Different requirements for α-galactosylceramide and recombinant IL-12 antitumor activity in the treatment of C-26 colon carcinoma hepatic metastases

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The glycolipid α-galactosylceramide (α-GalCer), ligand of NKT cells, has been recently shown to induce antitumor immunity in mice through the induction of IL-12 production by dendritic cells. In the present study we compared α-GalCer and rIL-12 antitumor activities in the treatment of hepatic metastases of the C-26 murine colon carcinoma. We show that in immunocompetent mice the two molecules display similar efficacy, whereas in mice knockout (KO) for β2-microglobulin (β2m), IFN-γ or IL-12p40, α-GalCer antitumor activity is severely impaired. Conversely, in all such KO mice, rIL-12 retains its efficacy. In this context, the IL-12 effect relies on NK cell function since it is abrogated by antibodies to NK1.1, expressed by both NK and NKT cells, but not in β2m KO mice that lack NKT and CD8 T cells, but have a perfectly functional NK cell population. Furthermore, in IFN-γ and IL-12p40 double KO mice, exogenous rIL-12 completely loses antitumor efficacy, suggesting the existence of an IFN-γ-independent IL-12 effect that does require the presence of endogenous IL-12p40 chain.

Key words: Tumor immunity / IL-12 / α-Galactosylceramide / NK cell / NKT cell

1 Introduction

The glycolipid α-galactosylceramide (α-GalCer) originally isolated from marine sponge, is a specific ligand for Vα14 NKT cells [1]. This recently identified lymphoid lineage is characterized by the expression of an invariant antigen receptor encoded by Vα14 and Jα281 gene segments, and the coexpression of the NK receptor NK1.1, a marker of NK cells. This unique T cell population has a strong preference for particular tissues, accounting for 20–30% of liver T cells, and has the potential to rapidly release large amounts of cytokines, such as IFN-γ and IL-4, without prior sensitization. Unlike conventional T cells, which recognize peptide antigens presented by classical MHC molecules, NKT cells bind glycolipids presented by CD1d, a non-classical MHC class I-like molecule, which is necessary for the development of the lineage (reviewed in [2]). Nevertheless, an NKT cell subset exists that does not express the invariant Vα14-Jα281 chain and is not positively selected by CD1d, and therefore is still present in CD1d-depleted mice, representing 10–20% of the number of NKT cells in wild-type tissue [3, 4].

α-GalCer, and its synthetic homologue KRN7000, have the ability to enhance NK activities of spleen cells *in vitro* and *in vivo* [5], and recently have been shown to be potent inducers of antitumor immunity in mice [1, 5–7]. The *in vivo* antitumor effect of such molecules highly resembles the antitumor activity of IL-12 [8, 9]; indeed it has been shown that Vα14 NKT cells are an essential target of IL-12 and Vα14 NKT cell-deficient mice can no longer mediate IL-12-induced rejection of the FBL-3 erythroleukemia or B16 melanoma tumors [8]. Supporting this idea, a recent paper from Kitamura et al. [10] showed that α-GalCer exerts its function through IL-12, and that NKT cells activation by α-GalCer requires both endogenous IL-12 produced by dendritic cells (DC), and direct contact between NKT cells and DC through CD40/CD40 ligand (CD40L) interaction.

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Despite the potent antitumor activity of IL-12 *in vivo* [11], its severe side effects hamper the clinical use [12]. Therefore, the search for substances able to induce endogenous release of IL-12, has gained importance.

We investigated α-GalCer and rIL-12 antitumor activity in the C-26 murine colon adenocarcinoma tumor model; the efficacy of α-GalCer in the treatment of hepatic metastases of such tumor model has already been shown by Nagakawa et al. [7]. These authors reported a similar potency of α-GalCer and rIL-12. We set our experiments starting from the conditions described by Nagakawa et al., in which the two molecules showed similar efficacy, and compared their antitumor activities in IFN-γ-knockout (KO), IL-12p40-KO, and β2m-KO mice to dissect and further clarify their mechanism of action.

