Cancer Therapy: Clinical

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Comparison of Clinical and Immunological Effects of Intravenous and Intradermal Administration of α-GalactosylCeramide (KRN7000)-Pulsed Dendritic Cells

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Abstract

Purpose: Human Vα24+Vβ11+ natural killer T-cells (NKT cells) have antitumor activity via direct cytotoxicity and by induction of antitumor actions of T and NK cells. Activation of NKT cells is crucial for their antitumor activity and is induced by α-galactosylceramide (α-GalCer, KRN7000) presented by CD1d on dendritic cells (DC). We conducted a phase I clinical trial of therapy with α-GalCer-pulsed DC to determine safety, tolerability, immune effects and an optimal dose, and administration route.

Experimental Design: Twelve subjects (3 cohorts) with metastatic malignancy received 4 treatments of α-GalCer-pulsed DC, 2 treatments intravenously (IV), and 2 treatments intradermally (ID). Each successive cohort received a log higher cell dose. Clinical and immunological outcomes were evaluated, including secondary effects on NK and T cells.

Results: Substantial effects on peripheral blood NKT cells were observed but were greater following IV treatment. Secondary immune effects including activation of T and NK cells, increases in T- and NK-cell cytoplasmic interferon-γ, and increases in serum interferon-γ levels were seen after IV but not after ID treatment. Therapy was well tolerated, but 9 of 12 subjects had tumor flares with clinical findings consistent with transient tumor inflammation. Disease response (minor) or stabilization of disease progressing up to enrollment was observed in 6 of the 12 subjects. Stabilization of previously progressive disease lasted for at least one year in three subjects.

Conclusion: We conclude that therapy with α-GalCer-pulsed DC induced clinically beneficial immune responses that are highly dependent on cell dose and administration route. *Clin Cancer Res; 17(15); 5140–51. ©2011 AACR.*

Introduction

Immunotherapy, aimed at stimulating tumor-antigen specific T cells *in vivo*, frequently induces a measurable immune response and clinical responses are seen in a proportion of patients (1–3). Improved response rates need to be shown before immunotherapy can be incorporated into mainstream clinical practice. Observations that malignant cells develop resistance to killing by conventional peptide–antigen specific cytotoxic T cells (CTL), for example by downregulation of the target antigen or by disruption of the antigen presentation mechanisms, suggests that activation of additional immune effector cells, with different pathways to cytotoxicity, is required to enhance clinical response rates (4–6).

One candidate for this role is the human lymphoid effector cell population, invariant Vα24Vβ11 T-cell receptor (TCR) expressing natural killer T (NKT) cells (Vα24+Vβ11+NKT cells). Human NKT cells have cytotoxic mechanisms and recognition pathways that are distinct from CTL (i.e., independent of target cell MHC-peptide expression) and natural killer (NK) cells (7). Tumor cells resistant to CTL through downregulation of antigen presentation to CTL would not be predicted to be resistant to NKT cell killing (8,9). Human NKT cells activated by α-galactosylceramide (α-GalCer, KRN7000), presented by CD1d on antigen presenting cells, particularly dendritic cells (DC), have been shown to exert significant antitumor activity *in vitro* and *in vivo*, using murine models (10, 11), against a variety of malignancies, including solid tumors (12–16) and myeloid leukemia (8).

Direct intravenous administration of α-GalCer to human subjects had less antitumor activity than expected from murine data and results in disappearance of peripheral blood NKT cells within 24 hours (17). With multiple α-GalCer doses administered weekly, NKT cell numbers remained below pretreatment levels. These observations suggest that alternative methods for activation of NKT cells are needed for antitumor effects in humans. Direct administration of α-GalCer to mice resulted in less antitumor activity than observed

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Preclinical data suggest that human V α 24Vβ11 NKT cells activated by α-galactosylceramide presented by dendritic cells induce antitumor immune responses. In a phase I clinical trial, we evaluated the effects of administration route and dendritic cell dose on α-galactosylceramide-pulsed dendritic cell stimulation of NKT cells and secondary immune effects consequent upon NKT cell activation.

We show that dendritic cell dose is critical to NKT cell-based immune responses and to secondary immune effects of NKT cell activation. We also confirm major differences in immunological effects between intravenous and intradermal administration. An important clinical observation was the high frequency of inflammatory flares involving observable tumor deposits. Our comprehensive evaluation of the effects of NKT activation on T and NK cells provides mechanistic data vital to the design of future studies using NKT cell stimulation as part of strategies to generate long lasting tumor-specific T-cell responses.

following administration of α-GalCer-pulsed monocyte-derived dendritic cells (moDC; ref. 18). As moDCs have high surface CD1d expression and marked capacity to functionally activate and induce proliferation of NKT cells *in vitro*, they have the potential to induce NKT cell activation and proliferation *in vivo*. We hypothesized that administration of α-GalCer pulsed moDC to human subjects would provide better control of NKT cell activation than that achieved by direct administration of α-GalCer and would improve strategies for combining NKT cell activation with enhancements to tumor peptide–antigen specific immunity and NK-cell activity. For example, it would be possible to repetitively stimulate both NKT and T cells in an optimal sequence and at an optimal time interval.

We have previously described preliminary observations from a phase I clinical trial involving intravenously administered α-GalCer-pulsed moDC, showing specific effects on NKT cells and secondary effects resulting in modulation of NK, T, and B cell numbers and increased serum interferon-γ (19). To optimally investigate the therapeutic potential of NKT cells, including via their potential to enhance NK- and T-cell–mediated immunity, further information is required about the optimal cell dose and route of administration. A number of clinical studies have evaluated immune and clinical responses to α-GalCer-pulsed dendritic cells (19–23). None have directly compared different routes of α-GalCer-pulsed DC administration or systematically evaluated the effects of different DC doses with a dose-escalation protocol.

Here we describe the substantial differences in immunological effects between intravenous (IV) and intradermal (ID) administration and the critical role of moDC dose in immunological outcomes. We also describe significant new data on clinical outcomes following administration of α-GalCer–pulsed moDC.

