α-GalCer, AN α-CANDIDATE IN TUMOUR SUPPRESSION

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ABSTRACT

Alpha-Galactosylceramide (α-GalCer, KRN7000) is an exogenous glycolipid ligand that is presented by CD1d molecules in antigen-presenting cells (APCs). It activates invariant natural killer T (iNKT) cells, characterized by semi-invariant T cell receptors (TCRs), which often leads to further downstream activation of the immune system. For example, iNKT cells release cytokines that regulate myeloid-derived suppressor cells (MDSCs) to promote tumor suppression. This critical review aims to clarify the observed effects of α-GalCer by examining recent studies, ranging from in vitro experiments with mice to in vivo clinical trials with humans. Within the current literature, α-GalCer has demonstrated beneficial effects toward tumour suppression. Most pre-clinical studies evaluating α-GalCer have seen success in suppressing tumour growth and increasing patient lifespan, although clinical trials yield inconclusive results. For example, the use of α-GalCer comes with severe limitations, including the induction of immune cell anergy amongst other unwanted side effects. Future studies and trials will be necessary to evaluate the full potential of α-GalCer. Nonetheless, α-GalCer may be a promising agent in combating cancer.

BACKGROUND

One of the most destructive leading causes of death worldwide is cancer, a group of diseases that involve the rapid and abnormal proliferation of cells beyond their usual boundaries.[1] These cells can grow and spread to other organs through metastasis, which is the primary cause of death from cancer.[1] Unfortunately, difficulty targeting cancer cells and the severe side effects of existing treatments make cancer difficult to cure, contributing to the annual death toll of over 10 million patients globally.[1,2] While cytotoxic immune cells are theorized to control tumor development, cancer cells have developed numerous strategies to avoid detection from the immune system.[3,4]

A promising field of immunotherapy research involves iNKT cells and α-GalCer. iNKT cells are a unique type of killer T cell with a semi-invariant TCR and they play important immunoregulatory roles by producing cytokines that influence the actions of other immune cells.[5,6] Almost all iNKT cells respond to α-GalCer, a potent glycolipid agonist that initiates a cascade of strong anti-tumor activity.[5,7] Agelasphins, including α-GalCer, were first isolated by the marine sponge *Agelas mauritianus* and exhibited high *in vivo* antitumour properties against murine B16 melanoma.[8,9] Further investigations showed that α-GalCer was not directly cytotoxic to tumour cells, but rather stimulated the immune system via activation of natural killer (NK) cells.[8,9]

While the fundamental mechanisms and interactions of iNKT cells and α-GalCer are documented, studies have shown mixed results. This critical review will analyze the strengths and areas for further research of *in vitro* studies, murine models, and human clinical trials in this field of research.

INKT MECHANISMS OF ACTION

iNKT cells commonly operate in the tumor microenvironment (TME) by recognizing glycolipid antigens most commonly presented on APCs or tumour cells by the CD1d protein.[10,11] This response mechanism allows for the upregulation of immunosurveillance and activation of downstream effectors.

Stimulated iNKT cells can rapidly produce cytokines and chemokines. One example is interleukin 12 (IL-12), an important cytokine for type 1 T helper (Th1) responses, including cancer protection.[12] It can be produced directly by iNKT cells or through intermediates, such as activated dendritic cells (DCs).[13,14] Cytokines activate or recruit immune cells including NK cells, CD8+ T cells, B cells, macrophages, neutrophils, and MDSCs.[13,15] In particular, NK cell and CD8+ T cell activation is critical for the targeting and lysis of tumour cells, including major histocompatibility complex (MHC) negative tumours.[13,15] These notoriously difficult targets lack peptide fragments that T cells recognize as pathogenic, enabling them to hide from the immune system.[16] Furthermore, iNKT cells can reprogram immunosuppressive tumor-associated macrophages to become inflammatory macrophages, reducing immunosuppression.[15,17] As a result of iNKT cells' wide range of functions, they have been coined the "Swiss Army knife" of the immune system.[14]

However, other cells in the TME can antagonize the antitumour response of iNKT cells. MDSCs secrete transforming growth factor beta (TGF-β), a potent immunosuppressive cytokine that promotes tumour growth.[15] MDSCs also induce anergy in NK and T cells, increasing the rate of metastasis.[15] Additionally, type II NKT cells, which lack the semi-invariant TCR, suppress tumour immunosurveillance.[13] An immunoregulatory axis exists between iNKT and type II NKT cells: these cells operate antagonistically and downregulate each other, which can explain why cancer therapies which neglect to target type II NKT cells have been largely ineffective.[13,15]

APPLICATIONS OF α-GALCER IN INKT PATHWAYS

α-GalCer is well-known as an exogenous glycolipid mediator for potent iNKT response.[6,13] α-GalCer(Bf), an α-GalCer compound, is produced at various sites within humans,

including by the gut bacteria *Bacteroides fragilis* and *Bacteroides vulgatus*.[18-20] Figure 1 summarizes the effects of α-GalCer as an exogenous iNKT cell ligand.

