Human natural killer T (NKT) cells are characterized by their expression of an invariant T cell antigen receptor α chain variable region encoded by a Va24Ju18 rearrangement. These NKT cells recognize α-galactosylceramide (α-GalCer) in conjunction with the MHC class I-like CD1d molecule and bridge the innate and acquired immune systems to mediate efficient and augmented immune responses. A prime example of one such function is adjuvant activity: NKT cells augment anti-tumor responses because they can rapidly produce large amounts of IFN-γ, which acts on NK cells to eliminate MHC negative tumors and also on CD8 cytotoxic T cells to kill MHC positive tumors. Thus, upon administration of α-GalCer-pulsed DCs, both MHC negative and positive tumor cells can be effectively eliminated, resulting in complete tumor eradication without tumor recurrence. Clinical trials have been completed in a cohort of 17 patients with advanced non-small cell lung cancers and 10 cases of head and neck tumors. Sixty percent of advanced lung cancer patients with high IFN-γ production had significantly prolonged median survival times of 29.3 months with only the primary treatment. In the case of head and neck tumors, 10 patients who completed the trial all had stable disease or partial responses 5 weeks after the combination therapy of α-GalCer-DCs and activated NKT cells. We now focus on two potential powerful treatment options for the future. One is to establish artificial adjuvant vector cells containing tumor mRNA and α-GalCer/CD1d. This stimulates host NKT cells followed by DC maturation and NK cell activation but also induces tumor-specific long-term memory CD8 killer T cell responses, suppressing tumor metastasis even 1 year after the initial single injection. The other approach is to establish induced pluripotent stem (iPS) cells that can generate unlimited numbers of NKT cells with adjuvant activity. Such iPS-derived NKT cells produce IFN-γ in vitro and in vivo upon stimulation with α-GalCer/DCs, and mediated adjuvant effects, suppressing tumor growth in vivo.

Keywords: NKT cells, adjuvant effects, clinical trial, induced pluripotent stem cells, artificial adjuvant vector cells
not conventional αβ T cells or NK cells developed (7). These and other studies confirmed that expression of Vα14/α18 in mice and Vα24/α18 in human is a unique NKT cell signature.

**DISCOVERY OF THE NKT CELL LIGAND**

The ligand for NKT cells was identified as α-galactosylceramide (α-GalCer), which is presented by the MHC class I-like CD1d molecule. However, unlike MHC class I molecule with polymorphic in nature, CD1d is monomorphic among species, indicating that α-GalCer can be used in any potential NKT cell therapy for all humans. The glycolipid nature of the NKT cell ligand was suggested by experiments using mice lacking the transporter associated with antigen processing (TAP), which is essential for translocation of cytoplasmic peptides generated by the ubiquitin-proteasome proteolytic pathway into the endoplasmic reticulum (ER) to make a stable complex with MHC class I molecules. The MHC peptide complex is required to select CD8 T cells, therefore, in TAP-KO mice, CD8 T cells are not generated. However, by RNase protection assays using the invariant Vα14/α18 as a probe, we could detect significant levels of protected bands in TAP-KO mice but not in β2M-KO mice, suggesting that the ligand is not a peptide, but likely to be a glycolipid in conjunction with a β2M-associated MHC-like molecule (8). The MHC-like molecule turned out to be CD1d, which has two large hydrophobic pockets, A′ and F′, that can bind the two long fatty acid chains of the ceramide portion of α-GalCer (9). Therefore, we screened various synthetic glycolipids and found the essential structure-function relationships critical for the NKT cell recognition, such as: (1) α-linkage between the sugar moiety and the ceramide portion of α-GalCer but not β-GalCer, (2) a 2′-OH configuration on the sugar moiety different from α-ManCer, and (3) a 3′-OH on the sphingosine of α-GalCer (10).

