tumor progression and metastasis [121,122]. A study by Hayakawa et al. indicated a critical role for iNKT cells in reducing tumor angiogenesis [123]. This study suggested that the inhibition of the subcutaneous tumor growth of B16-BL6 melanoma in α -GalCer-treated mice was caused by a reduction in angiogenesis mediated by IFN- γ secretion by both NKT and NK cells [123,124]. Furthermore, inhibition of endothelial cell (EC) proliferation was observed when ECs were co-cultured with splenic or hepatic mononuclear cells isolated from α -GalCer-treated mice in membrane-separated wells. This inhibitory effect was blocked when an anti-IFN- γ antibody was added to the culture [123]. In general, the two-way interaction between iNKT cells and other cellular components in the tumor microenvironment is still unclear and needs more investigation.

4. INKT AND MULTIPLE MYELOMA

4.1 Multiple Myeloma

MM is a monoclonal B-cell malignancy caused by the accumulation of malignant, terminally differentiated plasma cells in the bone marrow (BM); it is characterized by production of high levels of monoclonal antibodies detectable in the serum and urine, bone lesions, angiogenesis and kidney failure [125,126]. The MM cell most likely arises from a post switch memory cell or plasmablast. Whether this transformation occurs in the lymph nodes, peripheral blood or BM, the cells need to (re) enter and spread over the BM. The process of migrating from the vascular to the extravascular compartment of a tissue is called "homing". (reviewed in [127]). Once in the BM, MM cells interact with the microenvironment and induce signals for survival and proliferation. These signals mainly involve insulin-like-growth factor-1 and IL-6. [128]. Currently, different treatment strategies have been designed for MM. These regimens include different combinations of the conventional anti-MM agents (such as melphalan and dexamethasone) and novel agents (such as bortezomib, thalidomide and lenalidomide) [129]. At the moment, standard high-dose melphalan is conventionally used together with the novel agents as induction therapy to reduce the tumor burden, followed by autologous stem cell transplantation depending on the status of the patient [129]. Interestingly, the immunomodulatory drug lenalidomide was shown to enhance iNKT cell function and Th1 response and this effect was increased by combination with dexamethasone [95,130]. Despite the many clinical trials to eradicate the disease, MM is still incurable for most patients and more efforts are needed to find new immunotherapeutic targets. Combination of chemotherapy with immunotherapy could be an important tool for maximizing antitumor effects.

4.2 Preclinical Models of Multiple Myeloma

There is a need for preclinical immunocompetent mouse tumor models to study and improve novel therapeutic approaches and to predict the efficacy of such therapies. The obtained data can then be further translated into clinical settings. Below you can find an overview of currently developed immunocompetent myeloma mouse models, which can be used for the study of iNKT cell functionality and targeting in MM.

4.2.1 The 5TMM model

4.2.1.1 5T33MM and 5T2MM

The 5TMM models evolved from spontaneously developed MM in elderly C57BL/KalwRij immunocompetent mice and are propagated by intravenous injection of the 5TMMvv cells into young syngeneic recipient mice [131]. These immunocompetent models not only recapitulate the properties of the human disease, but they can also be used to examine the potency of new therapeutic targets [132]. The MM cells home to the BM and hematopoietic extramedullary sites [133]. Two important series of 5TMM models are the 5T2MM and 5T33MM models. They have been characterized in terms of homing, migration, proliferation, inducing angiogenesis and osteolytic lesions, spiking of serum M-protein and organ dysfunction [134]. The 5T2MM mice are more restricted to the BM and display osteolytic lesions while the 5T33MM model is similar to advanced MM and shows a more aggressive status [135]. The rapid tumor development in 5T33MM is reminiscent of a relapsed disease [136].

