

# Sustained expansion of NKT cells and antigen-specific T cells after injection of $\alpha$ -galactosyl-ceramide loaded mature dendritic cells in cancer patients

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**Natural killer T (NKT) cells are distinct glycolipid reactive innate lymphocytes that are implicated in the resistance to pathogens and tumors. Earlier attempts to mobilize NKT cells, specifically, in vivo in humans met with limited success. Here, we evaluated intravenous injection of monocyte-derived mature DCs that were loaded with a synthetic NKT cell ligand,  $\alpha$ -galactosyl-ceramide ( $\alpha$ -GalCer; KRN-7000) in five patients who had advanced cancer. Injection of  $\alpha$ -GalCer-pulsed, but not unpulsed, dendritic cells (DCs) led to >100-fold expansion of several subsets of NKT cells in all patients; these could be detected for up to 6 mo after vaccination. NKT activation was associated with an increase in serum levels of interleukin-12 p40 and IFN- $\gamma$  inducible protein-10. In addition, there was an increase in memory CD8<sup>+</sup> T cells specific for cytomegalovirus in vivo in response to  $\alpha$ -GalCer-loaded DCs, but not unpulsed DCs. These data demonstrate the feasibility of sustained expansion of NKT cells in vivo in humans, including patients who have advanced cancer, and suggest that NKT activation might help to boost adaptive T cell immunity in vivo.**

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Abbreviations used:  $\alpha$ -GalCer,  $\alpha$ -galactosyl-ceramide; ANA, antinuclear antibody; Flu-MP, influenza matrix peptide; ICS, intracellular cytokine secretion; IP, IFN- $\gamma$ -inducible protein; MIP, macrophage inflammatory protein; RF, rheumatoid factor.

Immune recognition of pathogens and tumors involves innate and adaptive arms of the immune system. NKT cells are innate lymphocytes that are implicated in the control of autoimmunity, and resistance to tumors and pathogens (1, 2). In contrast with conventional CD4/CD8<sup>+</sup> T cells that recognize peptide antigens, NKT cells respond to glycolipid ligands in the context of CD1d (2).  $\alpha$ -galactosyl-ceramide ( $\alpha$ -GalCer), first isolated from a marine sponge, is a synthetic ligand that is presented effectively on CD1d molecules to human and murine NKT cells (3). Activation of NKT cells in mice by injection of  $\alpha$ -GalCer is associated with a rapid release of cytokines within hours. Stimulation of NKT cells is followed by downstream activation of NK cells, DCs, and T cells (4–7).  $\alpha$ -GalCer-mediated NKT activation was shown to mediate tumor regression in several mouse models. The antitumor effects of NKT cells are

due to several mechanisms, including enhancement of immune effectors and antiangiogenesis (8–13). NKT cells also contribute to resistance against spontaneous and carcinogen-induced tumors in mice (13–15).

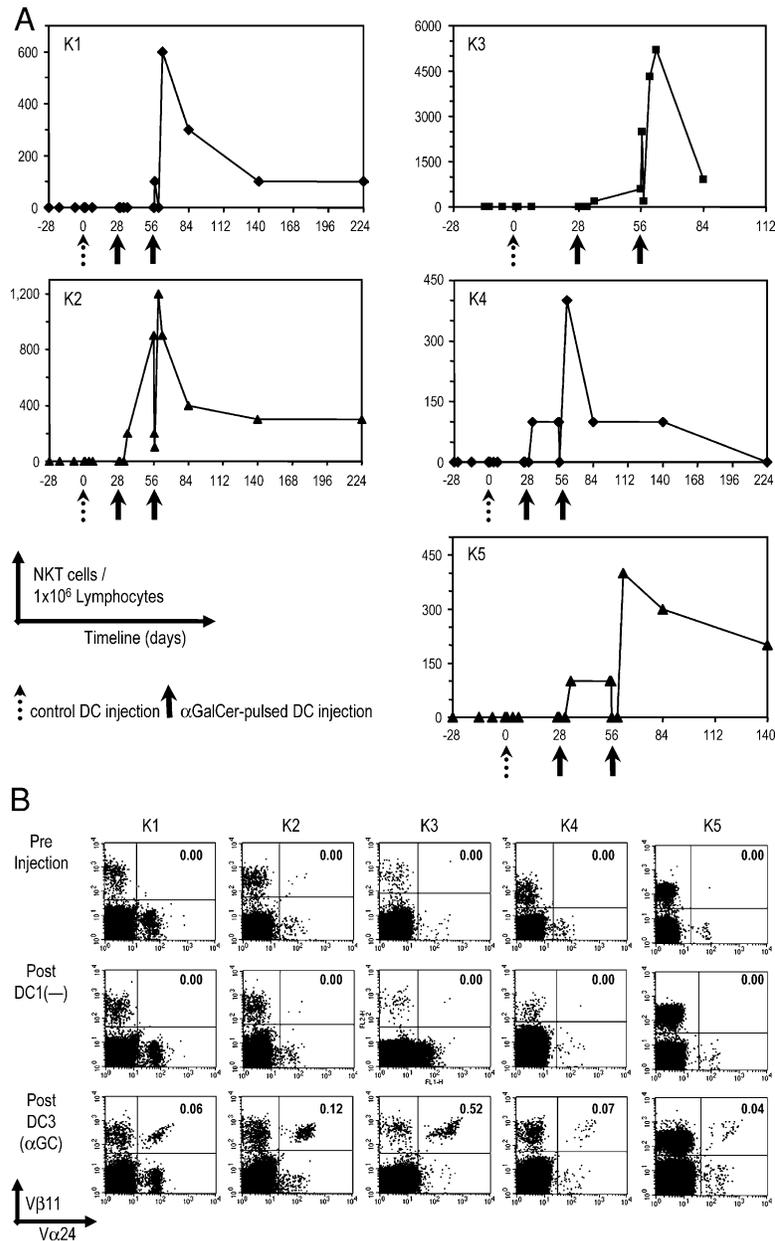
Human NKT cells have been analyzed on the basis of their expression of an invariant T cell receptor (V $\alpha$ 24/V $\beta$ 11) and their binding to CD1d- $\alpha$ -GalCer multimers (16). Human V $\alpha$ 24<sup>+</sup> NKT cells can mediate antitumor effects in vitro; a deficiency of NKT cells, or defects in their function, were described in cancer patients (9, 17–19). The availability of  $\alpha$ -GalCer as the pharmaceutical-grade drug, KRN-7000 (Kirin Breweries), has led to attempts to boost NKT cells in vivo in patients who had advanced cancer or healthy volunteers. However, the injection of KRN-7000 alone, or KRN-7000-loaded immature DCs, only leads to transient NKT activation in some individu-

als (20, 21). In previous studies, we observed that mature DCs are superior to immature DCs for activating human NKT cells in vitro (22). Here, we studied the immune responses to injection of KRN-7000-loaded mature DCs in patients who had advanced cancers.

RESULTS

Clinical characteristics

Six eligible patients were enrolled in this trial. One of the patients was removed from the study before receiving any  $\alpha$ -GalCer-loaded DCs to explore other options for progres-



**Figure 1. Expansion of NKT cells after injection of GalCer-pulsed DCs.** (A) Kinetics of V $\alpha$ 24<sup>+</sup>/V $\beta$ 11<sup>+</sup> NKT cells in blood. The proportion of V $\alpha$ 24<sup>+</sup>/V $\beta$ 11<sup>+</sup> NKT cells in blood was monitored before and after DC vaccination. The dates of DC injections are depicted by arrows. (B) Representative FACS plots showing the number of invariant NKT cells before and after DC vaccination. Numbers in upper right quadrant represent percentage of NKT/total lymphocytes. (C) Correlation between kinetics of  $\alpha$ -GalCer dimer binding cells and invariant TCR<sup>+</sup> cells. Dimer positive

NKT cells were analyzed based on the coexpression of V $\alpha$ 24 or V $\beta$ 11. The data shown are from K3. (D) Representative FACS plots showing the expression of V $\alpha$ 24/V $\beta$ 11<sup>+</sup> on  $\alpha$ -GalCer-CD1d dimer binding cells elicited after vaccination in a patient (K3). A sub-population of V $\alpha$ 24-CD1d dimer<sup>+</sup> cells is circled. Note that the V $\beta$ 11<sup>+</sup> dimer<sup>+</sup> populations consist of two distinct subsets. (E) Expansion of invariant NKT cells in the tumor bed. NKT cells in the blood or marrow tumor bed were monitored by flow cytometry.

sive tumor. This paper describes the results with the first five patients who received the entire set of three vaccines. Patient characteristics are shown in Table I. All had received exten-

sive therapy for their cancers with persistent disease; three of five patients had objective evidence of progressive disease at study entry. The characteristics of the DC preparations are