Furthermore, by using alanine substitution to mutate genize CD1d, we also identified important amino acids on CD1d, such as Ser76, Arg79, Asp80, Glu83, and Gln153, for activation of NKT cells in mice (11). In 2007, Borg et al. succeeded in crystallizing the triple complex of α-GalCer/human Vα24/α18/TRC/β11/human CD1d (12). Interestingly, the Vα24/α18 chain docks in parallell with the CD1d cleft without any direct contribution of the TRCβ chain to ligand binding. This configuration is quite different from the mode of ligand recognition by the TRCβ chain of conventional αβ T cells, in which only the TRCβ but not the TRCα chain recognizes the MHC bound peptide in a diagonal position.

Analysis of the structure also revealed that the first four amino acids (Asp94, Arg95, Gly96, and Ser97) of Ja18, which are conserved in mouse and human, are essential for binding with both CD1d and α-GalCer. The Ja18/α18 binds with CD1dArg79, Ja18/Arg95 with CD1dArg79/Ser76/Asp80 and the 3′-OH on the sphingosine, Ja18/Arg96 with the 2′-OH on galactose, and Ja18/Ser97 with CD1dGln150. Interestingly, the CD1d amino acid, Glu83, defined in important in functional assays with CD1d mutants, is important for binding with the TRCβ chain to make a stable complex with CD1d but has no direct contribution to the ligand binding itself. Moreover, the CD1d amino acids (Ser76, Arg79, and Asp80) important for binding with either α-GalCer or Ja18 are also well conserved among species such as mouse, rat, sheep, and human (10, 13–15). Thus, α-GalCer, identified as an NKT cell ligand in mice can also be used to activate human NKT cells.

**NKT CELL-MEDIATED ADJUVANT EFFECTS ON INNATE AND ADAPTIVE IMMUNITY AGAINST CANCER**

In general, tumor cells do not contain any adjuvant materials, so that it is difficult to induce proliferation of specific T cell clones to mount anti-tumor responses in patients. On this particular point, α-GalCer overcomes these problems by its intrinsic adjuvant activity, inducing clonal expansion of tumor-specific T cell cells as well as activating various innate cell types (16). In the initial anti-tumor response after stimulation with α-GalCer/DCs, NKT cells immediately produce large amounts of IFN-γ, which acts on DCs, NK cells, and neutrophils in the innate immune system to eliminate MHC negative tumor target cells and, at the same, also on CD8 cytotoxic T cells and CD4 Th1 cells to kill MHC positive tumor cells, resulting in tumor eradication (Figure 1) (1, 17, 18). Therefore, NKT cell-targeted therapy is expected to overcome the major problem of current anti-cancer immunotherapies – recurrent tumors – due to their targeting of only one type of effector cell (10, 19, 20). For example, in the immunotherapy using tumor peptide CTL or antibodies against PD-1 or CTLA4, the target is the CD8 killer T cell, which kills MHC positive but not negative tumor cells, resulting in tumor recurrence (21). Similarly, in the artificial cells recently developed by the forced expression of Rae1/H60 (NKG2D-L), Mult-1 (NKG2D-L), or CD70 (TNF-L), the target cells are NK cells, which will eliminate MHC negative, but not MHC positive tumor cells (22).

Tumors in general contain both MHC positive and negative cells. Therefore, for an optimal therapy, both MHC types of tumor cells should be eliminated simultaneously by activating both innate and adaptive immune responses (Figure 1A). Since only NKT cells, but not other immune cells, activate NK and CD8 killer T cells at the same time, thus eliminating both MHC positive and negative tumor cells, the NKT cell-targeted therapy is a promising strategy for cancer treatment (Figures 1B.C).