4.2.2 Genetically engineered models

*4.2.2.1 Vk*MYC model*

Vk*MYC is a preclinical murine model of MM in the C57/BL6 mouse strain genetically engineered to activate the c-myc oncogene under control of the kappa light chain (Vk) promoter. The ability of the C57/BL6Vk*MYC immunocompetent model to activate the MYC transgene in the germinal center of B-lymphocytes, albeit sporadically, results in the spontaneous development of MGUS, ultimately leading to MM [137,138]. It mimics the biology of human MM in several ways including BM homing and communication with the BM microenvironment, bone disease and anemia. It is used to test the activity of drugs used in MM therapy and to predict their clinical outcome efficacy. Interestingly, it can also be used as a model for relapsed refractory human MM resembling the aggressive end-stage of the disease by transplanting Vk*MYC tumor cells into congenic mice [133,138,139].

4.2.2.2 E μ XBP-1s transgenic model

This model represents a genetically engineered transgenic model that expresses X factor box binding protein-1 spliced isoform (XBP-1s) in B cells and plasma cells [140]. The XBP-1s transcriptional factor can regulate endoplasmic reticulum (ER) stress and plasma cell development [140]. The E μ XBP-1s is more prominent in human MM compared to healthy plasma cells [141]. Importantly, this model can spontaneously develop features similar to MGUS. Aging E μ XBP-1s mice have the potential to develop MM and so represent certain characteristics of MM such as the presence of bone lesions, serum M-spike and Ig deposition [140]. Preclinical studies using this model enable the investigation of dysregulated gene pathways involved in MM such as cyclin D1, gp130 and MAF [140].

4.3 Role of iNKT in Multiple Myeloma

We recently described the number and activity of iNKT cells during the development of MM in the murine 5T33MM model [142]. Our results clearly demonstrated a dramatic drop of iNKT number in the liver and spleen of 5T33MM mice at the end stage of the disease. A decline in iNKT number was also observed in the 5T2MM model. This was consistent to what was found in MM patients, as we saw a significant decrease in circulating iNKT in MM

patients of relapsed disease compared to healthy donors. We also showed the ability of murine iNKT cells to secrete IFN- γ in response to α -GalCer loaded mature DCs which was abrogated at the end stage of the disease. This reduction was due to a decline in NKT number and not due to the down regulation of CD1d expression. We furthermore observed that treatment with α -GalCer loaded DCs significantly increased the survival of 5T33MM mice from 22 days to 29 days when they were injected on the same day of tumor inoculation [142]. Mattarollo et al. has also shown in the Vk*MYC model that a single vaccination of irradiated Vk*MYC tumor cells pulsed with α -GalCer is efficient to inhibit MM development and prolong survival of mice [62].