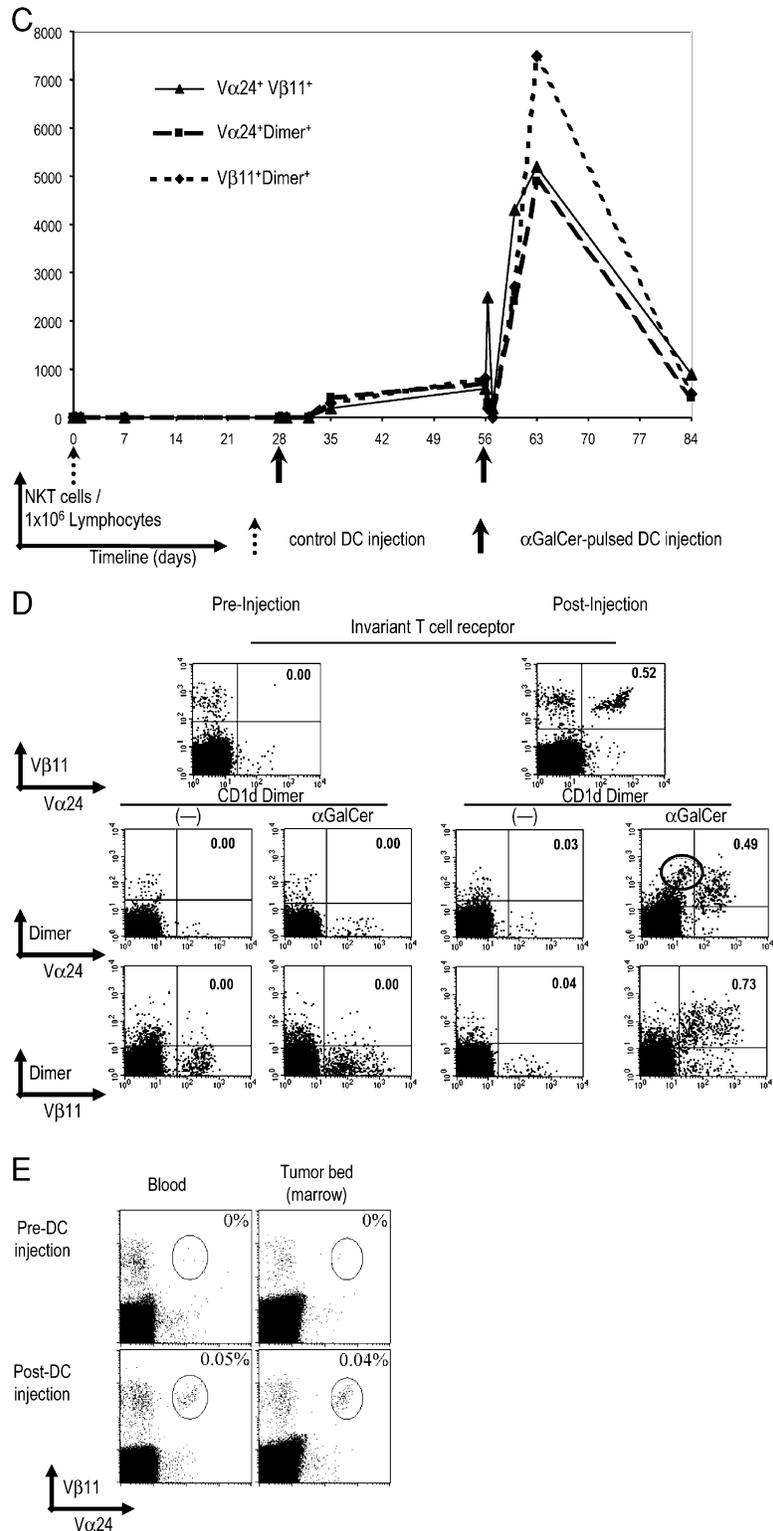


Figure 1 (continued)

**Table I.** Patient characteristics

Patient ID	Age	Sex	Diagnosis	Stage	Previous therapy	Disease status at entry	Disease at study entry	Best response on study	Duration on study	Current status
	<i>yr</i>								<i>mo</i>	
K-1	62	M	myeloma	III	chemotherapy, stem cell transplant	progressive	urine M spike 3.3 g/d	urine M spike 3.0 g/d	9	alive
K-2	41	M	myeloma	III	chemotherapy, stem cell transplant	stable	serum M spike 1.0 g/dl	serum M spike 0.4 g/dl	10	alive
K-3	57	M	anal cancer (squamous cancer)	IV	radiation, chemotherapy, surgery	progressive	metastases to liver, peritoneum	none	4	deceased
K-4	70	M	renal cell cancer	IV	surgery, IFN, TK inhibitor	progressive	metastases to lung, adrenal	stable metastatic disease	8+	alive
K-5	63	M	myeloma	III	radiation, chemotherapy, stem cell transplant	stable	urine M spike 116 mg/d	urine M spike 79 mg/d	10+	alive

shown in Table II. All DC preparations met release criteria and postrelease testing.

### Toxicity

The DC injections were well-tolerated in all patients with no greater than grade 1 injection-related toxicity (Table II). One patient developed rheumatoid factor (RF) and transient positive antinuclear antibody (ANA) at follow-up testing 1 mo after the third injection. This patient also incidentally was noted to have an increase in activated partial thromboplastin time after vaccination which was believed to be secondary to an inhibitor. There was no clinical evidence of autoimmunity or hepatic toxicity in any patient.

### NKT activation and expansion

None of the patients had detectable circulating NKT cells at baseline, which was likely due to extensive therapy of the underlying malignancy. The injection of unpulsed DCs did not lead to an increase in NKT cells in any patient at any time point. In contrast, the injection of  $\alpha$ -GalCer-pulsed DCs led to >100-fold increase in circulating NKT cells in all patients (Fig. 1 A). This was detectable as an increase in cells that were positive for invariant TCR and  $\alpha$ -GalCer-CD1d dimer binding (Fig. 1, B-D). NKT cells peaked at 7–30 d after vaccination and already were increased after the first injection of  $\alpha$ -GalCer-pulsed DCs in four of five patients. The numbers of NKT cells stayed above baseline for

**Table II.** Characteristics of dendritic cells

Patient ID	DC injection no.	Dose	Viability	Purity	Maturity	Injection-related toxicity
		$10^6$ cells			% CD83 <sup>+</sup>	
K-1	1 (unpulsed DCs)	5	100	83	94	fever (grade 1)
K-1	2 (KRN-7000 pulsed DCs)	5	99	83	94	none
K-1	3 (KRN-7000 pulsed DCs)	5	99	61	90	none
K-2	1 (unpulsed DCs)	5	100	52	94	none
K-2	2 (KRN-7000 pulsed DCs)	5	98	58	83	none
K-2	3 (KRN-7000 pulsed DCs)	5	99	53	89	none
K-3	1 (unpulsed DCs)	5	99	95	86	none
K-3	2 (KRN-7000 pulsed DCs)	5	96	92	66	none
K-3	3 (KRN-7000 pulsed DCs)	5	97	90	67	none
K-4	1 (unpulsed DCs)	5	100	86	93	none
K-4	2 (KRN-7000 pulsed DCs)	5	99	80	93	none
K-4	3 (KRN-7000 pulsed DCs)	5	98	89	94	ANA, RF positive; increase in aPTT due to inhibitor
K-5	1 (unpulsed DCs)	5	99	82	87	rash (grade 1)
K-5	2 (KRN-7000 pulsed DCs)	5	100	81	94	none
K-5	3 (KRN-7000 pulsed DCs)	5	100	80	92	none

aPTT, activated partial thromboplastin time.

>84 d in all patients, and were elevated above baseline for >6 mo in two patients with longer follow-up. There was a transient decline in circulating NKT cells on day 1 after  $\alpha$ -GalCer-pulsed DC injection. This decline was not due to TCR down-regulation, because the number of intracellular TCR<sup>+</sup> cells also was monitored and closely followed cell surface staining (unpublished data). Earlier studies in mice that showed TCR down-regulation in NKT cells after injection of  $\alpha$ -GalCer monitored splenic, but not blood, NKT cells, as measured here (23). In one patient who had myeloma, we looked for NKT cells in the marrow tumor bed before and after DC vaccination, and we could demonstrate sustained expansion of invariant NKT cells there for >3 mo after vaccination (Fig. 1 E). Thus, injection of  $\alpha$ -GalCer-loaded DCs leads to a sustained increase in NKT cells in blood and the tumor bed.

