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Review Article

iNKT cells and hematopoietic stem cell transplantation: Two-phase activation of iNKT cells may improve outcome

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A B S T R A C T

Invariant natural killer T cells (iNKT) produce large amounts of different cytokines which can influence differentiation, polarization and activation of immune cells, particularly NK and T cells. iNKT have been shown to suppress GvHD and promote anti-tumor and anti-pathogen immunity. There are highly specific and safe synthetic ligands such as alpha-galactosylceramide (α-GalCer) and C20:2 which activate iNKT cells toward relatively Th1 and Th2 pathways, respectively.

Bone marrow transplantation (BMT) or 'hematopoietic stem cell transplantation' (HSCT) is effective for leukemia and lymphoma through 'graft-versus-leukemia' (GVL) immunity. However, frequent serious complications include graft-versus-host-disease (GVHD), opportunistic infections and relapse. Both GVHD and GVL are mediated by T cells. Manipulating iNKT by different lipid analogues in early and late phases after transplantation may suppress GVHD and graft rejection and enhance GVL effect, as well as resistance to opportunistic infections and so, could be a novel and effective strategy for improving HSCT outcome.

1. Introduction

Leukemia and lymphoma remain among the most common life-threatening malignancies around the world and every year, many lives are taken by them despite therapeutic improvements. Significant improvements in the treatment of blood malignancies have been achieved in the latter part of the 20th century, which is largely due to advances in chemotherapy and radiotherapy methods. Several new drugs have been introduced as well as new uses for established drugs. Despite all this progresses, treatment outcome for advanced disease is still disappointing [1,2].

In the search for more effective treatments or even cures for hematological malignancies, bone marrow transplantation (BMT) and (with the introduction of mobilized peripheral blood stem cell products) the more recently adopted general term 'hematopoietic stem cell transplantation' (HSCT) have been introduced [3,4]. HSCT has improved survival in a number of hematologic diseases, including lymphomas and plasma cell dyscrasias such as Multiple Myeloma [4–6]. Regardless of all the achievements, allogeneic transplantation either using bone marrow or peripheral blood progenitor cell is associated with high risk of graft rejection, graft versus host disease (GVHD), opportunistic infections and recurrence of malignancy due to insufficient immune response against residual malignant cells, a response which is called graft versus leukemia (GVL) or graft versus tumor effect (GVT) [5]. Among all the problems which may occur after BMT or HSCT, GVHD and relapse are of most import.

Graft-versus-host disease (GVHD) is an immune-mediated reaction and a major complication following allogeneic hematopoietic stem cell transplantation (HSCT). It can affect between 40 and 60% of patients, depending on host and donor factors, and accounts for 15% of mortality after HSCT [7]. Although extremely rare, GVHD may also occur after transfusion of blood products, after solid organ transplantation, and even after autologous HSCT [8]. After BMT or HSCT, graft resident immune cells particularly T cells, recognize host as foreign and attack the recipient, who is usually in an immuno-compromised or "defenseless" state. In this situation, the invader T cells produce an excess of cytokines and cause inflammation and damage to organs, leading to poor prognosis among patients. On the other hand, GVL or GVT is an immune response against residual malignant cells, not the recipient.

In GVL/GVT, the grafted donor T cells attack the remaining malignant cells, thus this response is therapeutic and lowers the risk of relapse and improves prognosis. In both GVHD and GVL, T lymphocytes including CD4+ helper T cells (Th) and CD8+ cytotoxic T cells (CTL), are major players and both are classified into two functional subsets,

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Th1 and Th2 as well as Tc1 and Tc2 [9,10].

These subsets, originating from a common precursor, express different chemokine receptors and may perform different effector functions dependent on the cytokines they produce. Th1 cells mainly produce type I cytokines like IL-2 and IFNγ, whereas Th2 cells secrete type II cytokines such as IL-4, IL-5, and IL-10 [11,12]. In a similar manner, the cytotoxic CD8+ Tc1 and Tc2 cells differentially secrete the type I and type II cytokines, respectively [13]. Finally, NK cells also have a critical role in maintaining GVT in allogeneic bone marrow transplantation [14].