Our results show that α-GalCer and rIL-12 have different effect on C-26 hepatic metastases, and likely use different mechanisms for the induction of antitumor activity, at least in situations where key factors for the induction of an efficient immune response are unavailable. This study contributes to understand the role of IL-12 and IFN-γ in the outcome of DC-NKT cells interaction.

2 Results

2.1 α-GalCer and rIL-12 are equally effective in the treatment of wild-type mice bearing C-26 hepatic metastases

We examined the antitumor effect of α-GalCer and rIL-12 in mice bearing C-26 hepatic metastases: BALB/c mice were injected intrasplenically with C-26 cells to induce liver metastases and, starting 24 h later, treated with α-GalCer, rIL-12, or control compound (see Sect. 4). As shown in Fig. 1A liver weight of control mice was increased two- to threefold, while treated mice had only a slight augmentation in liver weight. Overlapping results were obtained when the experiment was performed in (BALB/c×C57BL/6)F1 hybrid mice (Fig. 1B and Table 1). Accordingly with this result, macroscopic observation of livers revealed extended metastatization in the control group, while no evident tumors were detectable in α-GalCer- and rIL-12-treated mice (Fig. 1C, left side, and Table 1). Microscopic analysis showed control livers fully metastatized, whereas those from α-GalCer- and rIL-12-treated mice were completely tumor free or, at worst, with a few, spared, microscopic metastases (Fig. 1C, right side, and Fig. 4, left panels). Such metastases were surrounded by infiltrating leukocytes, as shown by staining with Ab to CD45 (Fig. 1C). Biotin stains the liver parenchyma, but not areas of tumors (Fig. 1C).

The same schedule of treatment was also used to treat pulmonary metastases after intravenous injection of 10^4 tumor cells: 16 days later half of the animals were killed and the number of lung metastases counted, while the other half were evaluated for survival. rIL-12 therapy significantly reduced the number of pulmonary metastases, while α-GalCer treatment had only a partial effect (Fig. 2A); both substances prolonged the survival, but, again, rIL-12 was the most efficient (Fig. 2B). This result is in agreement with the idea that the efficacy of α-GalCer correlates with the presence of NKT cells in a particular tissue: indeed α-GalCer antitumor activity is optimal in the liver, where this T cell subpopulation is much more consistent than in other localizations, such as the lungs [13].

Table 1. Macroscopical detection of C-26 hepatic metastases

Strain	No treatment				α-GalCer				rIL-12			
	no	< 5[a)]	5–15	> 15	no	< 5	5–15	> 15	no	< 5	5–15	> 15
BALB/c	14%[b)]	–	8%	78%	79%	–	14%	7%	81%	–	13%	6%
C × B6[c)]	7%	–	–	93%	70%	5%	10%	15%	68%	–	20%	12%
IFN-γ-KO	7%	–	–	93%	–	–	–	100%	57%	–	6%	37%
IL-12p40-KO	10%	–	–	90%	–	–	10%	90%	70%	–	10%	20%
(IFN-γ × IL12p40)-KO	–	–	17%	83%	–	–	–	100%	29%	–	–	71%
β2m-KO	14%	–	7%	79%	–	–	–	100%	75%	–	9%	16%
BALB/c anti-CD8	–	–	–	100%	58%	9%	–	33%	35%	17%	17%	31%
C × B6 anti-NK1.1	20%	–	–	80%	14%	–	–	86%	25%	–	–	75%