Materials and Methods

Overview of study design

The study was a single center, phase 1, open-labeled, dose-escalation study in patients with metastatic solid tumors to investigate the safety, tolerability, and immunological effects of α-GalCer–pulsed autologous moDC. Twelve patients were enrolled in 3 consecutive cohorts with four patients in each cohort. Subjects in cohort 1 (KS101–KS104), cohort 2 (KS202–KS205), and cohort 3 (KS301–KS304) received 5×10^5, 5×10^6, and 2–5×10^7 α-GalCer–pulsed moDC, respectively. Each patient received two IV and two ID injections (days 0, 14, 42, and 56) with a crossover design to compare immunological outcomes resulting from different routes of administration. Subjects #1 and #2 in each cohort received two IV injections followed by two ID injections. Subjects #3 and #4 in each cohort received 2 ID injections followed by 2 IV.

Study subjects

Subjects with metastatic malignancy ($n = 12$) who had failed all standard therapies or in whom no standard therapy was considered suitable were enrolled after providing written informed consent. The study had human research ethics committee approval from all participating institutions. Clinical characteristics of study subjects are summarized in Table 1. Study subjects had performance status Eastern Cooperative Oncology Group 0–2 at the time of enrollment. Other inclusion criteria included acceptable renal, hepatic, cardiac, pulmonary, and hematologic function, presence of measurable tumor deposits of at least 2 cm, no history of autoimmune disease, no concurrent corticosteroid use or antitumor therapy and detectable peripheral blood Vα24+Vβ11+NKT cells. During the study, or in the 2 month period before enrollment, study subjects received no systemic therapy with potential antitumor- or immune-modulating effects. One subject (KS101) underwent palliative local radiotherapy and blood transfusions during the study period and was considered nonevaluable with respect to immune outcomes beyond day 35. One subject (KS205) withdrew before the final scheduled treatment because of complications of unrelated ureteric calculi.

Preparation and phenotypic analysis of α-GalCer-pulsed moDCs

Immature moDCs were generated for each patient treatment from aliquots of a single, cryopreserved-leukapheresis product by 5-day culture of adherent monocytes in the presence of GM–CSF (800 U/ml; Schering Plough) and IL-4 (500 U/ml; R&D Systems Inc.). For 24 hours before administration, the moDCs were pulsed with α-GalCer (α-GalactosylCeramide, Kirin Brewery Co. Pty Ltd.—now Kyowa Hakko Kirin Co. Ltd.) at 100 ng/ml. The immunophenotype of the moDC was determined by 3-color flow cytometry using the following monoclonal antibodies: CD14 PC5 IgG2a, CD3 PE IgG1, CD19 PE IgG1, CD86 PE IgG1, CD83 PE IgG2b, CD40 PE IgG1, HLA-DR PC5 IgG1, CD1a

Table 1. Patients characteristics

Study number	Age (Years)	Sex	Diagnosis	Site of malignancy at enrollment	Prior therapy	Baseline NKT-cell level ($\times 10^6$/L)	Disease response
KS101	53	F	Breast cancer	Bones, Liver	S,R,H	1.3	NE
KS102	61	M	Colon cancer	Colon, retroperitoneum	S,R,C	0.15	PR
KS103	27	F	Liver cancer	Liver, lungs	S	13.3	SD
KS104	64	M	Melanoma	Right groin	S,I	0.50	SD
KS202	64	M	Melanoma	Lungs	S	1.72	PR
KS203	39	M	Peritoneal adenocarcinoma	Peritoneum	S	1.54	PR
KS204	57	M	Renal cell carcinoma	Lungs, kidney, liver	S,R	12.79	SD
KS205	51	M	Peritoneal adenocarcinoma	Peritoneum	I	1.23	NE
KS301	65	M	Prostate carcinoma, Renal cell carcinoma	Prostate, bones, kidney	S	0.7	P
KS302	33	M	Lung carcinoma	Lungs, mediastinum, bones	C	0.8	P
KS303	49	M	Renal cell carcinoma	Lungs, mediastinum, adrenal gland	S	1.17	P
KS304	47	F	Lung carcinoma	Lungs, mediastinum, bones, liver	C	1.14	P

Abbreviations: S, Surgery; C, Chemotherapy; I, Immunotherapy; R, Radiotherapy; H, Hormonal antineoplastic therapy; P, Progression; SD, Stable disease; PR, Partial regression; NE, not evaluable.

PE IgG1 (Beckman Coulter), CD80 PE IgG1 (Beckton Dickinson), HLA- ABC PE IgG2a (DAKO Corporation), and CD1d 42.1 (gift from Steven Porcelli). Immunophenotyping was carried out on each batch of DCs to ensure consistency of the administered moDC. Administered immature moDCs were positive for HLA-ABC, HLA-DR, CD1d, CD80, CD83, and CD86 and negative for CD14 (Supplementary Fig. S1A). moDC with this phenotype were used in this study on the basis of *in vitro* data indicating their potent stimulation of NKT cells, evidence that CD1d expression was higher on immature moDC than on mature moDC (data not shown) and that subsequent interaction with NKT cells would lead to moDC maturation (24).

Clinical evaluations

Patients underwent clinical examination, computed tomography scanning, bone scanning, and if potentially informative, serial assessment of tumor markers before, during, and at the conclusion of the study period. For evaluation of safety, toxicity, and tolerability, patients underwent regular clinical review including physical examination, lung function testing, monitoring of biochemical parameters for renal and hepatic function, haematological testing with full blood counts, and basic screening for the development of auto-antibodies.

Immunological monitoring

Immunophenotyping of peripheral blood using 3-color flow cytometry was used to determine relative numbers of NKT cells (Vα24+Vβ11+CD3+), T-cell subsets (CD3+ CD4+ or CD3+CD8+), and NK cells (CD3−CD56+). Antibodies were anti-Vα24 TCR FITC IgG_1, anti-Vβ11 TCR PE IgG_1, anti-CD3 PC5 IgG_1 for NKT-cell assessment and anti-CD3 FITC IgG_1, anti-CD4 PE IgG_1, anti-CD8 PC5 IgG_1, anti-CD56 PC5 IgG_1 for T-cell, and NK-cell assessments (Beckman Coulter). Appropriate isotype controls were used. To ensure accuracy of flow-cytometric evaluation of Vα24+Vβ11+NKT cells, which are present at very low frequencies in peripheral blood, up to 1×10^6 cells were assessed to acquire >100 NKT-cell events. A representative flow-cytometry plot showing the method for enumeration of Vα24+Vβ11+NKT cells is shown in Supplementary Fig. S1B. Activation status of T and NK cells was determined by expression of surface CD69 (anti-CD69 IgG_{2b} PC5; Beckman Coulter) and cytoplasmic IFN-γ (anti-IFN-γ IgG_1 PE; Beckman Coulter) according to the manufacturer's protocol, with costaining for CD56 and CD3. As we aimed to determine whether *in vivo* activation occurred, an *in vitro* activation step (e.g., using phorbol 12-myristate 13-acetate) before analysis was not undertaken.