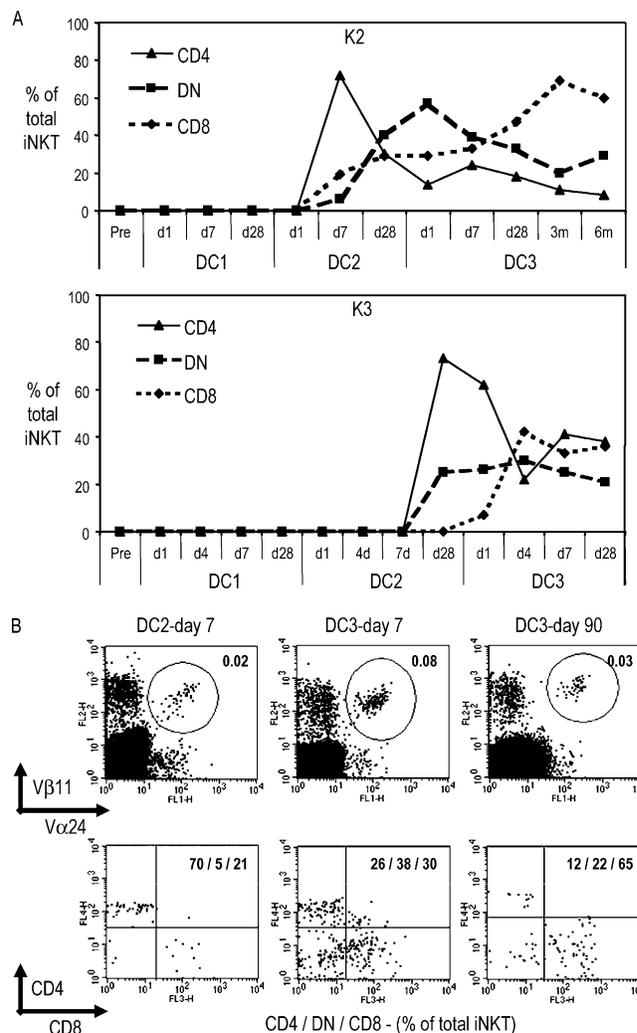
### Phenotype of NKT cells expanded in vivo

Recent studies showed that the subset of cells that binds to  $\alpha$ -GalCer-loaded CD1d tetramers can include invariant TCR<sup>+</sup> and invariant TCR<sup>-</sup> cells (24). In one study, many of the V $\alpha$ 24<sup>-</sup>, tetramer<sup>+</sup> cells were V $\beta$ 11<sup>+</sup>, which suggests alternate V $\alpha$  TCR usage by the CD1d multimer<sup>+</sup> cells (24). The kinetics of increase in CD1d-glycolipid dimer<sup>+</sup> and V $\alpha$ 24/V $\beta$ 11<sup>+</sup> cells in all patients were matched closely (Fig. 1, C and D). However, the numbers of dimer<sup>+</sup>/V $\beta$ 11<sup>+</sup> cells generally exceeded the numbers of V $\alpha$ 24/V $\beta$ 11<sup>+</sup> cells, or dimer<sup>+</sup>V $\alpha$ 24<sup>+</sup> cells. Indeed, the expansion of dimer<sup>+</sup>/V $\alpha$ 24<sup>-</sup> populations also could be demonstrated by flow cytometry (Fig. 1 D). Therefore, DC vaccination led to increases in dimer<sup>+</sup>V $\alpha$ 24<sup>+</sup> and dimer<sup>+</sup>V $\alpha$ 24<sup>-</sup>NKT cells in vivo, with similar kinetics.

Recent studies showed that human NKT cells consist of functionally distinct subsets that are based on the expression of CD4/CD8 (25, 26). In two patients who had postvaccination frequency of >10<sup>3</sup> NKT cells/10<sup>6</sup> PBMCs (K2, K3), the proportion of NKT subsets was analyzed by flow cytometry. In both patients, the phenotype of expanded invariant TCR<sup>+</sup> cells was predominantly CD4<sup>+</sup> at the early time points; however, at later time points, we noted an increase in double negative and CD8<sup>+</sup> NKT cells (Fig. 2, A and B). Thus, DC-mediated mobilization of NKT cells in humans is associated with expansion of all three human NKT subsets in circulation, which may, however, have different kinetics of mobilization.

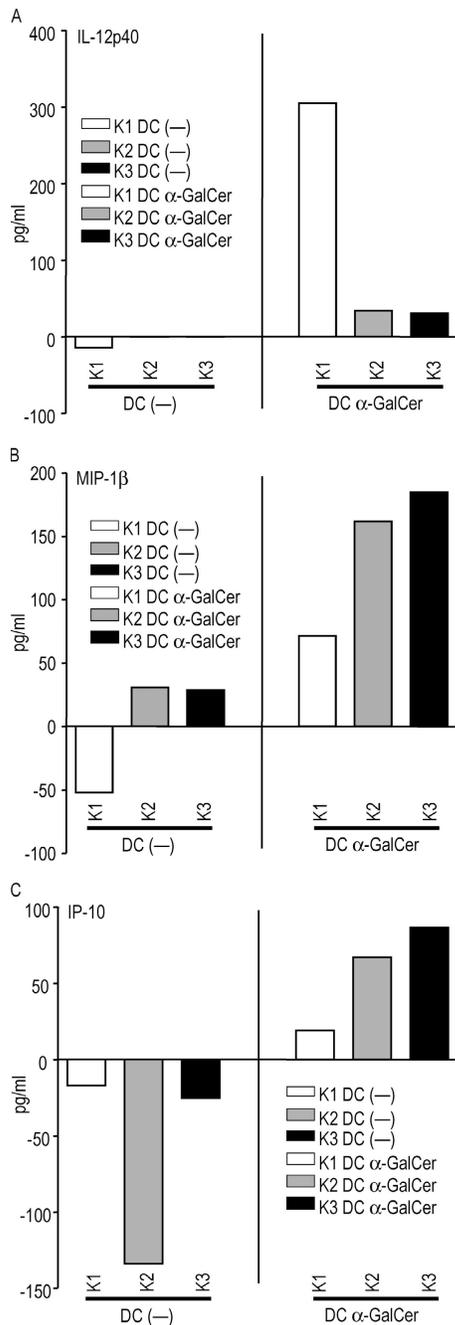
### Changes in serum cytokines

$\alpha$ -GalCer-mediated NKT activation in vivo can lead to changes in serum cytokines in mice (10). However, changes in serum cytokines also can occur as a result of DC vaccination alone and changes in the underlying disease. To distinguish these effects from those that are specifically due to  $\alpha$ -GalCer-mediated activation, we analyzed paired serum samples before and 24 h after each vaccine for 20 cytokines/chemokines using a cytokine multiplex method. Changes in the serum levels after  $\alpha$ -GalCer-pulsed DCs for each cyto-



**Figure 2. Kinetics of NKT subsets after the injection of  $\alpha$ -GalCer-pulsed DCs.** (A) Proportion of CD4<sup>+</sup>, double negative (DN) or CD8<sup>+</sup> subsets of NKT cells after  $\alpha$ -GalCer/DC vaccination in two patients (K2 and K3), as analyzed by flow cytometry. (B) Representative FACS plots from a patient (K2), showing subsets of mobilized NKT cells, at early, mid or later time points, as shown in (A). In the lower panel, numbers in the upper right quadrant are percentage of CD4<sup>+</sup>/double negative/CD8<sup>+</sup>, respectively of total NKT cells.

kine in each patient were analyzed in the context of the changes after unpulsed DCs as a control. The sera from one patient (K4, who also developed ANA/RF positivity after vaccine; Table I) showed a high background from baseline and postvaccine samples that was consistent with autoimmune phenotype and were not analyzable by this method. The following cytokines were analyzed in this assay: IL-1 $\beta$ , -2, -4, -5, -6, -8, -10, -12 p40, -13, -15, -17; IFN- $\gamma$ ; TNF- $\alpha$ ; GM-CSF; macrophage inflammatory protein (MIP)-1 $\alpha$  and -1 $\beta$ ; IFN- $\gamma$ -inducible protein (IP)-10; monocyte chemoattractant protein-1; eotaxin; and regulated on activation, normal T cell expressed and secreted. Of this panel, three cytokines (IL-12 p40, IP-10, MIP-1 $\beta$ ) consistently showed



**Figure 3. Cytokine multiplex analysis.** Increase or decrease in serum levels before and 24 h after unpulsed (first DC injection) or  $\alpha$ -GalCer-pulsed DC vaccine (third DC injection) were analyzed separately for each patient by Luminex. (A) Changes in serum IL-12p40. (B) Changes in serum MIP-1 $\beta$ . (C) Changes in serum IP-10.

a statistically significant increase after injection of  $\alpha$ -GalCer-loaded DCs (third vaccine), but not unpulsed DCs (first vaccine) in all subjects tested (Fig. 3, A–C). Similar findings were noted when first and second vaccines were compared (unpublished data). This, coupled with a transient, but

$\alpha$ -GalCer-specific decline in circulating NKT cell numbers on day 1 (Fig. 1 A), suggests *in vivo* NKT activation and homing to tissues with IFN- $\gamma$  release *in situ*; this accounts for an increase in IP-10. The other two cytokines (IL-12 and MIP-1 $\beta$ ) were likely to be released *in vivo* by antigen-presenting myeloid cells (27, 28).