After HSCT, distinct subsets of T cells which are different in their cytokine spectrum can mediate and regulate immune responses related to transplantation and residual malignant cells. GVHD and graft rejection are mediated by Th1/Tc1 T cells and IFNγ cytokine [15], but in contrast, alloreactive Th2/Tc2 T cells and cytokines such as IL-4, 5, 10, TGF-β can suppress GVHD and graft rejection and at the same time mediate a beneficial GVL effect [16,17].

Separation of GVHD from GVL is the “Holy Grail” of HSCT because the former should be suppressed while the later should be strengthened [18–20]. To date, several strategies have been used to overcome this paradoxical problem including the elimination of graft T cells [21], using NK cells [22,23], delayed administration of T cells after primary graft and so on, but none of them have made a significant difference.

Indeed, we need a modality that gives us the capability of controlling T cell differentiation, because they are the major players of almost every immune response. Changing or influencing the cytokine milieu around T cell precursors can be a smart way because differentiation of Th and Tc precursors depends on the cytokines they are exposed to [24].

In this regard, invariant natural killer T cells (iNKT) can be a very helpful tool. iNKT cells are a rare subset of T cells that share properties of both T cells and NK cells and express a highly restricted T cell receptor which recognizes glycolipid antigens presented by CD1d [25–28]. After activation, iNKT cells rapidly produce large quantities of Th1 and Th2 cytokines such as IFNγ, IL-4, GM-CSF, IL-2, and TNF- β [28–30]. iNKT cells are activated early in an immune response and therefore can influence differentiation, polarization, and activation of a wide array of other immune cells such as dendritic cells, B, NK and T cells (Fig. 1). Beside their immunomodulatory roles, they have some important effector functions and play a pivotal role in a variety of immune responses including host defense, malignancies, autoimmunity, inflammation and graft response [11,12,25].

There is another interesting aspect of iNKT cells which make them an attractive target for immunotherapy and immunomodulation. There are some synthetic and natural glycolipid ligands such as alpha Galactosylceramide (α-GalCer) and its chemically modified analogues, such as OCH and C20:2, which can stimulate iNKT cell differently [11,31–35]. α-GalCer induces a broad immune response with relatively high ratio of Th1 type cytokines such as IFNγ and indirectly IL-12, not only directly from iNKT cells but also subsequently through bystander downstream effector cells such as NK cells (IFNγ) and antigen-presenting / myeloid cells (IL-12). α-GalCer also promotes expression of CD40 ligand and therefore enhances the inherent killer activity of iNKT cells [31]. In contrast, OCH and C20:2, Th2-inducing iNKT ligands, selectively induce iNKT Th2 cytokines, particularly IL-4 and IL-13, while reducing the production of IFNγ and IL-12 by iNKT cells (31–35) (Fig. 1). Interestingly, iNKT can be divided into CD4+ (~ > half), rare CD8+ (undetectable to up to ~ 10%) and double negative (DN) subsets (remainder). In general, the CD4+ subset has the widest cytokine spectrum, DN are more Th1 biased and CD8+ the most Th1 biased, although all respond to the polarizing ligands in the same direction [11,29–39].

There are also some newer modified forms of glycolipids which can induce more potent and/or preferential secretion of Th1 or Th2 cytokines than the parent compound. For example, KRN7000, a synthetic form of α-GalCer used clinically selectively induces relatively large amounts of both IFNγ and IL-4 [11,36], However, some highly Th1-biasing α-GalCer derivatives can induce maximal IFNγ to IL-4 ratios. For example, α-GalCer analogues containing a phenyl group in their acyl tail are more effective than α-GalCer in inducing Th1 cytokines/chemokines as well as human iNKT cell expansion [11,37].