a) Number of superficial metastases.
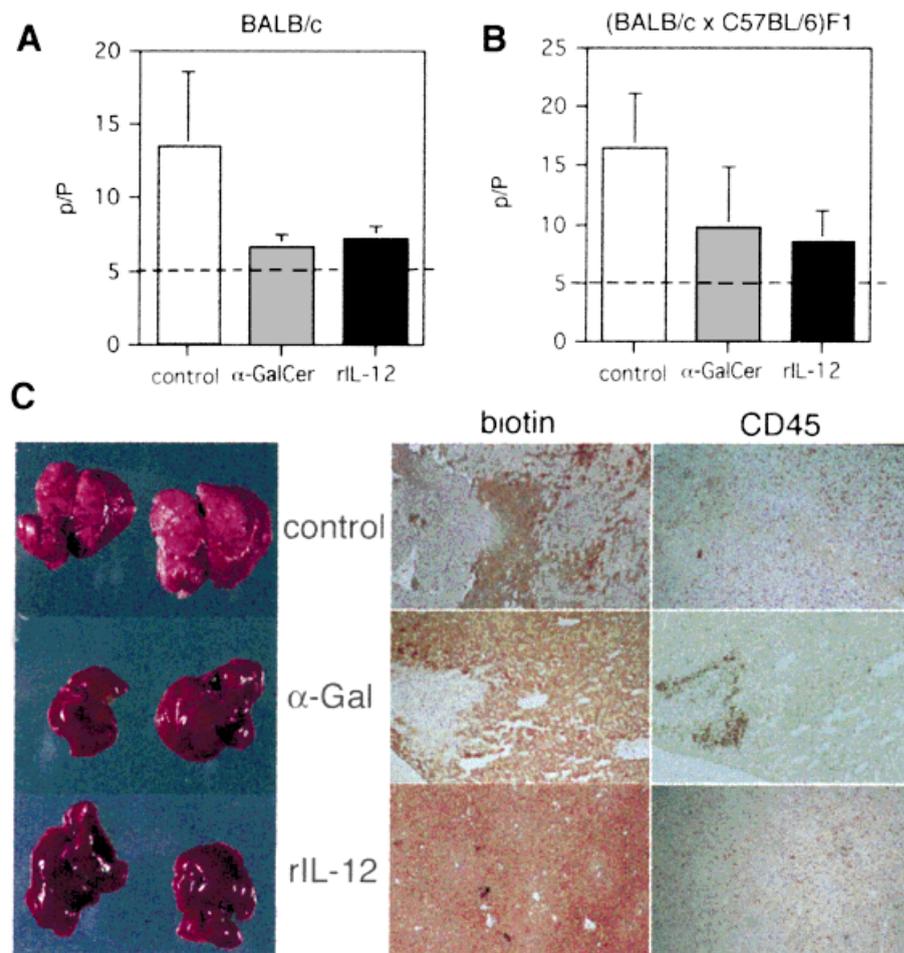b) Percentage of mice (*n* = 15–25).
c) (BALB/c × C57B/6)F1.

Fig. 1. Efficacy of α-GalCer and rIL-12 in the treatment of C-26 hepatic metastases in wild-type mice. For the induction of liver metastases mice were injected intrasplenically with 5×10^5 tumor cells, and spleen immediately removed; treatments with α-GalCer and rIL-12 are the following: α-GalCer (100 μg/kg) on days 1, 5, and 9; rIL-12 (1 μg/mouse) on days 1, 3, 5, 7, and 9, by i.p. injection. On day 12 after inoculum mice were killed, and livers removed for weighing and detection of metastases. (A) Liver weight evaluated in relation to mouse total body weigh (p/P) in BALB/c mice. (B) Liver weigh in (BALB/c×C57BL/6)F1 hybrid mice. (C) Left side: representative photographic views of treated livers; right side: immunostaining for biotin, endogenously produced by hepatocytes; tumor cells and areas of metastases do not show any staining; anti-CD45 Ab stains infiltrating leukocytes.

2.2 α-GalCer, but not rIL-12, requires host produced IFN-γ or IL-12 to exert antitumor activity

Since α-GalCer and rIL-12 demonstrated equivalent antitumor activity in the treatment of liver metastases in wild-type mice, we investigated their mechanism of action using mice knockout for IL-12p40 or IFN-γ genes, whose products have been proved critical for α-GalCer and IL-12 antitumor activity, respectively. In both IFN-γ- and IL-12-deficient mice, rIL-12 still showed antitumor activity against C-26 hepatic metastases, whereas α-GalCer did not: livers from mice treated with α-GalCer were enlarged (Fig. 3A, B) and fully metastasized (Table 1), as for control mice.