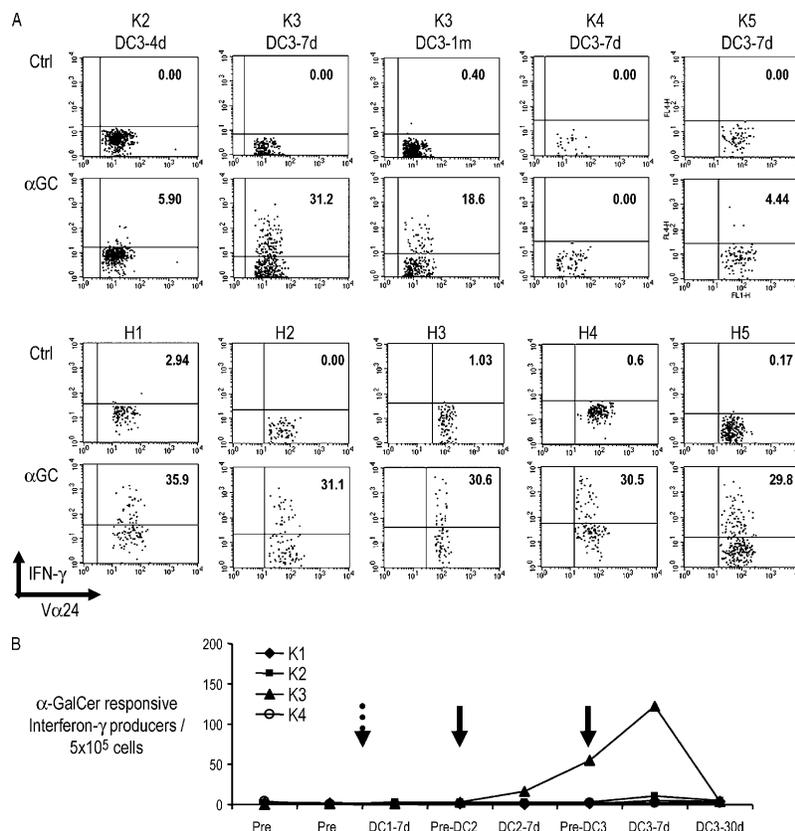
#### Cytokine production by *in vivo* expanded circulating NKT cells

Circulating NKT cells from healthy humans respond to  $\alpha$ -GalCer with rapid production of IFN- $\gamma$  *in vitro* (29). As DC vaccination led to an increase in NKT cells to within the range that is seen in healthy donors, we analyzed the ability of these cells to secrete IFN- $\gamma$  *in vitro* using Elispot and intracellular cytokine secretion (ICS). In both assays, freshly isolated NKT cells that were mobilized *in vivo* produced little detectable IFN- $\gamma$ , with the exception of some time points in one patient (K3; Fig. 4, A and B). Therefore, freshly isolated circulating NKT cells expanded *in vivo* seemed to be impaired in their ability to secrete IFN- $\gamma$ , similar to that reported for naturally occurring NKT cells in patients who had myeloma and other advanced cancers (17, 19).

A lack of IFN- $\gamma$  production may be associated with the production of other cytokines. For example, in mice, IL-13-producing NKT cells have immune regulatory properties (30). Therefore, production of alternate cytokines was analyzed by two methods. An analysis of supernatants from overnight cultures of PBMCs before and after vaccination confirmed the lack of increase in IFN- $\gamma$  or the chemokine, IP-10 (normally produced in response to IFN- $\gamma$ ; unpublished data). No increase in IL-4, -10, -13, and -2 was noted in these supernatants (unpublished data). RNA that was isolated from these cells also was analyzed for IFN- $\gamma$ , IL-4, and IL-13 transcripts by TaqMan, and again confirmed the data from other assays (unpublished data). Therefore, NKT cells that were mobilized *in vivo* in blood after  $\alpha$ -GalCer DC injection were less efficient at rapid IFN- $\gamma$  production in response to  $\alpha$ -GalCer restimulation in short-term assays, compared with those from healthy donors.

#### Proliferative capacity of mobilized NKT cells

Human NKT cells readily undergo several fold expansion *in vitro* after stimulation with  $\alpha$ -GalCer-pulsed mature DCs to yield NKT cells with a Th1 phenotype. To test the proliferative capacity of mobilized NKT cells, pre- and postimmunization PBMCs were cultured with  $\alpha$ -GalCer-loaded DCs. Minimal NKT expansion was seen in preimmunization samples, which was consistent with a severe deficiency of NKT cells at baseline. In contrast, NKT cells could be expanded readily from postvaccine samples in just 1-wk cultures (Fig. 5 A). The expanded NKT cells again were  $V\alpha 24^+/\alpha$ -GalCer-dimer $^+$  and  $V\alpha 24^-/\alpha$ -GalCer-dimer $^+$ , as in the case of freshly isolated NKT cells (Fig. 5 B). Even when these expanded NKT cells were stimulated *in vitro* with  $\alpha$ -GalCer-loaded DCs, they exhibited reduced capacity for IFN- $\gamma$  secretion when compared with their counterparts from healthy



**Figure 4. Function of freshly isolated NKT cells mobilized in vivo.** (A) Intracellular cytokine staining for  $\alpha$ -GalCer reactive IFN- $\gamma$  production. Freshly isolated PBMCs from postvaccination samples (K2–K5) containing NKT cells or healthy donors (H1–H5) were cultured overnight with anti-CD28 alone (as control [Ctrl]), or with 100 ng/ml  $\alpha$ -GalCer ( $\alpha$ GC) in the presence of monensin. The percentage of IFN- $\gamma$ -producing  $V\alpha 24^+V\beta 11^+$

cells was quantified by flow cytometry. The data shown are gated on  $V\alpha 24^+V\beta 11^+$  cells. (B) Detection of  $\alpha$ -GalCer reactive IFN- $\gamma$ -producing cells by Elispot. Freshly isolated PBMCs from pre- or postvaccination samples were cultured overnight with 100 ng/ml  $\alpha$ -GalCer. The presence of IFN- $\gamma$ -producing cells was quantified by ELISPOT.

controls (Fig. 5 C). This was confirmed again by TaqMan analysis, wherein the  $\alpha$ -GalCer-reactive increase in IFN- $\gamma$  was significantly lower in patients versus healthy controls (Fig. 5 D).