2. The hypothesis

Manipulating iNKT cells by synthetic analogues could be a novel and effective strategy for improving HSCT outcome and with initial clinical safety of KRN7000 [31,35,38,39] and future clinical data on analogues expected, it seems that we are now close to such position.

HSCT is associated with some early complications, including graft rejection and acute GVHD as well as some late problems such as weak or suboptimal GVL/GVT immune response and chronic GVHD. These problems are primarily mediated by different T lymphocytes and downstream by a variety of effector cells. iNKT cells have important modulatory and effector functions in the immune system. Regarding the unique characteristics of iNKT cells and their potent synthetic ligands, manipulation of immune responses involved in GVHD and GVL by iNKT cells is a possible and promising strategy for improving HSCT outcome. In our hypothesis, administration of C20:2 early after HSCT, when rejection and acute GVHD are the most serious complications, activates iNKT cells and induces secretion of substantial Th2 cytokines such as IL-4, which subsequently promote differentiation of Th0 toward Th2 helper cells. IL-4 and Th2 cells actively suppress Th1 pathways and therefore the rate and severity of GVHD and graft rejection decreases/will decrease. In the later stages, when the graft is established and the risk of GVHD and graft rejection decreases, treatment with broad-spectrum α-GalCer clinical form ‘KRN7000’, induces IFNγ and CD40L and promote a powerful Th1 response, which would eliminate either malignant cells or infections agents, while potentially also maintaining some Th2 ‘flavor’ as well, which could ameliorate chronic GVHD. At such a time as chronic GVHD was no longer an issue and/or stronger Th1 response was required such as the common issue of various viral reactivations, even purer Th1 polarizing iNKT ligands could be deployed to further tip the balance toward anti-tumor / anti-pathogen immunity (Fig. 2).

3. Evaluation of the hypothesis

We believe that two-step stimulation of iNKT cells in early and late phase after HSCT with two different synthetic analogues (e.g. C20:2, and α-GalCer or KRN7000 respectively), is a safe and feasible method and will improve the outcome of HSCT in hematological malignancies (Fig. 2). Below, we present some evidence supporting our idea.

3.1. iNKT cells as potential modulators of GVHD

GVHD is mediated by Th1/Tc1 T cells, and reports show a relation between the donor CD_{4+} T cells, Interleukin 2 and GVHD. Th_1 cells induce activation of APCs, which consequently promote GVHD [40,41]. In addition, Tc1 cells can play a role in GVHD via FasL-dependent mechanisms, resulting in increased IFNγ. Th1 polarization of donor T cells predominantly plays a role in inducing the cytokine burst seen in GVHD. In contrast, Th2 polarization mostly suppresses inflammation and reduces the severity of GVHD. One study reports that graft iNKT cells can suppress GVHD, but this cannot occur in IL-4 deficient mice, confirming that iNKT cells mediate this phenomenon through IL-4 and the Th2 pathway. In fact, iNKT cells are able to deviate Th0 polarization toward Th2 cells. Other studies also showed the role of Th2 cytokines in the suppression of GVHD [42,43]. It was reported that treatment with KRN7000, which is a potent inducer of Th1 and IFNγ, exacerbates GVHD, while C20:2, a Th2 inducer analog, suppress both GVHD and GVL responses [44]. Direct injection of IL-4, an inducer of Th2 iNKT cells, to mice receiving allogeneic HSCT, creates a significant reduction