α-GalCer can serve as a "jump start" molecule for iNKT antitumour immune responses.[21] It interacts with iNKT cells primarily through APCs, particularly DCs.[18] Clinical trials involving free α-GalCer or α-GalCer-pulsed DC (DCs with α-GalCer loaded in their CD1d proteins) administration in humans have shown that detection of the α-GalCer antigen by iNKT cells rapidly stimulates their proliferation and production of granzymes (Gzms) A and B.[13,15,18] In this pathway, Gzms A and B promote lysis and apoptosis of CD1d-expressing tumours.[15,18] Furthermore, α-GalCer-pulsed DC reduces the inhibitory nature of MDSCs.[15] DC-mediated T and B cell activation are well-established strategies for effective immune memory development, protecting against tumor relapses.[15] Although there is no dose-limiting toxicity level, free administration of α-GalCer induces anergy in iNKT cells, greatly decreasing the ability of restimulation for up to two months.[13,15,22]

CRITICAL ANALYSES OF α-GALCER STUDIES

α-GalCer studies related to various cancers have been conducted at in vitro and in vivo levels, including human clinical trials. However, the complicated nature of immune pathways in humans has yielded largely inconclusive results; murine studies are often more insightful due to higher levels of experimental control.

In 2022, Li et al. conducted an *in vitro* and *in vivo* study on melanoma and lung cancer tumour-bearing humanized NSG mice.[6] In the *in vitro* approach, α-GalCer administration enhanced cytotoxic function and Gzm B levels in PBMC-iNKT cells, confirming α-GalCer's antitumour capabilities in cell lines.[6] In the *in vivo* approach, transferring human iNKT cells with a single dose of localized α-GalCer resulted in rapid iNKT cell recruitment into solid tumours within 24 hours, causing reduced tumour growth.[6] The treatment group with α-GalCer had higher iNKT cell counts and enhanced cytokine production compared to the PBMC-iNKT and PBMC-T cell negative controls and the phosphate-buffered saline (PBS) placebo control.[6] However, the sample sizes for each test group ranged between 3 and 4 mice, resulting in decreased confidence in the results.[6]

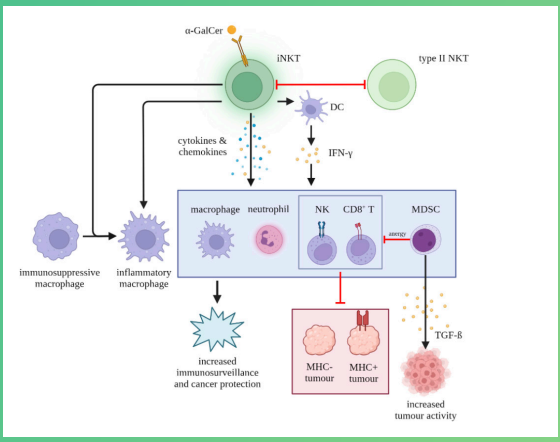

Figure 1: α-GalCer presentation by APCs with CD1d proteins activate iNKT cells, inducing a cascade of downstream cell behavior, resulting in increased immunosurveillance and cancer protection. iNKT cells also upregulate MDSCs, which contribute to NK and CD8+ T cell anergy. iNKT and type II NKT cells are antagonistic to each other.

These results correspond with another murine study conducted by Haghighi et al., which concluded that the combination of α-GalCer and *Lactobacillus casei*, a probiotic species of bacteria, was highly effective against cervical cancer.[23] *L. casei* is a safe probiotic that exhibits anti-cancer properties and may modulate immune responses against solid tumours in humans.[23] The test group received heated cytotoxic type 1 T cells (Tc1s) with α-GalCer and *L. casei* and was compared against a PBS placebo control, a Gardasil human papillomavirus (HPV) vaccine positive control, and various combination treatment groups, allowing for high confidence in results.[23] The treatment group receiving both α-GalCer and *L. casei* with heated Tc1s experienced the greatest increase in pro-inflammatory cytokine stimulation and nitric oxide levels, activating both innate and adaptive immune branches.[23] Further testing should seek to establish the method by which α-GalCer and *L. casei* interact to better understand this combination treatment and investigate additional anticancer probiotics.