#### Activation of adaptive T cell immunity: changes in virus-specific memory T cells

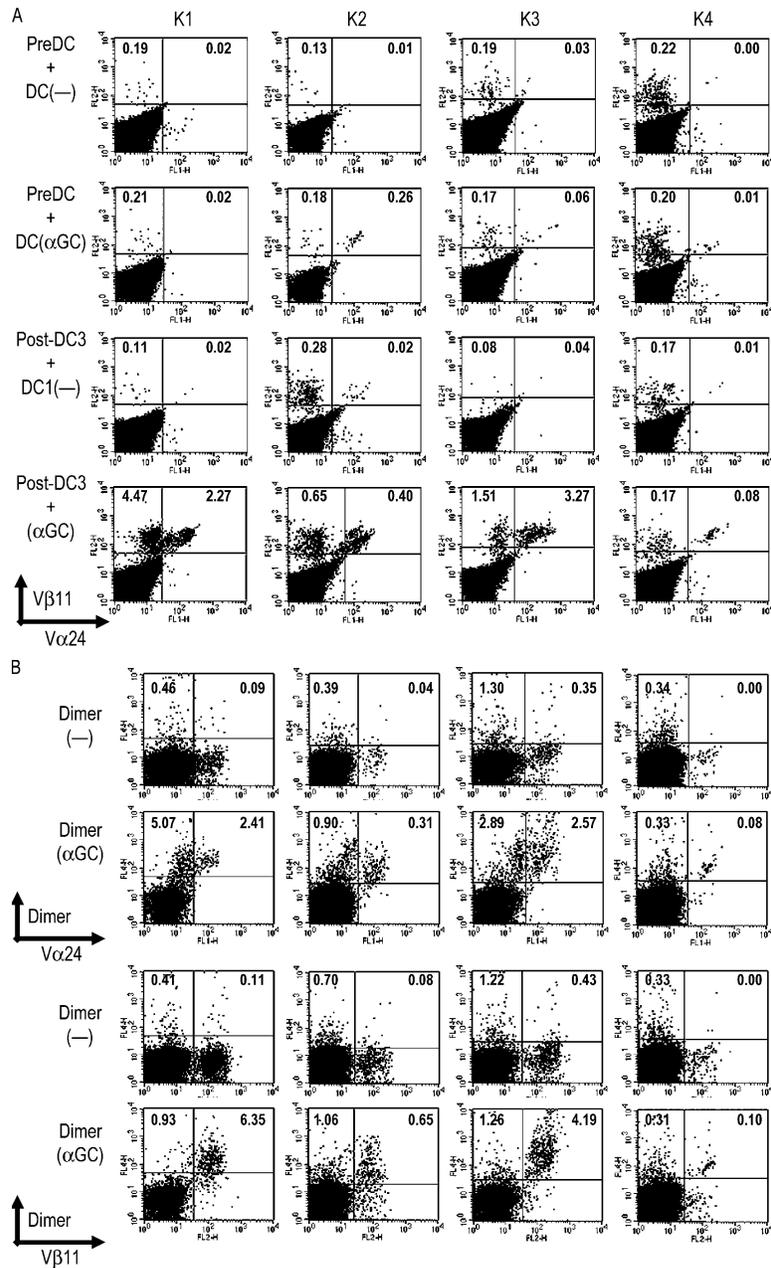
The increase in serum IL-12 after  $\alpha$ -GalCer/DC vaccination suggested the possibility that NKT activation might lead to the activation of APCs in vivo, which, in turn, might enhance antigen-specific CD8<sup>+</sup> killer T cell responses (31–33). To assess changes in antigen-specific T cells, viral antigen-specific T cells against influenza matrix protein and CMVpp65 were monitored by Elispot and MHC tetramers. This was feasible because all patients were HLA A2<sup>+</sup>. An increase in CMVpp65-specific, but not influenza matrix peptide (Flu-MP)-specific IFN- $\gamma$  producers in fresh PBMCs was observed in three of four patients who were tested following the injection of  $\alpha$ -GalCer-pulsed, but not unpulsed, DCs (Fig. 6 A). This also was accompanied by a modest increase in peptide-MHC tetramer binding cells (unpublished data). To

assess memory T cells, PBMCs from before and after immunization were thawed together and expanded using peptide-loaded DCs. Antigen-specific T cell expansion was monitored using MHC tetramers. Injection of  $\alpha$ -GalCer-loaded DCs was associated with a significant increase in CMV-specific memory T cells—but not Flu-MP specific T cells—in all three individuals who were tested (Fig. 6, B and C).

One of the patients (K2) received an inactivated influenza vaccine as a part of routine care, shortly after the injection of  $\alpha$ -GalCer-loaded DCs. Traditionally, these vaccines are believed to be poor at activating CD8<sup>+</sup> killer T cell responses and mostly boost only humoral immunity (34). Surprisingly, there was a significant expansion of Flu-MP-specific, IFN- $\gamma$ -producing, and memory T cells in this patient which was consistent with enhancement of vaccine-induced immune response with  $\alpha$ -GalCer-pulsed DCs (Fig. 6 D).

#### Expansion/activation of NK cells

Activation of NKT cells in mice can lead to rapid activation of NK cells in vivo (35). DCs also can lead directly to activation of innate effectors, including NK cells (36). We ob-



**Figure 5. Proliferative capacity and function of mobilized NKT cells.** (A) DC-mediated expansion of mobilized NKT cells. Pre- and postimmunization PBMCs were thawed together and cultured with autologous monocyte-derived mature DCs with or without pulsing with 100 ng/ml  $\alpha$ -GalCer ( $\alpha$ GC; DC/responder ratio of 1:20), in the presence of 50 U/ml IL-2. After 7 d of culture, the presence of  $V\alpha 24^+V\beta 11^+$  invariant NKT cells was quantified by flow cytometry. Numbers in the quadrant indicates the percentage of cells in the quadrant of the total lymphocyte population. (B) Binding of iNKT cells expanded in vitro from postvaccine samples to CD1d- $\alpha$ -GalCer dimers. Dimer(-) refers to unloaded dimers. Expression of  $V\alpha 24$  or  $V\beta 11$  on dimer-binding cells was analyzed by flow cytometry. (C) IFN- $\gamma$  production by ex vivo expanded NKT cells. NKT cells expanded ex vivo as in (A) were stimulated overnight

with unpulsed or  $\alpha$ -GalCer-pulsed autologous DCs in the presence of monensin. NKT cells expanded from healthy donors (H6-H8) were used as a control. The presence of IFN- $\gamma$ -producing cells was quantified by flow cytometry. (D) TaqMan analysis of cytokine secretion in expanded NKT cells. NKT cells expanded ex vivo as in (A) were stimulated overnight with unpulsed or  $\alpha$ -GalCer-pulsed autologous DCs. NKT cells expanded from healthy donors (H9-H11) were used as a control. The presence of mRNA for selected cytokines (IFN- $\gamma$ , IL-4, IL-10, and IL-13) was analyzed by real time RT-PCR. Data were normalized to the expression of a housekeeping gene, GAPDH.  $\alpha$ -GalCer reactive transcription was analyzed by comparing the expression in control versus  $\alpha$ -GalCer-stimulated samples. \*P < 0.05 for comparison with healthy donor control.

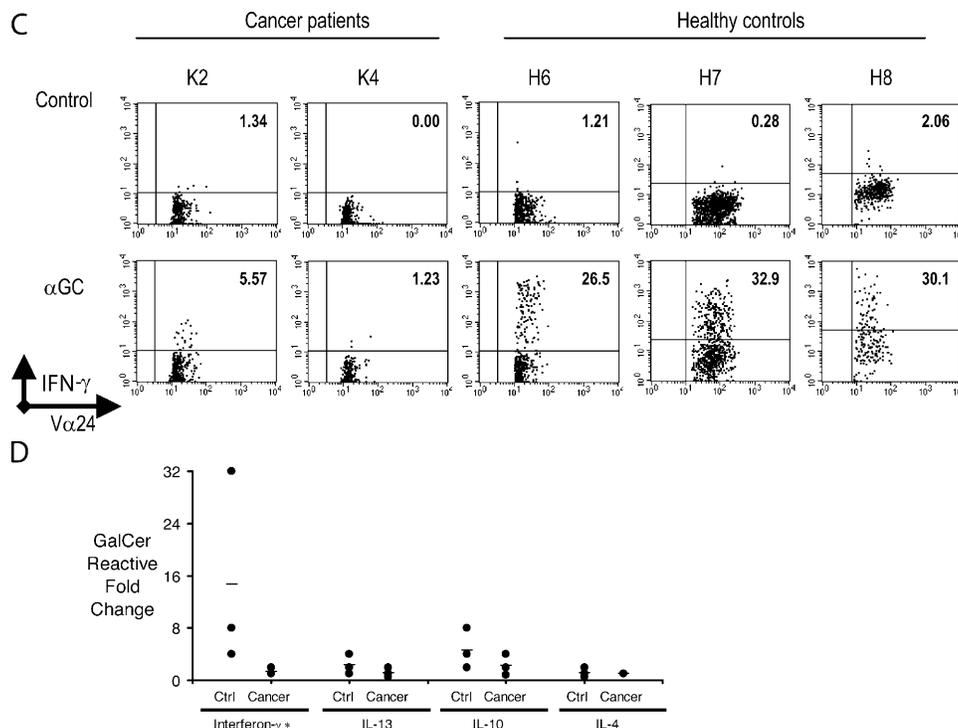


Figure 5 (continued)

served a mild transient decline in circulating NK cell numbers at 6 h or 24 h after all vaccines. The cause of this decline is not known, but it did not differ between unpulsed or  $\alpha$ -GalCer-pulsed DCs. In two patients (K2 and K3), there was a transient spike in NK numbers (and CD16<sup>+</sup> and CD16<sup>-</sup>CD56<sup>high</sup> subsets) after  $\alpha$ -GalCer-pulsed DCs. However, this was within the interassay variance that was seen at baseline (Fig. 7). There was no detectable up-regulation of CD69 on NK cells/subsets, and no changes in the expression of natural cytotoxicity receptors p30, p44, and p46 on NK cells/subsets after DC vaccination (unpublished data). There also was no detectable increase in IFN- $\gamma$  production in the CD3 population that was enriched in NK cells after vaccination. These studies only monitored blood NK cells; further analysis of changes in NK cell function, particularly in lymphoid tissues, is needed to assess fully the impact of DC vaccines on NK cells in vivo in humans (37).