**NKT CELL-MEDIATED ADJUVANT EFFECTS ON DC MATURATION**

Another important NKT cell function is their ability to interact with immature DCs in the presence of α-GalCer to induce DC maturation (17). Therefore, NKT cell-targeted therapy is also useful for advanced cancer patients, who often suffer from severe immunodeficiency. DCs in these advanced cancer patients are usually immature because of the presence of immune suppressive cytokines, such as IL-10 or TGFβ, produced by tumor cells (Figure 1A) (23). The immature DCs are able to capture tumor antigens, but unable to activate specific T cells. However, immature DCs presenting α-GalCer are activated by NKT cells through CD40–CD40L interactions to produce IFN-γ, which induce full DC maturation (24). This leads to a robust interleukin (IL)-12 response to further activate NKT cells, followed by activation of CD8 T cells and NK cells (17, 24).

The DC maturation by activated NKT cells is a prominent strategy for the enhancement of protective innate and acquired immune responses. To investigate the mechanisms of bystander potential of α-GalCer-activated NKT cells, an experimental system
FIGURE 1 | Natural killer T cell-mediated adjuvant effects on anti-tumor protective responses and clinical trial outcomes. (A) Mechanisms of NKT cell-targeted adjuvant cell therapy: upon NKT cell activation in patients by α-GalCer/DCs, immature DC become mature, and both MHC positive and negative tumor cells will be killed by CD8 killer T cells and NK cells, respectively. (B) Clinical trials of NKT cell-targeted adjuvant cell therapy on advanced non-small lung cancer: 60% of patients (***) showed significant prolonged median survival time of 29.3 months compared with best supportive care group with a MST of 4.6 months. The response to NKT cell therapy correlated with clinical efficacy (median survival time) and IFN-γ levels; patients with high (**H) levels responded significantly better than those with low (*L) levels. (C) Clinical trials of NKT cell-targeted adjuvant cell therapy for head and neck tumors: all 10 cases treated with the combination therapy of α-GalCer/DCs and activated NKT cells showed significant clinical efficacy (SD or PR). (D) Correlation between clinical efficacy (PR in red, SD in black) of head and neck tumors and NKT cell numbers in the tumor in situ.

Using immunization with OVA-loaded TAP-deficient spleen cells loaded with OVA after permeabilization by osmotic shock was developed. In this system, OVA was used as an artificial tumor antigen to induce OVA-specific CD8 T cells to kill OVA-bearing tumor cells. Only after α-GalCer administration, IFN-γ production by NK and CD8T cells was observed (see Figure 2A). Under these conditions, the clonal expansion of OVA-specific CD8 T cells and strong anti-tumor responses develop in the mice, and the response requires co-administration of α-GalCer (17).

CLINICAL TRIAL OF NKT CELL-TARGETED THERAPY FOR ADVANCED LUNG CANCER AND HEAD AND NECK TUMORS

For effective NKT cell activation, α-GalCer/DC has distinct advantages to induce significant expansion of NKT cells and to inhibit in vivo tumor growth in a mouse model of metastatic lung cancer and liver metastasis in melanoma (25, 26). In a preclinical study, we used mouse melanoma cells, which were injected into the spleen to induce liver metastasis. Treatment of tumor-bearing mice by intravenous administration of α-GalCer/DCs (3 × 10^9) resulted in complete eradication of the liver metastasis within 7 days after treatment (27).

Based on the dramatic effects of α-GalCer/DCs in the preclinical studies, a clinical trial of NKT cell-targeted immunotherapy was conducted at Chiba University hospital in patients with advanced non-small cell lung cancer to evaluate the safety, feasibility, immunological responses, and clinical outcomes (28). Seventeen patients with advanced or recurrent non-small cell lung cancer refractory to the standard treatments, including surgery, chemotherapy, and radiation therapy, completed the protocol. The patient’s peripheral blood mononuclear cells (PBMCs) obtained by apheresis were cultured with GMP grade GM-CSF and IL-2 for 7 days and then pulsed with α-GalCer (29). The α-GalCer-pulsed PBMCs were then intravenously administered (1 × 10^9 cells/m^2/injection) back into autologous patients twice with a 1-week interval followed by two courses with a 1-month interval between the second and third administration.