Automated full blood counts were carried out on all samples to determine absolute peripheral blood lymphocyte counts to calculate the number of NKT-, NK- and T-cell subsets per liter of peripheral blood. To establish a pretreatment baseline, samples were collected on at least 3 occasions for more than at least a 2-week period before the first treatment. During posttreatment, samples were collected immediately before treatment, 6 hours after treatment administration, then days 1, 2, 5, 7, and 10

after each treatment, and then weekly until four weeks after the final treatment.

Serum IFN-γ was assessed before (time point 0) and at intervals (6 hours, day 1, 2, and 7) after each treatment. Serum was separated from clotted peripheral blood within 10 minutes of collection and cryopreserved at −80°C until analyzed using ELISA (BD OptEIA ELISA Kits, Beckton Dickinson) according to the manufacturer's instructions.

Trafficking of moDCs

To compare distribution of moDC following IV and ID administration, Indium^{111}-oxine labeled moDC (20% of the total moDC dose) was infused immediately after the unlabeled cells (25). The proportion of indium-labeled moDC within different organs was determined immediately after administration and 4, 6, 24, and 48 hours later. Control injections of free Indium^{111}-oxine were administered several weeks later to confirm that labeled DC was being tracked rather than free indium released from the DC.

Statistical analysis

The frequency of events following administration of therapy by the IV and ID routes was compared using the McNemars chi-squared test using exact methods for cross-over data.

Results

Safety and tolerability (including dose and route information)

Study therapy was administered on schedule for 47 of the planned 48 treatments (4 per subject) and no treatments needed to be withheld due to treatment related toxicity. Ten out of the 12 subjects enrolled were fully evaluable for clinical and immunological parameters. The therapy was well tolerated and suitable for outpatient administration. Minor systemic side effects, unrelated to the malignancy, including malaise and lethargy, occurred in the days following study therapy in 6 out of 12 patients (Table 2). Symptoms generally lasted 1 to 3 days but occasionally persisted longer. Fever was uncommon but occurred in 2 patients with a total of 4 episodes. Systemic symptoms were not dose related, occurring in patients in all cohorts (2 patients in cohort 1, 7 of 16 treatment episodes; 3 patients in cohort 2, 7 of 15 treatment episodes; and 1 patient in cohort 3, occurring in 2 of 16 treatment episodes). Administration

Table 2. Adverse events and treatment related symptoms

	Cohort			Number of episodes		
	1 $n = 4$	2 $n = 4$	3 $n = 4$	Total $n = 47$	Following IV $n = 23$	Following ID $n = 24$
Subjects with systemic symptoms (total)	2	3	1			
Episodes/Courses administered	7/16	7/15	2/16	16	11/23	5/24
Subjects with treatment related fever	0	0	2			
Fever episodes/Courses administered	0/16	0/16	4/16	4/47	4/23	0/24
Patients with injection site reactions	0	1	4			
Episodes/Number of ID treatments administered	0/8	1/8	8/8	9/47	NA	9/24
Patients with tumor symptom flare (total)	2	3	4			
Episodes/Evaluable treatments	10/16	5/15	10/16	25/47	16/23	9/23
Patients with tumor infiltrated lymph node pain and swelling (4/4)	0	0	4			
Patients with symptom flare/Patients with involved lymph nodes			10/16	10/47	5/23	5/24
Patients with lung nodules or pulmonary infiltration with tumor	2	2	3			
Patients with new or aggravated respiratory symptoms (4/7)	1/2	1/2	2/3	11/47	9/23	2/24
Patients with bone scan evidence for bone metastasis (4/5)	1	0	3			
Patients with new or aggravated bone pain	1/1	0	3/3	7/47	2/23	5/24
Patients with elevated serum tumor markers	0	1	4			
Patients with tumor marker flare	0	0	4	NA	NA	NA
Patients with peritoneal metastases	0	2	0			
Patients with treatment associated abdominal or gastrointestinal symptoms	0	2	0	4/47	2/23	2/24

route influenced the frequency of systemic symptoms, occurring after 11 of 23 IV treatments and after only 5 of 24 ID treatments ($P = 0.004$, excluding KS205 who only received 3 treatments). Some subjects experienced systemic symptoms only after IV treatment, but all subjects experiencing symptoms after ID therapy also had symptoms after IV therapy. These clinical observations are consistent with the immunological observations that NKT-cell activation was consistently greater following IV than ID therapy (see below) and there was also a close temporal relationship between systemic symptoms and immunological responses. Local injection site reactions, clinically similar to delayed type hypersensitivity (DTH) responses with erythema and induration, were observed following ID therapy but only at higher moDC doses occurring after 0/8, 1/8, and 8/8 ID treatments in cohorts 1, 2, and 3 respectively.

Tumor-associated symptoms

Of particular interest, the majority of patients (9 of 12) experienced temporary exacerbations of tumor symptoms, 3 of whom were unaware of disease at sites at which flares occurred until the symptoms developed. Tumor flares occurred after both ID and IV therapy but were more common after IV therapy (16 episodes compared with 9 episodes). These flares are interpreted as evidence of inflammatory responses to the tumor, because they had a strong temporal relationship to study therapy, were reproducible in timing and nature with subsequent treatment episodes, were transient (generally lasting only 1 to 3 days), and did not occur in any subjects before or after the study period. Rapidly developing events (biochemical changes, pain or tumor enlargement) that did not spontaneously resolve were considered progression rather than a flare.

Four subjects had clinically palpable, tumor-infiltrated lymph nodes. All assessable, affected nodal groups for each of these patients were transiently enlarged above baseline size, coinciding with pain and tenderness, in the days following administration of study therapy. One subject (KS301) with retroperitoneal lymphadenopathy developed temporary elevations in urea and creatinine and computed tomography scan evidence for ureteric obstruction, in parallel with swelling and transient tenderness of inguinal and axillary lymph nodes beginning the day after study therapy and lasting for several days. These changes spontaneously resolved.