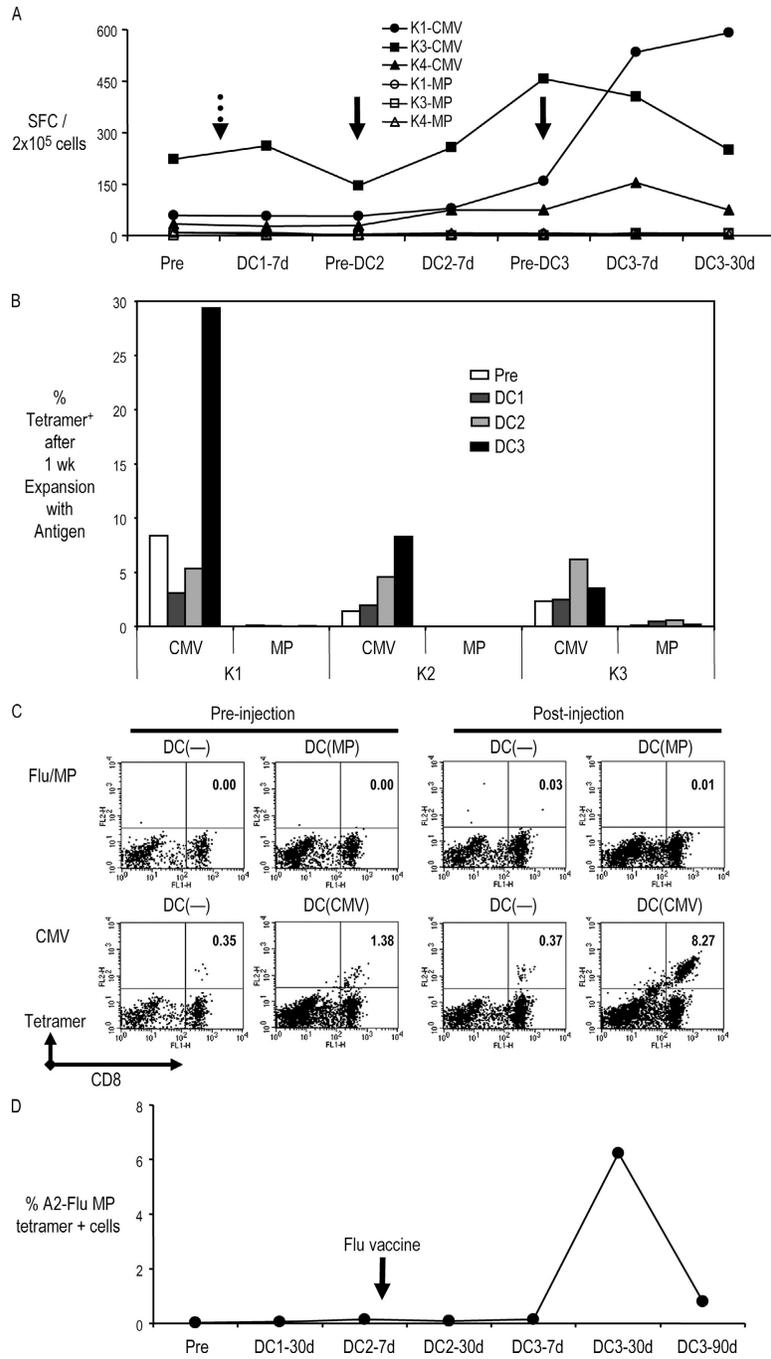
## DISCUSSION

These data reveal that the injection of  $\alpha$ -GalCer-pulsed mature DCs can lead to sustained expansion of glycolipid-reactive NKT cells in vivo in humans. The NKT expansion that was induced by only two injections of  $\alpha$ -GalCer-loaded mature DCs lasted for several months. This is in contrast with the reported transient NKT activation that was observed in response to KRN-7000 alone, or pulsed on immature DCs (20, 21). NKT cells expanded in all patients we studied, even though these patients had severe NKT deficiencies at baseline, and the patients were not preselected based on a minimum

NKT cutoff, as in earlier studies (21). NKT expansion was feasible in vivo, even when NKT cells could not be expanded in vitro from the preimmunization samples of these patients. Therefore, as with peptide antigens, targeting glycolipid antigens to mature DCs leads to enhanced immune activation.

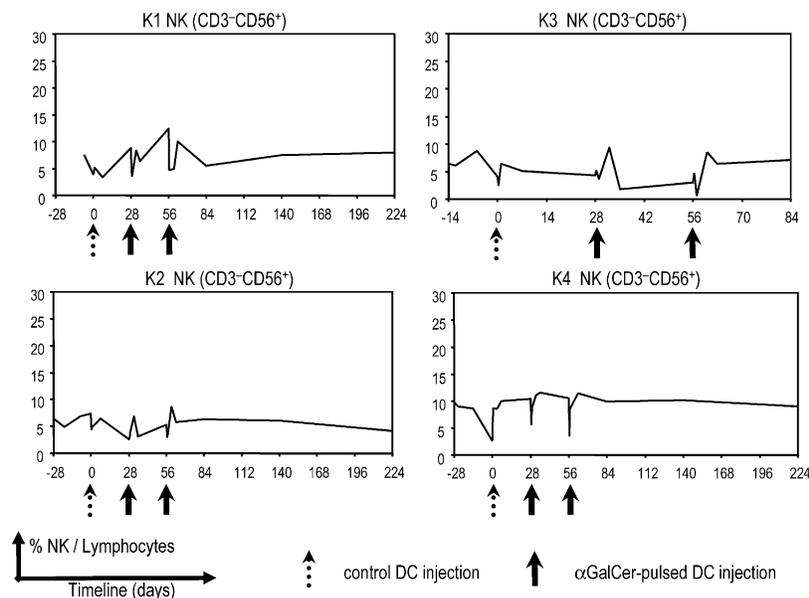
The feasibility of a sustained expansion of NKT cells provides the opportunity to study the biology of mobilized human glycolipid-reactive T cells in vivo. Recent studies have shown that human NKT cells can be divided into distinct subsets (CD4<sup>+</sup>, double negative, CD8<sup>+</sup>) with distinct functional properties (25, 26). Our data show that all three subsets can be expanded with DCs that are loaded with  $\alpha$ -GalCer in vivo. However, at least in some patients, we observed that the proportions of these subsets changed over time, which suggests different kinetics of mobilization, tissue homing, or survival. Additional patients will need to be studied to characterize this further. A similar kinetics of different NKT subsets recently were observed after in vitro expansion (38). Also, it is now clear that the repertoire of glycolipid-reactive T cells—as detected using glycolipid-CD1d multimers—includes standard invariant TCR<sup>+</sup>ve and TCR<sup>-</sup>ve cells (24). In this study, we also observed an expansion of V $\alpha$ 24<sup>-</sup>CD1d dimer<sup>+</sup> cells; this suggests that both populations of glycolipid-reactive T cells are expanded in vivo with this ligand.

Activation of NKT cells by  $\alpha$ -GalCer in vivo leads to rapid release of cytokines and activation of other immune effectors. Recent studies in mice suggest that  $\alpha$ -GalCer-mediated NKT activation can provide an adjuvant function, and enhance T cell immunity to coadministered antigen, which



**Figure 6. Changes in antigen-specific T cell responses.** (A) Changes in CMVpp65<sup>+</sup> and Flu-MP-specific IFN- $\gamma$ -producing T cells. Freshly isolated PBMCs were cultured overnight with defined A2-restricted peptides from Flu-MP or CMVpp65. The number of antigen-specific IFN- $\gamma$ -producing T cells was measured using an Elispot assay. (B) Expansion of virus-specific memory T cells (recall tetramer assay). Samples from pre- or postimmunization samples were thawed together and cocultured with autologous mature DCs that were pulsed with specific peptides. After 7 d of culture, the number of peptide-specific MHC tetramer binding T cells was quantified by flow cytometry. (C) Repre-

sentative FACS plots showing staining for CMVpp65-HLA A2 tetramer and Flu-MP-A2 tetramer, from an expansion as in (B). Number indicates percent of tetramer positive cells in the quadrant. (D) Changes in Flu-MP-specific memory T cells in a patient who also received an inactivated influenza vaccine during the course of the trial. Pre- and postimmunization T cells were thawed together and expanded with autologous peptide-pulsed DCs as in (B). After 7 d of culture, the presence of Flu-MP-A2 tetramer binding T cells was monitored by flow cytometry. SFC, spot-forming cells.