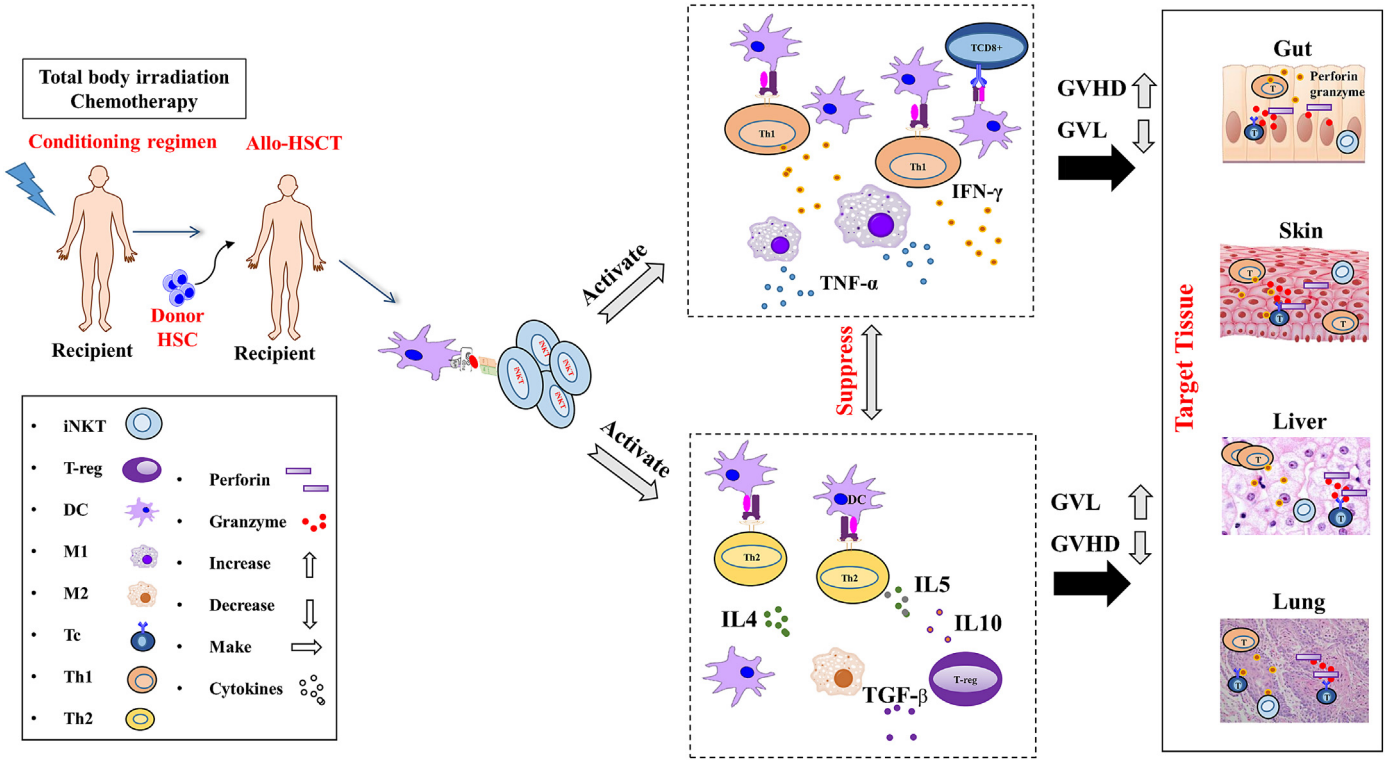

Fig. 1. Balance of GVHD and GVL in allogeneic stem cell tranplant. Th1 and/or Th2 and regulatory cytokines, thereby influencing other immune cells. Th1 (type I) cytokines include IL-2 and IFN-γ, whereas Th2 (type II) cytokines include IL-4, IL-5, and IL-13 and Regulatory cytokines include IL-10. GVHD and graft rejection are mediated by Th1/Tc1 T cells and IFNγ cytokine, but suppressed by allo-reactive Th2/Tc2 T cells and cytokines such as IL-4, IL-5, IL-10 can suppress GVHD and graft rejection and at the same time mediate or at least not inhibit a beneficial GVL effect.