Microscopic analysis of livers from wild-type and IFN-γ-KO mice confirmed those observations. Fig. 4 shows leukocyte infiltration detected by immunostaining with an Ab against CD45: in both wild-type and IFN-γ-KO untreated mice, the number of CD45+ cells was low and cells were localized in the areas of metastatization (Fig. 4A, B); in wild-type mice treated with α-GalCer or rIL-12, infiltrating leukocytes were more abundant, and were dispersed in the hepatic parenchyma (Fig. 4C, E); in case of rIL-12, they localized mainly in the portal spaces; metastases, if present, were microscopic and heavily infiltrated (see insert in Fig. 4E). In IFN-γ-KO mice treated with α-GalCer livers were fully metastatized, and leukocyte infiltration was less abundant than in wild-type mice; leukocytes were mainly in areas of metastases and
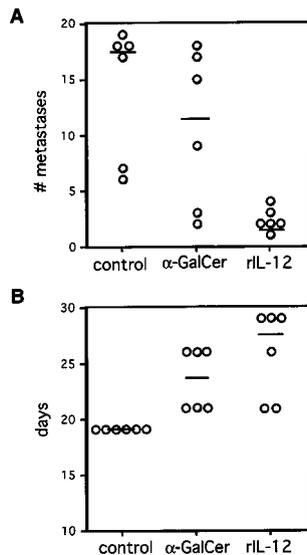
Fig. 2. α-GalCer and rIL-12 have different effects on pulmonary metastases. Lung metastases were induced by i.v. injection of 10^4 tumor cells in the tail vein and mice treated following the treatment schedule used for the hepatic model. After 16 days half of the mice were killed and lung metastases counted (A); the other mice were evaluated for survival (B). Median value for the respective groups of six mice each are represented by bars.

along the portal vein (Fig. 4D); IFN-γ-KO mice treated with rIL-12 showed a strong infiltration, and leukocytes were both dispersed in the liver parenchyma and localized in the portal spaces (Fig. 4F). Immunostaining with Ab to CD8 overlaps the results obtained with Ab to CD45, indicating that many cells of the inflammatory infiltrate are CD8^{+} T lymphocytes (data not shown).

Performing the experiment in the IFN-γ and IL-12p40 double-KO mice worsened the situation: neither α-GalCer nor rIL-12 was able to reject C-26 hepatic metastases (Fig. 3C). This may suggest that IL-12 given exogenously is sufficient to exert antitumor activity in absence of endogenous IL-12 if IFN-γ can be produced, whereas an IFN-γ-independent effect (as in IFN-γ–KO mice) requires endogenous local production of IL-12.

Preliminary *in situ* hybridization experiments (data not shown) indicated that IL-12 treatment increased the number of infiltrating cells that express IL-12p40, especially in absence of endogenous IFN-γ (IFN-γ-KO mice).
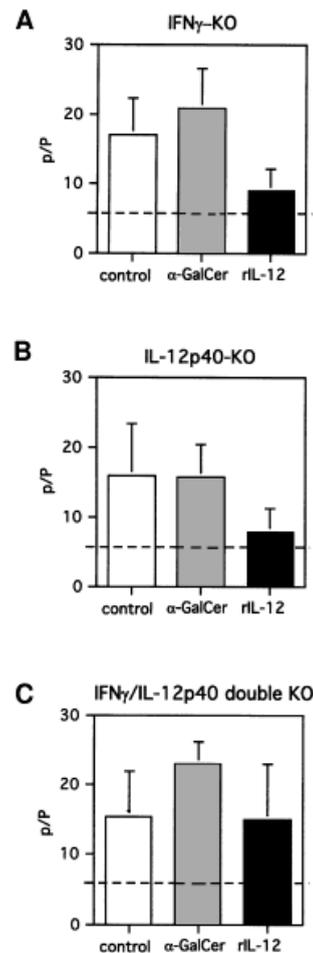

Fig. 3. α-GalCer and rIL-12 inhibit hepatic metastases differently in KO mice. In IFN-γ-KO (A) and in IL-12p40-KO mice (B) α-GalCer becomes completely inefficient, while rIL-12 retains full antitumor activity. In mice deficient for both cytokines (IFN-γ×IL-12p40-KO mice) neither α-GalCer nor rIL-12 are able to inhibit C-26 liver metastases (C). Liver weigh over total body weigh is shown (p/P) (*n*=10–15 mice/group).