α-GalCer is not limited to murine environments. In 2020, Toyoda et al. examined the effects of α-GalCer on an *in vivo*, phase II human trial of non-small cell lung cancer cases.[24] 34 patients were given four doses of α-GalCer-pulsed APCs.[24] Overall, the median survival increased to 21.7 months from the expected 17 months for untreated patients.[24] In addition, only nine patients reported minor side effects, indicating that α-GalCer is a relatively safe treatment for tumour suppression.[24] Despite these positive results, the single-arm design does not provide sufficient confidence in this treatment compared to traditional protocols.[24] Furthermore, the open label design and lack of a placebo group could result in biases in physician and patient reporting of results.[25]

In contrast to these findings, a 2019 study by Biagioli et al. found that α-GalCer induced acute hepatitis in mice, which reduces the viability of α-GalCer as a treatment.[26] However, this study also demonstrated that selective agonists of GPBAR1, a G protein-coupled receptor activated by bile acid, were able to diminish liver damage.[26] Since GPBAR1 regulates liver iNKT activities, there may be additional opportunities to mitigate the negative effects of α-GalCer elsewhere in the body.[26] Furthermore, the concentration of α-GalCer used to induce acute hepatitis was 0.01g/L[26], whereas the concentration used in Toyoda et al.'s human trial was 0.0002g/L.[24]

Overall, α-GalCer shows strong potential as a cancer treatment following *in vitro*, murine, and human models. However, studies should seek to compare α-GalCer-based treatment against traditional approaches in a double-arm design.[27] Appropriate negative control and test groups should be selected to illustrate the relative efficacy of proposed treatments.

NEXT STEPS

Current studies focus on the short-term effects of single doses of α-GalCer, which neglects the long-term impact of anergy. Since cancer patients generally require treatments for up to six months, anergy would result in significantly decreased effectiveness of immune and cellular responses to α-GalCer.[28] Since free α-GalCer induces anergy in iNKT cells, the efficacy of subsequent doses is diminished.[17] To combat this, Fujii et al. found that α-GalCer-pulsed DCs reduced tumor growth and metastasis with minimal induction of anergy.[15,29] α-GalCer analogs are another

alternative avenue of study; analogs such as β-mannosylceramide (β-ManCer) induce almost no long-term anergy of iNKT cells.[30] However, this glycolipid uses a nitric oxide synthase (NOS)-dependent mechanism, raising the need for further research.[13] Because of α-GalCer's broad range of interactions, there is no standard delivery method. For example, the adoptive transfer of iNKT cells may complement α-GalCer treatments since cancer patients often have decreased function and number of iNKT cells.[15,31] Alternatively, while α-GalCer-pulsed DCs are a highly effective delivery method, it is difficult to obtain sufficient quantities of DCs. While this lack of a standard delivery method has opened up various intriguing research pathways to pursue, it clouds objective assessments and comparisons of different trials. Some notable areas for investigation to increase α-GalCer effectiveness include alternative delivery methods, combination treatments, and iNKT cell production. Delivery vectors such as nanoparticles, artificial APCs, exosomes, and liposomes should be explored to address the low efficacy of free α-GalCer administration.[15,32,33]Since chemotherapy continues to be a major component of cancer treatment, studies need to consider the interactions between them. Early studies of head and neck carcinoma suggest that this chemotherapy-α-GalCer combination treatment results in increased iNKT cell and IL-12 production at the cost of increased adverse events.[34] Since α-GalCer modulates iNKT activity, the low quantity of endogenous iNKT cells in peripheral circulation presents a barrier to α-GalCer treatment efficacy in humans.[6] Various pluripotent stem cell-derived iNKT cell production methods should be researched.[15]

CONCLUSION

α-GalCer therapy is a promising treatment pathway for a variety of cancers. α-GalCer's effects have been demonstrated in cell lines, murine models, and human clinical trials, increasing iNKT cell-mediated anti-tumour activity. However, additional research is required to further investigate improved α-GalCer delivery methods and strategies to increase patient iNKT cell counts. Within the broader scope of cancer treatment, α-GalCer treatment will likely be coupled with traditional chemotherapy; the potential adverse side effects of the co-administration of the two therapies should be investigated in the future.

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