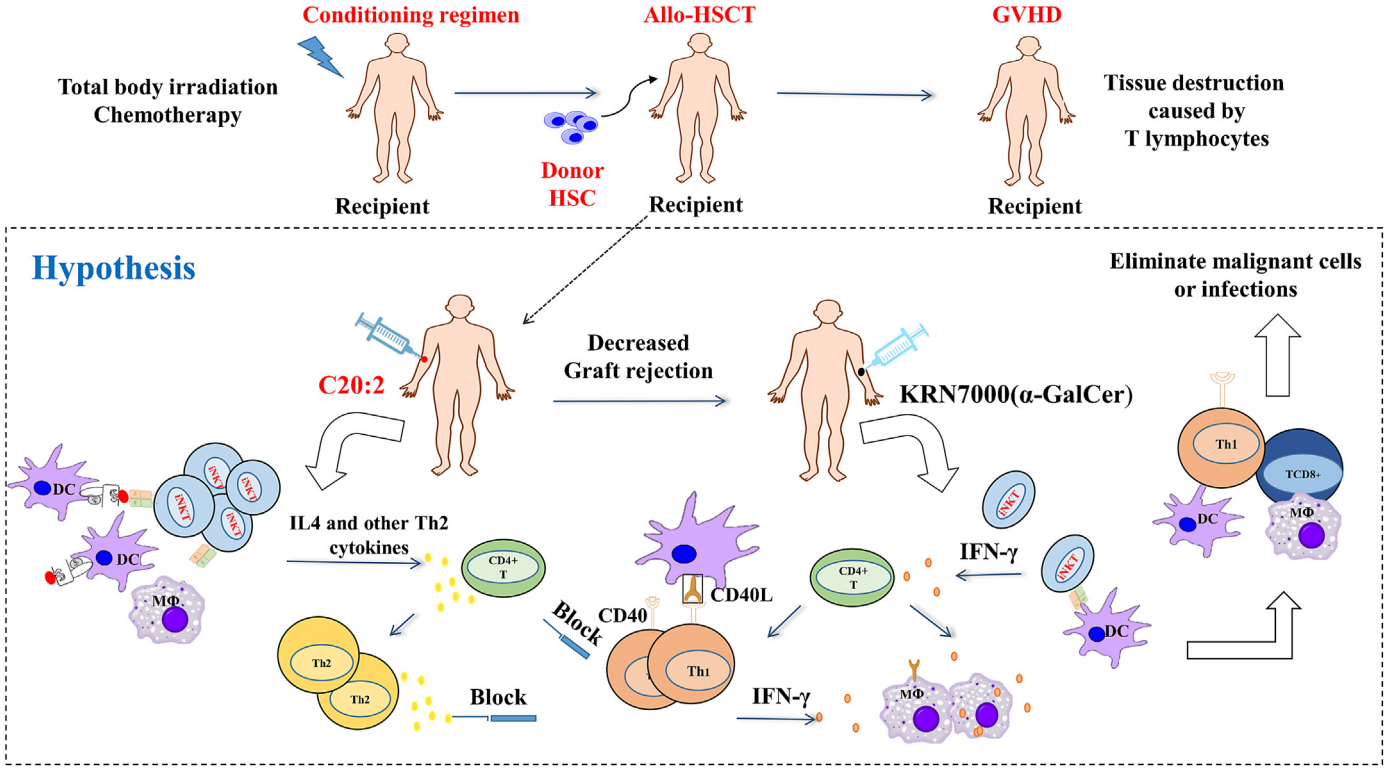

Fig. 2. Novel Approach to exploiting Invariant NKT Cells in hematopoietic stem cell transplantation. iNKT cells are able to secrete large amounts of Th1 and/or Th2 and regulatory cytokines, thereby influencing other immune cells. There are highly specific and safe synthetic ligands such as alpha-galactosylceramide (α-GalCer) and OCH (or for human use, C20; 2), which activate iNKT cells toward relatively Th1 and Th2 pathways, respectively. Manipulating the iNKT by two different analogues in early and late phases after transplantation may suppress GVHD and graft rejection and subsequently enhance GVL effect as well as resistance to opportunistic infections and so could be a novel and effective strategy for improving HSCT outcome.

in the severity of GVHD due to induction of TH2 responses [45].