Four of five subjects, shown in pretreatment bone scans to have bone metastases, developed bone pain at sites of known disease in the days following study therapy, two of whom had transient exacerbations of preexisting pain and two of whom developed transient pain in areas previously asymptomatic. Four of 7 subjects with pulmonary involvement developed transient respiratory symptoms, including cough and dyspnoea, in the days following study therapy. This was severe in one patient (KS101) with microscopic pulmonary infiltration with breast cancer, who experienced significant dyspnoea and hypoxia at rest following each treatment despite being asymptomatic at other times. Of interest, this patient had abnormal liver function tests related to microscopic metastatic infiltration of the liver, which transiently improved after each dose of ID study therapy (Supplementary Fig. S2).

One patient (KS104) with melanoma developed tenderness, redness, warmth, and acute swelling of subcutaneous melanoma deposits after each of 4 treatments. Subjects KS203 and KS205, both of whom had adenocarcinoma metastatic within the peritoneal cavity, developed abdominal pain and gastrointestinal symptoms on the day following administration of study therapy with subsequent spontaneous resolution.

The three subjects who had no flare of tumor symptoms had no definite tumor-related symptoms at any time during the treatment and evaluation period and only had tumors in sites unlikely to cause symptoms (e.g., intraabdominal enlarged lymph nodes or tumor masses).

Clinical outcomes

Disease outcomes are summarized in Table 1. During the study period of three months, six of the ten patients evaluable for disease response had stable disease defined as no sustained increase in tumor masses or tumor markers. Of these six, three had minor objective improvement defined as reduction in tumor masses on radiological criteria (by <25%) or by reduction in tumor markers by 25% to 50%. Of the 6 patients with stable disease or minor improvement, 5 had clearly progressing disease before initiation of study therapy. Three subjects had elevated serum lactate dehydrogenase (LDH) levels, indicating high tumor burden during the study period, all of whom had transient increases in LDH following treatment associated with tumor associated pain (Supplementary Fig. S3A). This is consistent with an increase in destruction (apoptosis or necrosis) of tumor cells at these times, associated with inflammation of the tumor causing pain. One subject (KS203) with peritoneal adenocarcinoma had transient increases (double) in CA19.9 during the study period, however this was followed by a decrease in CA19.9 levels, in addition to decrease in carcinoembryonic antigen (CEA) levels to below pretreatment levels during the 4 months after cessation of study therapy (Supplementary Fig. S3B). These biochemical changes were associated with radiological and clinical examination evidence for a minor reduction in disease bulk (<25% decrease) and cessation of previous requirements for regular drainage of ascitic fluid for more than 1 year (after which the patient was lost to follow up with stable disease).

One subject with rectal carcinoma (KS102) had a sustained decrease in serum CEA levels for the 12 months posttreatment to 60% of the levels at the conclusion of the study (Supplementary Fig. S3C). No therapy of any kind was administered during this period. Subsequent to this 12-month posttreatment observation period, the patient developed progressive disease and rising CEA levels, suggesting that the decrease in CEA was not simply related to decreased capacity of the tumor to produce CEA. Responses or stabilization lasted up to at least one year in KS102, KS104, and KS203.

All 4 patients in cohort 3 had progressive disease during the study period, all of whom had advanced and bulky disease with greater tumor burdens or worse initial performance status than the patients in the first 2 cohorts. Two other patients had progressive disease (KS101 and KS205) but were considered not fully evaluable as described above.

Immunological effects of administration of α-GalCer pulsed moDCs

Effect of route on immunological responses. Highly reproducible changes in numbers of peripheral blood NKT-, NK-, and T-cell subsets were observed following IV therapy. In the 24 to 48 hours post administration, the number of all of these immune cells decreased in peripheral blood to nadir levels as low as 20 fold less than baseline in the case of NKT cells and 9- and 3-fold less than baseline in the case of NK and T cells, respectively. Subsequently, levels of NKT and NK cells rose to above baseline levels while T cells returned to the pretreatment baseline (Figs. 1 and 2, Supplementary Fig. S4). In contrast, responses to ID therapy were less reproducible. In some cases, a pattern closely resembling that following IV administration was observed. In these cases, there was a marked decrease in peripheral blood levels of NKT cells and to a lesser extent NK and T cells, followed by a return to baseline levels (in the case of NK and T cells) and above baseline levels in the case of NKT cells. However, the posttreatment nadir for NK, NKT, and T cells was less marked following ID therapy than after IV therapy. Unlike IV therapy, ID therapy generally did not increase peripheral blood NK cell levels above baseline (Fig. 3). The pattern of response of the CD4+ and CD8+ T-cell subsets mirrored that of the overall