**Figure 7. Changes in NK cells.** The number of CD3<sup>+</sup>CD56<sup>+</sup> NK cells in fresh PBMCs was monitored by flow cytometry, before and after DC vaccination.

is mediated by induction of DC maturation (31–33). In this study, NKT activation was associated consistently with a transient loss of circulating NKT cells and an increase in the serum levels of IP-10, IL-12 p40, and MIP-1 $\beta$ . Generally, these cytokines are believed to be derived from myeloid APCs. Therefore, these data are consistent with the possibility that NKT activation led to downstream activation of myeloid antigen-presenting DCs in vivo.

The activation of NKT cells also was associated with an expansion of antigen-specific memory T cells, as might be expected if the DCs that matured in vivo in response to NKT cells also were capturing antigens (31–33). Changes in antigen-specific T cell function were not tested in earlier clinical studies that targeted NKT cells via  $\alpha$ -GalCer. The increase in memory T cells was observed only for those for which the antigen was persistent (CMV), but not for influenza, for which the antigen is lacking. The only setting in which a clear increase in Flu-MP-specific memory T cells was observed was after concurrent injection of an influenza vaccine in one patient. These data do not allow us to establish a causal relationship between NKT and T cell activation, but suggest a correlation. In previous studies of DC immunotherapy, we and others had not observed a nonspecific increase in virus-specific memory T cells (39). Therefore, we believe that this likely is due to the inclusion of KRN-7000 as an adjuvant with the DCs. However, these data do not distinguish between direct effects of NKT cells on APCs and indirect effects via release of cytokines. In view of these findings, the breadth of T cell analysis in future NKT-based human trials needs to be extended.

In recent studies, NKT cells from myeloma and other advanced cancers were defective in their ability to secrete IFN- $\gamma$  after  $\alpha$ -GalCer stimulation in vitro (17, 19). The circulat-

ing NKT cells that were mobilized in this trial had similar defects in their ability to secrete IFN- $\gamma$  in vitro, when compared with those that were isolated from healthy donors. This was true even after these cells were expanded in culture using  $\alpha$ -GalCer-loaded DCs. One possibility is that NKT cells in blood may be distinct from those in the tissues (40). The mechanism behind the paucity of IFN- $\gamma$  secretion by circulating NKT cells in cancer is not known, but may relate to the effects of suppressor cytokines or to binding of self- or tumor-derived CD1d ligands in vivo (41–43). Understanding the mechanism of loss of NKT effector function and pharmacologic restoration of this function in vivo might improve the clinical efficacy of NKT targeting against human cancers.

In this trial, we observed a rapid, but transient, decline in circulating NK cell numbers after each DC vaccine. However, this was similar between unpulsed and KRN-7000-pulsed DCs, and therefore, could not be ascribed to KRN-7000. The cause for this decline in NK cells is not known. However, it is consistent with the recent observations of Martin-Fontecha and colleagues of DC-mediated transient NK recruitment to lymphoid tissues in mice (37), and had not been tested previously in human DC vaccine studies (44). This loss of NK cells from the blood also may have limited our ability to detect NKT-mediated NK activation, because we are unable to access NK cells in lymphoid tissues or spleen at these time points in the human setting.

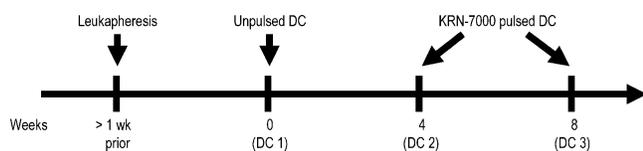
In summary, these data provide evidence that the presentation of  $\alpha$ -GalCer by mature DCs allows reliable and sustained expansion of glycolipid-reactive NKT cells in vivo in humans. Given the importance of NKT cells in microbial and tumor immunity,  $\alpha$ -GalCer-pulsed DCs may be a useful clinical therapeutic approach for targeted immune restoration of NKT cells, analogous to the current use of granulo-

cyte colony-stimulating factors for boosting neutrophils in humans. The lack of NKT expansion with unpulsed DCs was somewhat surprising, because they would be expected to present endogenous NKT ligands. These data suggest that this time-consuming control may be omitted in future studies. DC-mediated human NKT activation also leads to downstream DC activation and enhanced memory T cell function *in vivo*. Defects in DC maturation have been proposed to underlie immune paralysis in cancer and HIV infection (45, 46). Therefore, NKT cell-mediated DC activation *in vivo* may be an approach to help alleviate cancer-associated immune paresis. Enhancement of T cell memory also may be of benefit in patients who have persistent viral infections, such as HIV or hepatitis. The data also provide a framework for combining this approach with other vaccines that target T cells, particularly against tumors and chronic viral infections. Further study of this approach in larger numbers of patients is needed to better understand the impact of interindividual variability in NKT cells on the efficacy of NKT targeting in humans. In our view, such studies will require the use of cryopreserved DCs that are generated from progenitors using closed systems. Earlier studies for harnessing the adjuvant function of DCs in humans focused solely on directly activating T cell immunity via loading antigens on DCs that were generated *ex vivo* (44); however, the experiments reported here show that DCs can be effective adjuvants for boosting innate effectors in humans as well, and that this can lead to an increase in adaptive immunity to antigens that are being presented *in vivo* in the treated patients.

## MATERIALS AND METHODS

**Study design and eligibility.** The study design was a single-arm open-label trial to test the feasibility and tolerability of injection of unpulsed and KRN-7000-pulsed DCs in patients who had advanced cancer (Fig. 8). Patients who had relapsed or persistent myeloma after initial therapy, metastatic renal cell cancer, unresectable or metastatic hepatocellular cancer, or metastatic cancer of any histologic type that had failed standard therapy were eligible. Other eligibility criteria were: Eastern Cooperative Oncology Group performance score of 0–2, age >18 yr, and adequate organ function (hemoglobin >8 g/dl, white blood cells >3,000/ $\mu$ l, serum transaminases <4 $\times$  upper normal limits, and serum bilirubin <3 mg/dl). Patients who had active or chronic infections, known clinical autoimmune disease, or Gaucher's disease, and pregnant or lactating females were excluded. All patients signed an informed consent that was approved by the Rockefeller University Institutional Review Board, and the study was conducted under an Investigational New Drug application that was approved by the U.S. Food and Drug Administration.

**Treatment and monitoring schema.** After screening and registration, patients underwent a 2–3 h steady-state leukapheresis for the collection of



**Figure 8.** Schema for clinical trial.

DC progenitors. All patients received an initial intravenous injection of 5 million unpulsed monocyte-derived mature DCs, followed at monthly intervals by two additional injections of mature DCs that were pulsed with  $\alpha$ -GalCer (KRN-7000). Unpulsed DCs were injected as a control because of the possibility that mature DCs alone might stimulate NKT cells *in vivo* (47). After each injection, patients were observed overnight at the Rockefeller University General Clinical Research Center. Patients were evaluated at 4 and 7 d after each injection, 30 d after the last injection, and every 3 mo thereafter, until removal from the study. Complete blood count and serum chemistries, including liver function tests, were monitored at baseline, 1 d, 7 d, and 1 mo after each injection. Patients also were monitored for the development of RF or ANAs.

**Generation of DCs.** DCs were generated from blood monocytes using a protocol that was adapted from Thurner et al., in a dedicated facility under good tissue practices guidelines (48). In brief, mononuclear cells were isolated from leukapheresis specimens using Ficoll Hypaque density gradient centrifugation and cryopreserved in aliquots. For generating DCs, the mononuclear cells were thawed and allowed to adhere in tissue culture plates. Adherent monocytes were cultured in RPMI 1640 (Biowhittaker) supplemented with 1% autologous plasma, in the presence of 20 ng/ml GM-CSF (Immunex) and 20 ng/ml IL-4 (Cell Genix). The cultures were supplemented with cytokines on days 1, 3, and 5 of culture. On day 5, immature DCs were transferred to fresh six-well plates, and induced to mature using an inflammatory cytokine cocktail that consisted of 10 ng/ml IL-1 $\beta$ , 1,000 U/ml IL-6, 10 ng/ml TNF- $\alpha$  (all obtained from Cell Genix), and 1  $\mu$ g/ml prostaglandin E<sub>2</sub> (Amersham Biosciences). This maturation stimulus was chosen based on preclinical data which showed that maturation cytokines were comparable to other stimuli, such as CD40 ligand or LPS (29), and because it yields a uniform population of phenotypically mature DCs. For KRN-7000 loading, DCs were pulsed with 100 ng/ml of KRN-7000 at the time of DC maturation. The DCs were harvested for injection ~20 h after the induction of maturation, and reconstituted in 5% autologous plasma in normal saline at 1 million DCs/ml before intravenous injection. Release criteria for DCs were viability >80%, purity >50%, maturity (expressed as percentage of CD83<sup>+</sup> cells) >60%, negative gram stain, endotoxin and mycoplasma PCR, and negative in process cultures from samples sent 48 h before release. Postrelease tests included negative extended sterility cultures, and mycoplasma PCR. DC preparations also were monitored for the ability to stimulate allogeneic mixed lymphocyte reactions. Effective loading of the final product with  $\alpha$ -GalCer was monitored by testing the ability of these DCs to expand NKT cells in culture, without further *ex vivo* loading. All products met all release criteria and postrelease testing, and the DCs had a typical phenotype of CD14<sup>-</sup>, HLA<sup>-</sup>DR<sup>+</sup>, and CD83<sup>+</sup> cells (48).