On the other hand, treatment with C20:2, a potent inducer of IL-4 decrease GVHD drastically (possibly because IL-4 secretion by iNKT cells) promoted the expansion of IL-10–secreting donor regulatory T cells and polarized donor T cells toward a Th2 phenotype thereby decreasing damage in the target organs of GVHD [35,46].

The role of iNKT in suppressing GVHD has been clearly demonstrated in recently published studies [27,45,47]. Thus, stimulation of iNKT cells by OCH, C20:2 or other analogs that induce IL-4 cytokines in early phases after HSCT, can regulate acute GVHD by inducing Th2 polarization and expansion of regulatory T cells. Together, these findings confirm our idea and suggest that stimulation of iNKT cells may be an effective adjunct to other prophylactic regimens against GVHD (Fig. 2).

3.2. NKT cell regulation of the GVL effect

NKT cells have a fundamental role in tumor immunity. Their antitumor effects mediated through three main mechanism including: (i) direct cytotoxic activity against tumor cells through perforin/granzyme pathway (ii) production of pro-inflammatory cytokines such as IFNγ and TNF-α which activate bystander cells like NK cells and/or CTL cells and (iii) activation of antigen presenting cells (APCs) to start anti-tumor immunity.

Several studies reported that treatment with α-GalCer could be beneficial in the prevention of tumor growth and metastasis in mice. This effect may be the consequence of their NK-like cytotoxic activity against tumors or more likely, an indirect action through IL-2 mediated activation of NK cells. A series of studies suggest that iNKT activation by their ligands induce IFNγ and upregulates CD40L; which consequently stimulates dendritic cells to produce IL-2, and finally, IL-12 triggers a second burst of IFNγ secretion by iNKT cells and NK cells and these all together enhances Th1 responses against tumors [46,48,49].

iNKT cells activated by α-GalCer show a potent antitumor activity which is partly mediated by NK cells [50–52]. In vitro studies have revealed that human leukemia cell lines are the most sensitive to killing activity of iNKT cells. On the other hand, some forms of leukemia such as Myelomonocytic leukemia, are also very sensitive targets for iNKT cells due to the expression of CD1d by them [53]. It is also reported that the GVL effect is most effective when iNKT cells are present [54].Dendritic cells loaded with α-GalCer could also induce significant anti-tumor responses in metastatic tumors by activating iNKT and therefore in vitro proliferation and activation of iNKT cells considered a therapeutic strategy in the early stages of some tumors [55]. Taraban has shown that administration of α-GalCer can induce CTL and enhances antitumor responses as well [56]. It is also reported that there is an inverse relationship between a number of iNKT around the tumor and likely of metastasis [57]. Altogether, this evidence confirms that activation of iNKT α-GalCer in the late phase after HSCT can enhance GVL effect by different direct and indirect mechanisms (Fig. 2).

3.3. NKT cells and infectious diseases

Infections are one of the most important causes of post-transplant morbidity and mortality. After HSCT and because of conditioning regimen and/or delay in recovery of B cell and T cell functions, different types of infections including bacterial, viral and fungal occur [58,59]. The risk of mortality is even higher in the post-engraftment period in comparison to the early short post-transplant neutropenia period [57].