iNKT cells and their role in MM was also profoundly described by the group of Dhodapkar [143]. They showed that V α 24⁺V β 11⁺ iNKT cells were detectable in the blood and tumorbed of MM patients at either early or progressive stages of the disease. Unfortunately, the cells lose their ability to produce IFN- γ at later stages. Many reasons can lead to this dysfunction such as presentation of α -GalCer by non-professional APCs or presentation of tumor-derived glycolipids by CD1d-expressing tumor cells or APCs [143]. iNKT dysfunction was also demonstrated in other patients with advanced cancers, indicating the proliferative deficiencies of iNKT cells in cancer [144,145]. α -GalCer loaded DCs in vitro were shown to induce expansion of iNKT cells from MM patients and to improve their functions [143]. They furthermore demonstrated that iNKT cells have a direct cytotoxic effect by killing CD1d-expressing primary myeloma cells and myeloma cell lines in an α -GalCer dependent manner [143]. Spanoudakis et al. and others have shown that CD1d expression is down-regulated in patients with advanced MM and this would impact negatively on tumor survival [146]. However, we observed that the expression of CD1d in the 5T33MM model was still high at the end stage of MM [142] and despite their loading with α -GalCer they lacked the potency to activate iNKT cells and cause tumor cell lysis. We also found that the 5T33MM cells seem to miss the necessary co-stimulatory molecules such as CD40, CD80 and CD86 [142]. Another study by Chang et al., introduced α -GalCer loaded DCs to 3 MM patients at stage III. The intravenous injection of α -GalCer loaded mature DCs but not unloaded DCs led to more than 100 fold expansion of circulating iNKT cells and sometimes BM iNKT cells in patients who had received chemotherapy and stem cell transplantation, resulting in a sequential decrease in the serum or urine levels of M-protein [94]. In one MM patient there was a sustained expansion of iNKT cells that lasted for more than 3 months after vaccination [94]. The activity of iNKT cells led to an increase of IL-12 p40, IP-10 and MIP-1B in the patient's serum levels. This IFN- γ production suggests in vivo iNKT activation which in turn is important in activating more myeloid DCs and attracting more T-cells [94]. Another group succeeded in activating and expanding CD1d-restricted iNKT cell lines isolated from MM patients at different stages of the disease [95]. Their results clearly indicate the ability of iNKT cells to secrete Th1-biased cytokines in response to α -GalCer loaded DCs or primary MM cells and to display a direct cytotoxic effect against primary MM cells. Furthermore, combination of iNKT immunotherapy with immunomodulatory drugs such as lenalidomide, led to increased Th1/Th2 cytokine secretion [95]. The group of Dhodapkar has shown that lenalidomide increases NKT expansion in the presence of α -GalCer in both healthy donors and MM patients and stimulates more IFN- γ secretion by NKT cells [130]. Recently, they investigated the synergistic effect of α -GalCer loaded DCs and lenalidomide on NKT cells and other cells of the innate immunity in MM patients [147]. They found that combination therapy enhances iNKT cell activity and provides more pharmacologic costimulation to the downstream NK cells, monocytes and eosinophils by upregulating surface receptors such as NKG2D, CD56 and CD16, ultimately leading to tumor suppression [147]. All these data indicate the feasibility of using iNKT cells as a tool to predict the clinical outcome in MM

patients with emphasis on the possibility of using MM cells as a target of iNKT cell immunotherapy [143].

5. FUTURE PERSPECTIVE AND CHALLENGES

Activation of iNKT cells is important to harness the innate characteristics of these cells toward immunotherapeutic approaches. The role of iNKT cells in immunosurveillance is not completely understood particularly because the participation of other players such as TAMs, MDSCs and type II NKT cells can affect the final portrait of iNKT cell-cell talk. Moreover, the presence of several endogenous self antigens that can activate different pathways can also confound the cell-cell talk. Several successful preclinical trials have been performed but the challenge is to translate these benefits into clinical trials. Human iNKT cells are less frequent than murine iNKT cells and are more variable between individuals. Their variable distribution among tissues, the different populations and subpopulations, their versatile reactivity against ligand agonists and the different APCs involved in Ag presentation add more to this complexity and can explain the paradoxical role of iNKT cells. However, great efforts have been made to amplify the outcome of iNKT activity by using many methods including vaccination using appropriate APCs, ex-vivo and in vivo expansion of iNKT and in some cases adoption. Combination therapy using α -GalCer and activating monoclonal antibodies against costimulatory molecules such as CD40, CD28 and 4-1BB [79] could be promising. α -GalCer is a powerful agonist but on the other hand, it causes anergy after activation which hampers immune responses. The discovery of new potent derivatives of α -GalCer therefore seems to be a fruitful goal for the coming future. Specifically, agonists with less affinity that can in conjugation amplify the proper immune response, either a Th1 or Th2 response, would be very useful. As angiogenesis also plays an important role in tumor progression, NKT therapy might reduce tumor burden through secretion of cytokines such as IFN- γ . To fully understand the biology of iNKT cells and their relationship with various components of the neighbourhood, preclinical animal models that mimic the human disease, are still needed. This would enable the study of the mechanisms behind iNKT activity and would open the door to extensive treatment studies. By this strategy, better understanding of the nature of these cells can lead to new therapeutical approaches in the treatment of cancer.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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