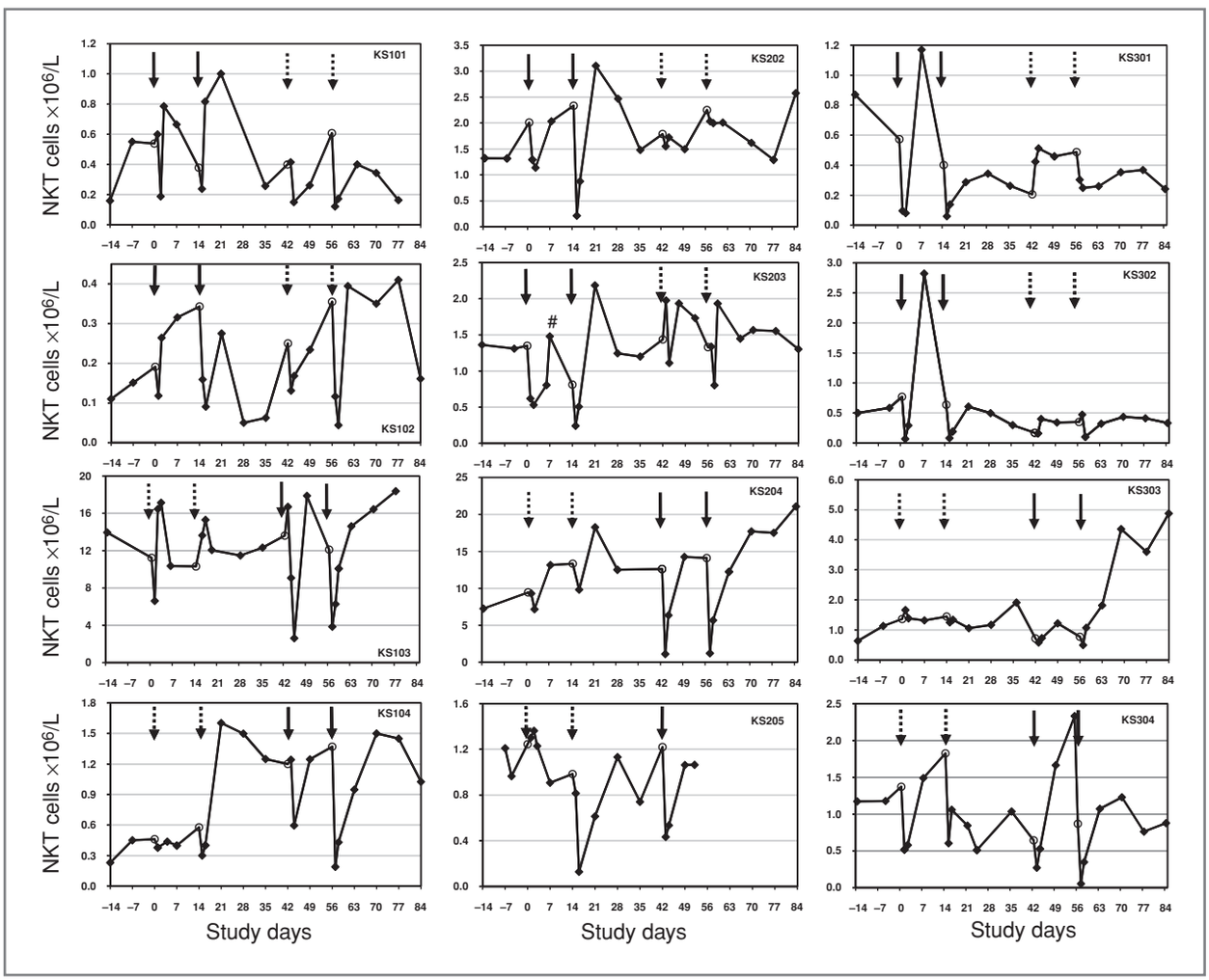

Figure 1. Peripheral blood levels of Vα24+Vβ11+NKT cells before and after treatment with α-GalCer-pulsed moDCs. Absolute levels of Vα24+Vβ11+NKT cells per liter of blood at the time points indicated determined by flow cytometry and automated blood cell counting. Statistically significant increases ($P < 0.03$) were observed in all patients evaluated. Subject KS203 contracted a viral illness, indicated with hash marker. Treatment with α-GalCer-pulsed moDC is indicated on the figures as open circles. Full arrows indicate IV administration and dashed arrows ID.