NKT cells act rapidly in the early phases of immune response and several pieces of evidence support their participation in the regulation of innate immune responses [60,61]. They play an important role in defense against bacterial and viral infections through induction of the Th1 pathway. They can recognize glycolipid structures in Mycobacterium, Protozoa, *Pseudomonas aeruginosa*, and Listeria. Glyco-Ceramides structures in gram-negative bacteria that lack LPS, also activate iNKT cells [62,63]. iNKT cells are also involved in defense against *Cryptococcus neoformans*, which is an important problem in immunocompromised patients [64,65]. Some Toll-like receptors (TLR) such as TLR 4,7,9 on dendritic cells which recognize lipopolysaccharides and bacterial nucleic acids also indirectly stimulate iNKT cells and induce a strong anti-microbial Th1 response [66]. iNKT cells also play a role in defense against viruses. The role of these cells in response to HSV, which is frequently seen after transplant, has also been demonstrated [67,68]. Some viruses reduce expression of CD1d to protect themselves, indicating the importance of iNKT in defense against these viruses. CMV activates iNKT cells through interaction with TLR9 on DC cells and induction of IL-12 and this is an important phenomenon because CMV infection is a great problem after transplantation and associated with significant morbidity and mortality [69,70].

The protective role of iNKT cells against infections either mediated by their ability to promote Th1 response through secretion of IFNγ or by their indirect effect on NK cells. Many studies have shown that treatment with α-GalCer and its analogs can increase the level of protection against different types of infections [71–73].

This information supports our hypothesis and adds another application for it. Therefore, treatment with α-GalCer or KRN7000 in the late phase after HSCT not only boosts GVL/GVT response but also increase the level of protection against infections (Fig. 2). More highly Th1-biasing α-GalCer derivatives (e.g. 37) might be even more effective, should this be needed.

4. Summary & proposal

Hematopoietic stem cell transplantation is a curative procedure for a number of hematologic malignancies including leukemia and lymphoma. However, GVHD and weak GVL responses are among the main issues in HSCT failure.

Homing, transplant survival and prevention of GVHD are very important after HSCT. Inducing Th2 responses in this period not only can help with grafting but also should decrease the possibility of GVHD. The other concern is to prevent infections and relapses through enhancing the Th1 pathway. Infections typically occur in the several months after transplant, hence the use of extensive anti-infective prophylaxis post-transplant, whereas acute GVHD typically occurs within the first 2–4 weeks.

iNKT cells are critically involved in the development and suppression of various immune responses (Fig. 1). The classic ligand for iNKT cells has been identified as the glycolipid, α-GalCer, which is presented by CD1d molecules both in humans and in mice. Recognition of α-GalCer presented by CD1d molecules stimulates iNKT cells to produce high levels of cytokines, particularly IL-4 and IFNγ. Alternative ligand C20:2 stimulates Th2 pathway induction and should lead to enhanced graft survival, and should also suppress Th1 mechanisms through which GVHD may be induced. Treating with C20:2 or other iNKT Th2 ligands during this critical period of time, ensuring transplant engraftment and suppressing GVHD, we propose to subsequently induce the anti-tumor and anti-infection Th1 pathway by means of broad spectrum α-GalCer. In this phase, this analogue activates Cytotoxic T cells and NK cells indirectly and inhibits infections and promotes GVL immune response. Therefore, we propose that C20:2 will be administered to the patient shortly after transplant; this will activate iNKT and will cause secretion of Th2 cytokines, especially IL-4. Approximately one month after transplant α-GalCer, that stimulates iNKT through more of the Th1 pathway, particularly IFNγ, will be administered. Amounts of iNKT cells, IL-4, and IFNγ serum-levels, inflammation markers, and also liver enzymes, will be measured before and after. Should this approach prove safe, enhance engraftment, reduce GvHD, but not have as potent anti-tumor and anti-infection effects as desired, more Th1 polarizing iNKT ligands (e.g. 37) could be employed in subsequent trials. Consequently, this procedure may not only lessen the main complications due to the transplant but could also improve survival and lessen the recurrence of

malignancy (Fig. 2).

Conflict of interests

Mark Exley receives financial support from and contributes to AgenTus Inc., a cancer immunotherapy company. The potential therapeutic approaches presented are not AgenTus controlled or planned and Dr. Exley has no intellectual property related to clinical use of α-GalCer or derivatives. The other authors have no potential conflict of interest to declare.

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