In the 17 patients who completed the protocol of a phase IIa clinical trial, the treatment was well-tolerated, and no severe adverse events related to the cell therapy were observed (28, 30). To monitor IFN-γ production by NKT cells from the patients, an enzyme-linked immunospot (ELISPOT) assay was performed (31). The results demonstrated that a significant increase in the number of IFN-γ-producing PBMCs was detected in 10 out of
17 patients, which was correlated with a significantly prolonged median survival time (MST; 29.3 months) in comparison with the group with no increase compared to the pretreatment status in IFN-γ-producing cells (MST of 9.7 months) (Figure 1B) (32). The α-GalCer-reactive IFN-γ spot forming cells appeared to include both NKT cells and NK cells (31, 33), consistent with the notion that α-GalCer-activated NKT cells subsequently stimulate NK cells to produce IFN-γ (34, 35). We also investigated NKT cell infiltration in the surgically resected tumor samples and found a significant increase (25- to 60-fold) in the number of NKT cells in the tumor in situ (36). Because of the clinical correlation between increased IFN-γ production and prolonged overall survival, we conclude that IFN-γ may be a good biological marker for predicting clinical efficacy of this treatment. Although this prediction cannot be made prior to α-GalCer/DCs administration, the monitoring of IFN-γ production would still be valuable for patients receiving this immunotherapy. Although none of the cases showed significant tumor regression, the overall MST of all 17 patients (18.6 months) was superior to that of patients with best supportive care (4.6 months) or those treated with other types of therapies (average 10 months) in Figure 1B (37–40).

In the case of the head and neck tumors, we used a combination therapy with α-GalCer/DCs (10⁸) and activated NKT cells (5 × 10⁷) and completed 10 cases, including patients with pharyngeal, laryngeal, esophageal, maxillary, and oral carcinomas, who had advanced or recurrent disease after standard treatments (41). All treated patients showed either a partial response or achieved a stable disease state, indicating significant clinical efficacy (Figure 1C), which was associated with significant NKT cell infiltration into the tumor in situ (Figure 1D). To evaluate
clinical efficacy, a computed tomography (CT) scan was performed a few days before enrollment and also after the treatment. In some cases with partial responses, we observed that the enhanced area decreased in size, and necrosis appeared at the center of the tumor.

These encouraging clinical studies on advance lung cancers and head and neck tumors warrant further evaluation of NKT cell-targeted immunotherapy for survival benefit. In general, the immunotherapy may be more effective in patients with low tumor burden. Currently, we have been conducting α-GalCer/DC therapy for stage IIA to IIBA lung cancer patients with small tumor foci, including remaining micro-metastasis after radical surgery or after receiving the established first-line therapy in collaboration with National Hospital Organization.

**FUTURE DIRECTIONS FOR NKT CELL-MEDIATED CANCER THERAPY USING iPS-DERIVED NKT CELLS**

Although an NKT cell-targeted therapy has been shown to have significant clinical efficacy, only one third of patients are eligible in the case of advanced non-small lung cancer patients; the frequency of NKT cells in the other patients is too low. To overcome this problem, we established in vitro methods for generation of unlimited numbers of functional NKT cells, which then can be transferred into the patients whose endogenous NKT cell numbers are limited.

Induced pluripotent stem (iPS) cells were generated from mature NKT cells using Oct3/4, Sox2, Klf4, and c-Myc genes and then were developed into functional NKT cells in vitro in the presence of IL-7 and Flt3L according to the conventional protocol (42–44). The NKT cells generated in vitro from iPS-NKT cells were functional in the in vivo setting using the experimental model of OVA as an artificial tumor antigen (44). When NKT-KO mice were reconstituted with iPS-derived NKT cells followed by immunization with OVA and α-GalCer, we detected a 70-fold increase in the number of OVA-specific IFN-γ producing CD8+ T cells above that seen in the control mice (Figure 2A). Under these conditions, the growth of the OVA-expressing EL4 (EG7) tumor cells was suppressed (Figure 2B). Thus, the iPS-derived NKT cells are able to function in vivo.