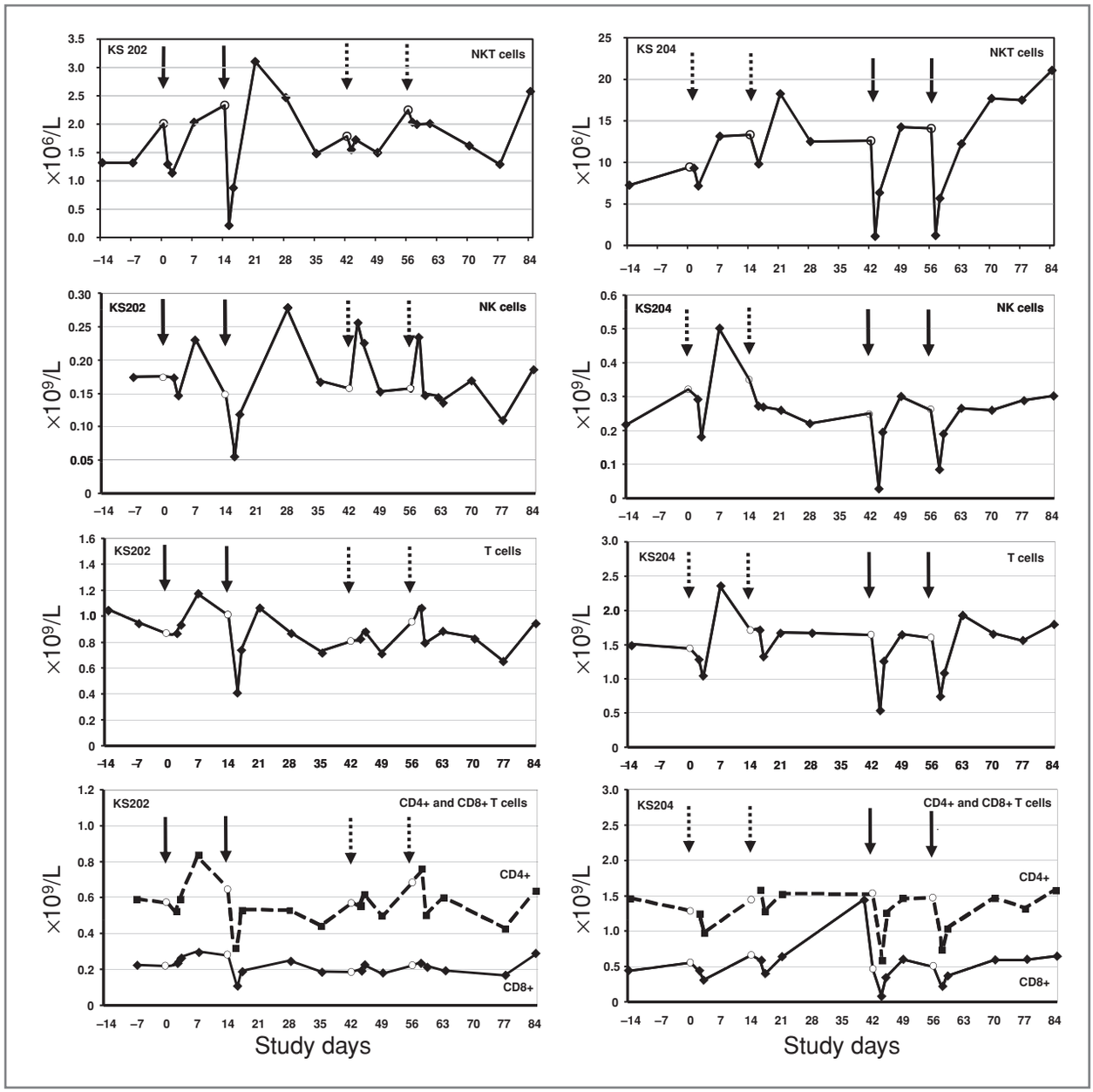

Figure 2. NKT, NK, and T-cell levels. Peripheral blood levels of NKT cells, NK cell, T-cells, and T-cell subsets (CD4+ and CD8+) in 2 representative subjects (KS202 and KS204) showing effects on peripheral blood levels of immune effector cells following IV and ID administration of α-GalCer-pulsed moDC.

T-cell population, and there was no difference in the responsiveness of CD4+ or CD8+ T cells to the vaccinations (data not shown).

Other secondary immune effects were also substantially less after ID than after IV treatment. The percentage of NK and T cells in peripheral blood that expressed cytoplasmic interferon-γ and the absolute number of interferon-γ-producing NK and T cells in peripheral blood increased after IV treatments but not after ID treatments (Fig. 4A). Similarly, increases in serum interferon-γ levels were regularly seen after IV administration but were not observed after ID administration (Fig. 4B). Transient upregulation of the early activation marker CD69 on NK cells and T cells occurred following IV treatment but this was infrequent after ID therapy (data not shown).

Effect of dose on immune responses. The 8 subjects in the lower-dose cohorts and one subject in the higher-dose cohort had almost identical patterns of immune responses following administration of study therapy (Fig. 1, Supplementary Fig. S4). A key feature of this was that the second

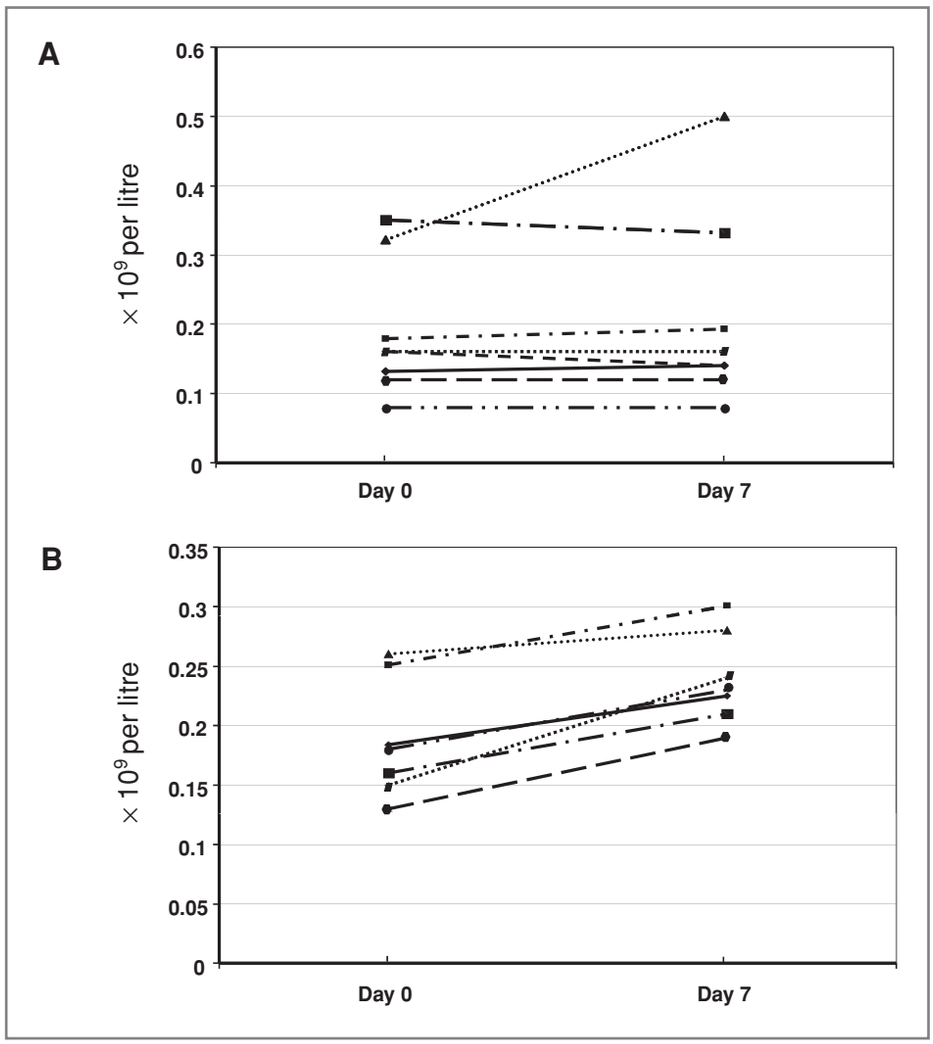

Figure 3. Peripheral blood NK cell pre- and posttreatment levels. NK cell numbers in peripheral blood before administration of α-GalCer-pulsed moDC (Day 0) and 7 days after the treatment (Day 7) for patients enrolled in the second study cohort (5×10^6 α-GalCer-pulsed MoDC per injection). A, NK-cell levels pre- and post-ID treatments (8 treatments). B, NK-cells levels pre- and post-IV treatments (7 treatments).

IV treatment resulted in a significant increase in peripheral blood levels of NKT cells with the level 7 days after the second treatment being significantly above baseline levels ($P < 0.05$). In contrast, at higher doses peak NKT cell levels 7 days after the first IV treatment were more marked than that observed at lower doses but was followed by a blunted response to the second IV dose. There was no difference in the direct immunological outcomes between the three different dose levels following ID administration. However, ID administration at only the two higher-dose levels was able to prime for induction of responses to the first of the subsequent IV DC administrations. Even at the highest-dose level, ID administration did not seem to induce anergy to the subsequent first IV dose of DC.