**Immunologic monitoring.** Immune monitoring was performed predominantly using blood samples. In all patients, at least three baseline samples were obtained before the first injection in order to establish a baseline. Follow-up samples for immune monitoring were obtained at 6 h, 24 h, 4 d, 7 d, and 1 mo after each DC injection, and every 3 mo after the last injection. When possible, samples of bone marrow aspirates in myeloma patients were used to assess the changes in NKT cells in the tumor bed before and after vaccination.

**Number and subsets of NKT cells.** The numbers of NKT cells in freshly isolated blood or bone marrow mononuclear cells were quantified by multi-parameter flow cytometry, based on the expression of invariant T cell receptor ( $V\alpha 24/V\beta 11$ ) on the cell surface and intracellularly after permeabilization using saponin, and binding to CD1d dimer (DimerX, BD Biosciences) loaded with  $\alpha$ -GalCer using the manufacturer's recommendations. At least  $2 \times 10^5$  lymphocyte-gated events were acquired to allow reliable estimation of NKT cells. Subsets of expanded NKT cells were monitored based on the expression of CD4 or CD8 on invariant T cell receptor positive cells.

**Functional studies on NKT cells.** Functional aspects of NKT cells were assayed based on cytokine production and proliferation in response to  $\alpha$ -GalCer. Cytokine production was measured in fresh PBMCs and after in vitro expansion using  $\alpha$ -GalCer-loaded DCs, and analyzed by Elispot, intracellular cytokine flow cytometry, quantitative RT-PCR by TaqMan, and multiplex cytokine analysis.

**Stimulation of NKT cells in culture.** To assess the ability of NKT cells to proliferate in culture, pre- and postimmunization PBMCs were thawed together and cultured in the presence of autologous  $\alpha$ -GalCer-pulsed monocyte-derived mature DCs (on unpulsed DCs as a control), at a DC/PBMC ratio of 1:20 in the presence of 50 U/ml of IL-2 (Chiron), as described (19, 22). Expansion of NKT cells was monitored by quantifying V $\alpha$ 24<sup>+</sup>/V $\beta$ 11<sup>+</sup> or CD1d<sup>-</sup>  $\alpha$ -GalCer dimerX-binding NKT cells by flow cytometry.

**Elispot assay for IFN- $\gamma$ -producing T/NKT cells.** To detect IFN- $\gamma$ -producing NKT cells, freshly isolated PBMCs ( $5 \times 10^5$  cells/well) were cultured overnight in the presence of 100 ng/ml  $\alpha$ -GalCer in Elispot plates that were precoated with anti-IFN- $\gamma$  antibody (Mabtech), as described (19). For detecting influenza-specific T cells, PBMCs were infected with influenza (MOI = 2) before plating in the Elispot assay. Staphylococcal enterotoxin A (50 ng/ml) was used as a positive control. In HLA A2<sup>+</sup> patients, antigen-specific T cells against A2-restricted peptides from influenza matrix protein and CMVpp65 also were monitored using this assay, as described previously (49).

**Intracellular cytokine detection by flow cytometry.** For the detection of ICS, freshly isolated PBMCs were cultured overnight with 100 ng/ml  $\alpha$ -GalCer in the presence of anti-CD28 (3  $\mu$ g/ml; BD Biosciences), or anti-CD28 alone as a control in the presence of 0.7  $\mu$ g/ml Golgi Stop (from Cytofix/Cytoperm Plus kit from BD Biosciences). Stimulation with PMA (50 ng/ml) and ionomycin (1  $\mu$ M) was used as positive control. The presence of IFN- $\gamma$ -producing V $\alpha$ 24<sup>+</sup>/V $\beta$ 11<sup>+</sup> cells was quantified by flow cytometry, as described (19). For the detection of intracellular cytokine production by expanded NKT cells (after 1-wk culture with DCs as described above; recall ICS), these cells were restimulated overnight with unpulsed or  $\alpha$ -GalCer-loaded DCs (DC/responder ratio 1:10), before staining and analysis, as described (19).

**TaqMan RT-PCR quantitation of mRNA.** Analysis of  $\alpha$ -GalCer-reactive cytokine production in the Elispot and ICS assays was limited to IFN- $\gamma$ . Therefore, we used cytokine multiplex analysis and TaqMan RT-PCR to assay the production of alternate cytokines in these cultures. For the TaqMan analysis, PBMCs were cultured with  $\alpha$ -GalCer as described earlier. For some assays, pre- and postimmunization samples expanded with DCs were restimulated with unpulsed or  $\alpha$ -GalCer-loaded DCs (DC/responder ratio of 1:10) for 8 h. Stimulated cells were pelleted and total RNA was isolated using the QIAGEN RNeasy kit. The primers and probes for IFN- $\gamma$ , and IL-4, -10, and -13 were purchased from Applied Biosystems/PerkinElmer. RT-PCR reactions were performed in duplicate samples according to the manufacturer's directions (EZ PCR Core Reagents; TaqMan and Applied Biosystems), using an Applied Biosystems PRISM 7700 Thermal cycler, as described previously (50). mRNA levels for a housekeeping gene GAPDH were used to normalize each gene from each sample.

**Cytokine multiplex analysis.** Serum was analyzed for 20 cytokines and chemokines using the Protein Multiplex Immunoassay kit (Biosource International) as per the manufacturer's protocol. Briefly, Biosource's Multiplex beads were vortexed and sonicated for 30 s and 25  $\mu$ l was added to each well and washed twice with wash buffer. The samples were diluted 1:2 with assay diluent and loaded onto a Millipore Multiscreen BV 96-well filter plate; 50  $\mu$ l of incubation buffer had been added previously to each well. Serial dilutions of cytokine standards were prepared in parallel and added to the plate. Samples were incubated on a plate shaker at 600 revolutions/min in the dark at room temperature for 2 h. The plate was applied to a Milli-

pore Multiscreen Vacuum Manifold and washed twice with 200  $\mu$ l of wash buffer. 100  $\mu$ l of biotinylated Anti-Human Multi-Cytokine Reporter was added to each well. The plate was incubated on a plate shaker at 600 revolutions/min in the dark at room temperature for 1 h. The plate was applied to a Millipore Multiscreen Vacuum Manifold and washed twice with 200  $\mu$ l of wash buffer. Streptavidin-phycoerythrin was diluted 1:10 in wash buffer, and 100  $\mu$ l was added directly to each well. The plate was incubated on a plate shaker at 600 revolutions/min in the dark at room temperature for 30 min. The plate was applied to the vacuum manifold, washed twice, and each well was resuspended in 100  $\mu$ l wash buffer and shaken for 1 min. The assay plate was transferred to the Bio-Plex Luminex 100 XYP instrument for analysis. Cytokine concentrations were calculated using Bio-Plex Manager 3.0 software with a five parameter curve-fitting algorithm applied for standard curve calculations.

**Analysis of NK cell subsets and activation.** The numbers of NK cells and their subsets (CD16<sup>neg</sup> CD56<sup>hi</sup>; CD16<sup>pos</sup> CD56<sup>lo</sup>) in blood mononuclear cells before and after vaccination were monitored by flow cytometry. The activation status of NK cells was monitored based on the expression of CD69 and natural cytotoxicity receptors (p30, p44, and p46).

**Analysis of antigen-specific T cell responses.** Influenza virus-specific T cells were monitored by Elispot and intracellular cytokine staining, as described earlier (19). In HLA-A2<sup>+</sup> patients, T cell responses to A2-restricted epitopes that were derived from influenza matrix protein and CMVpp65 were monitored by ELISPOT in fresh PBMCs. Antigen-specific T cells against these epitopes also were quantified by MHC tetramers, as described (39). To assess the ability of memory T cells to proliferate in culture, pre- and postimmunization samples were thawed and cultured with peptide-pulsed DCs, as described previously (39). After 1 wk of culture, antigen-specific CD8<sup>+</sup> T cells were quantified using MHC tetramers.

**Statistical analysis.** Data from pre- and postimmunization assays were compared using the Student's *t* test, or Mann-Whitney test. Significance was set at  $P < 0.05$ .

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