Before any clinical application of iPS-derived NKT cells, two immunological issues need to be addressed, one is whether GvHD is induced by NKT cells and the other is whether semi-allogeneic NKT cells will work in vivo, because of the clinical use of iPS-derived NKT cells under semi-allogeneic conditions. To address the first question, iPS-derived NKT cells on a B6 background and B6 or BALB/c CD4 T cells were injected into BALB/c RAG-KO mice. The results were very clear: only B6 CD4T cells, but not iPS-derived B6 NKT cells or BALB/c CD4 T cells, induced GvHD characterized by weight loss, diarrhea, skin disease development, or death after cell transfer. Concerning the second issue of the functional potential of semi-allogeneic NKT cells in vivo (129xB6) F1 NKT cells derived from cloned ES cells established by nuclear transfer of mature NKT cells into unfertilized eggs were injected into B6 NKT-KO mice and analyzed for their adjuvant activity in the OVA model. Significant proliferation of OVA-specific CD8 killer T cells was detected, even though these cells are eliminated in a few days. The ability to generate NKT cells using a simple in vitro culture system offers a powerful approach for the establishment of optimal NKT cell therapy. Our clinical application of the iPS-derived NKT cell therapy program has now been selected as a Center for Clinical Application Research on Specific Disease/Organ (Type B) in the Research Center Network for Realization of Regenerative Medicine, Japan.

**FUTURE DIRECTIONS FOR THE NEXT GENERATION OF NKT CELL-TARGETED THERAPY**

For the establishment of the next generation of NKT cell-targeted therapy, we developed artificial adjuvant vector cells to induce both innate and long-term memory CD8T cell responses against cancer. In this system, allogeneic NIH3T3 fibroblasts were used as a vector cell, into which tumor antigen mRNA and CD1d with α-GalCer were introduced. In the model experiment, we used OVA mRNA as an artificial tumor antigen together with α-GalCer/CD1d to induce the NKT cell-mediated adjuvant effects in vivo in situ (Figure 2C) (22). The allogeneic artificial vector cells were destroyed by the host immune system soon after inoculation and all materials carried by the cells were taken up by the host DCs, which immediately stimulated host NKT cells followed by induction of DC maturation and also by activation of innate NK cells and adaptive OVA-specific CD8 killer T cells. Surprisingly, long-term memory CD8 T cell responses were induced in an antigen-specific manner and persisted even 1 year after the initial single injection and suppressed OVA-expressing tumor cell metastasis (Figures 2D,E) (45). To test if this method could be generalized, we used TRP-2, tyrosinase related protein-2, which is a weak tumor antigen expressed by both mouse and human melanoma cells as the tumor antigen, and successfully suppressed tumor growth in vivo. Therefore, the artificial vector cells should be useful in the future for vaccines against various tumors.

**SUMMARY**

Natural killer T cells bridge innate and adaptive immunity, which enhances protective immune responses and also establishes long-term memory responses. Therefore, NKT cells have important therapeutic potential. In support of this notion, clinical trials on NKT cell-targeted therapy have demonstrated clinical safety and significant clinical efficacy in terms of prolonged median overall survival time in lung cancer patients and achieved stable disease status or partial responses in head or neck cancer patients.

The powerful treatment options for the future are to establish iPS cells that can generate unlimited numbers of NKT cells with adjuvant activity in vitro and suppress tumor growth in vivo. The other option is to establish the artificial adjuvant vector cells containing tumor mRNA and α-GalCer/CD1d, which have been shown to induce tumor-specific long-term memory CD8T cell responses and to inhibit tumor growth even 1 year after single injection. Thus, these could be therapeutic candidates for the next generation of NKT cell-targeted therapy.

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