Trafficking of α-GalCer pulsed moDCs using Indium-111-oxine

As previously reported, trafficking studies indicated that following IV administration there was highly coordinated movement of administered moDC with the majority trafficking to and then remaining in the lung for 4 to 6 hours but with subsequent movement, almost complete by 24 hours, to the liver and spleen. moDC remained in these sites for at least the 48 hours of follow up. Control injections of indium without moDC clearly show that tracer distribution is not related to free indium. In contrast to the observations following IV administration, a minority (<2%) of moDC administered ID trafficked to regional nodes, with the majority of administered cells remaining at the injection site (data not shown). There appeared to be ongoing gradual movement of moDC from the injection site to draining lymph nodes. Migration to lungs or spleen was not observed; however, small amounts of tracer were observed in the liver (<2% of total administered) in some subjects. The destination of α-GalCer-pulsed moDC was similar to that observed after ID and IV administration of protein- or peptide-pulsed DC [unpublished data, (25)], indicating that α-GalCer does not significantly alter trafficking properties of the DC.

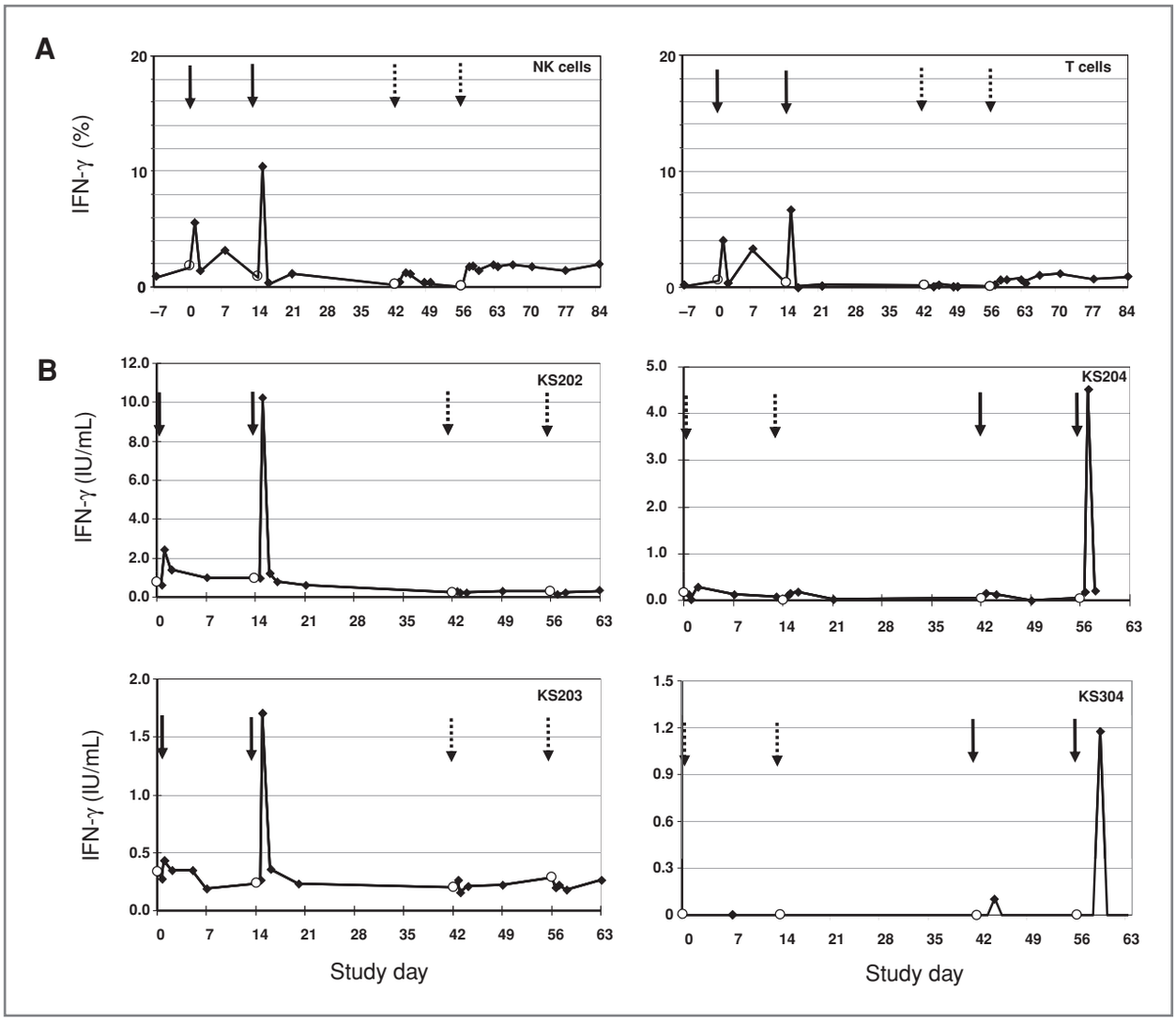

Figure 4. A, increase in cytoplasmic IFN-γ. The percentage of peripheral blood NK cells and T cells expressing IFN-γ in one representative patient (KS202). B, increase in serum IFN-γ. Serum IFN-γ levels following therapy with α-GalCer-pulsed MoDC in 3 patients receiving medium dose (5×10^6) and 1 patient (KS304) receiving high dose (5×10^7) α-GalCer-pulsed moDC.

Discussion

Overview and immune related clinical effects

This clinical study shows that therapy with moDC pulsed with the specific NKT cell ligand, α-GalCer, is well tolerated clinically and that even low numbers of treatments may have some clinical antitumor activity. The study was not designed to determine which administration route or treatment dose had the greatest clinical activity. However, α-GalCer-pulsed moDC at doses of 5×10^6 per injection by the IV route (rather than the use of higher or lower dose or the ID route) most reproducibly produces repetitive activation of NKT cells resulting in increased peripheral blood NKT cell levels and secondary immune effects, including NK- and T-cell activation, increased peripheral blood NK cells and increased serum IFN-γ.

Disease outcomes

Conclusions about disease outcome are preliminary, as this was a small heterogenous group of patients given small numbers of treatments. However, the high frequency of therapy-induced, clinically apparent inflammatory responses at tumor sites provides compelling evidence for clinically relevant antitumor responses. The clinical evidence for tumor inflammation was temporally associated with immunological changes detected in peripheral blood. In a proportion of patients, these changes translated into objective clinical responses and disease stabilizations, although the extent of clinical responses was predictably minor in view of the few treatments administered. The absence of tumor flares outside the therapeutic period and the reproducible temporal relationship to study therapy is strong corroborating evidence that these effects were a

result of the study therapy. We did not undertake tumor biopsies to determine the nature of the inflammatory responses. These clinical observations are very encouraging as they suggest that with prolonged or additional therapeutic maneuvers (such as protein or peptide antigen pulsing of DC's or other immunotherapy modalities), increased rates of objective tumor reduction may be seen. The immune responses to α-GalCer-pulsed DC, according to evaluation of peripheral blood immune cells and clinical evidence of inflammatory responses, were transient, therefore for maximal antitumor effect repetitive activation is likely to be required. In view of this, it is significant that our results show that the immune stimulation can be repeated and is enhanced with subsequent treatments, except at high doses of moDC. A small number of subjects have been treated with between 6 and 16 total doses of IV α-GalCer-pulsed DC without loss of immune reactivity (data not shown).

Effects of route

Many factors potentially contribute to the greater immunological effects observed post IV treatment. One is the effective cell dose. The majority of ID administered moDC did not leave the administration site in the skin to traffic even to regional lymph nodes and there was no or minimal movement to lungs, liver, or spleen. This may reflect the use of immature moDCs which do not express CCR7. There is other evidence suggesting that immature DC do not migrate effectively to regional nodes (26) and DC from mice lacking CCR7 fail to migrate (27). In contrast, IV administered moDC have the potential to interact with NKT cells in peripheral blood, lung, liver, and spleen. As there are NKT cells in human lung (28), where the majority of IV administered moDC reside for at least 4 hours after administration (19), there is the potential for direct and early interaction between administered moDC and NKT cells. Furthermore, this prolonged period in the lung exposes the moDC to the whole blood volume many times over, maximizing the opportunities for moDC to interact with circulating NKT cells in addition to interaction with resident lung NKT cells.

We previously described *in vivo* evidence that human NKT cells, with a key role in early, innate immune responses, display immunological memory manifest as more rapid, vigorous, and sustained effects following a second stimulation with IV α-GalCer-pulsed DC (19). Administration of α-GalCer-pulsed DC via the ID route, had less effects on peripheral blood lymphoid cell levels (Figs. 1–3) but interestingly did induce NKT-cell memory, resulting in more rapid effects of subsequent IV treatments (Supplementary Fig. S4). This was only observed after intermediate and high doses of ID α-GalCer-pulsed DC.

Effects of dose

As few as 5×10^5 α-GalCer-pulsed moDC administered IV induced changes in peripheral blood levels of NKT cells, secondary effects on NK cells and T cells, clinical symptoms consistent with treatment-induced tumor immune responses and systemic symptoms. However, at this dose, secondary immune effects occurred less frequently than at higher doses and were more likely to require a priming injection, suggesting that this dose is near the lower threshold for reproducible induction of immune responses. In contrast, at the highest doses administered (5×10^7 moDC), the greatest immune effects were seen after the first IV treatment and there seemed to be blunted immune responses after a second IV dose. Possibly the higher doses result in overstimulation or persistent stimulation of NKT cells, resulting in decreased rather than enhanced immunological effects. Prior ID therapy at the lowest dose did not prime for subsequent responses to the first dose IV but this was seen at the higher two dose levels. Prior ID therapy at the highest dose (5×10^7) did not seem to induce anergy to the subsequent IV treatment. Further escalation to much higher doses (e.g., 5×10^8 or beyond) would be needed to determine whether this is a route effect or purely a dose effect.

The high dose cohort had the worst clinical outcome with all patients progressing. The physiological effects and failure to induce disease responses with administration of larger numbers of α-GalCer-pulsed DC are reminiscent of the observations seen with direct IV administration of α-GalCer (17). As described above, multiple IV treatments of 5×10^6 moDC or less could be administered without loss of immune reactivity. We conclude that for further evaluation of α-GalCer-pulsed DC, doses above 5×10^5 and below 5×10^7 cells per treatment are probably optimal, particularly if recurrent bursts of immune activity are required for antitumor efficacy.

Future directions

The possibility of copulsing DC with α-GalCer and peptide or protein antigens is of particular interest as this may allow additive or synergistic antitumor activity by NKT-, NK-, and tumor-antigen specific CTL. The optimal route of administration for such therapy is currently unclear. The data presented here clearly suggest superiority for the IV route with respect to increases in peripheral blood numbers of NKT- and NK-cells and secondary immune effects, including nonspecific activation of CD8+ T cells. It is not known whether these benefits of IV administration will extend to the induction of peptide–antigen specific CTL for which there is currently a preference for the ID route (29) but with some studies attesting to the potential efficacy of the IV route (30, 31). There is increasing evidence that α-GalCer induced NKT-cell activation enhances tumor antigen specific cytotoxic T cells (20, 32–37) but further studies are required to determine how best to achieve this goal in the clinical setting. The simplest strategy of pulsing DC with both α-GalCer and peptide antigens warrants clinical evaluation. Alternative strategies are also being evaluated (38). Immature DC were used in the study described here because immature DC had higher CD1d expression than mature DC and our *in vitro* studies showed immature DC had greater potency for NKT cell activation and proliferation than mature DC. As we wanted to establish proof of principle, unavailable at the time the study was initiated, that NKT

responses would be observed in response to α-GalCer-pulsed DC, we used the DC maturation state that seemed most likely to show this. However, for future studies aiming to show antigen specific T-cell responses, the use of mature DC would be warranted, albeit with additional evaluations to confirm their effect on NKT cells.

In conclusion, we have shown that administration of α-GalCer-pulsed moDC is well tolerated and produces substantial, readily detectable effects on immune effector cells with potential antitumor activity (Vα 24+NKT cells, NK cells, and T cells). Transient tumor flares of a presumed inflammatory nature are frequent and provide indirect support for a clinically meaningful immune response. Immunological effects are more pronounced following IV administration, which delivers the majority of the moDC to lungs, liver, and spleen than following ID administration. Dendritic-cell dose is critical to the immune effects and high doses of moDC result in dampening of immune responsiveness to subsequent treatments.

Disclosure of Potential Conflicts of Interest

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