2.3 α-GalCer and rIL-12 antitumor activity in β2m-KO mice

We further analyzed the molecular requirements for α-GalCer and rIL12 activity in β2m-KO mice. β2m-deficient mice have impaired expression of CD1, and therefore a reduced population of NKT cells in thymus, spleen and liver [14]. As expected α-GalCer-antitumor activity was completely abolished in these mice (Fig. 5). Conversely, rIL-12 still inhibited C-26 hepatic metastases indicating that, in absence of β2m, and therefore of both CD1 and MHC I expression, rIL-12 does not require NKT cells, and/or CD8^{+} T lymphocytes, to exert its activity against liver metastases. These data suggest that NK cells are sufficient to mediate such activity.
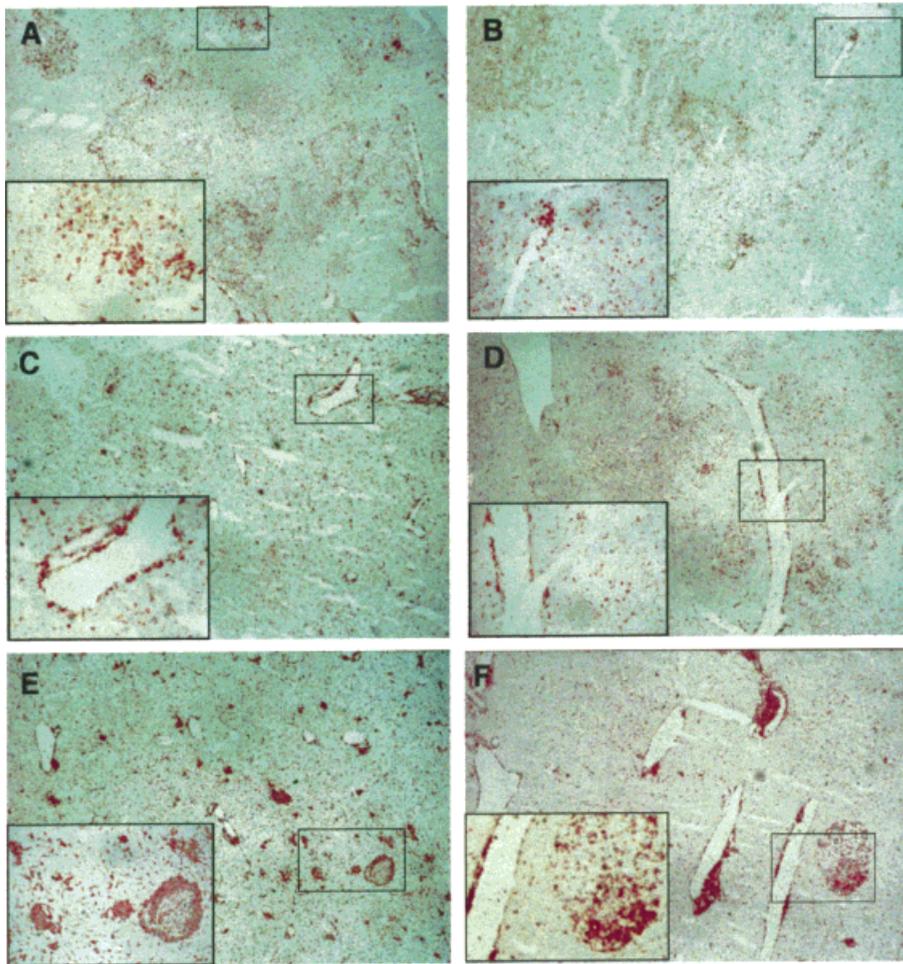
Fig. 4. Immunohistological analysis of liver leukocyte infiltration (CD45^{+} cells). Wild-type BALB/c mice (A, C, E) and IFN-γ-KO mice (B, D, F), untreated (A and B) or treated with α-GalCer (C and D) or rIL-12 (E and F). In untreated mice, both wild-type and IFN-γ-KO, the number of CD45^{+} leukocytes is low and localized in the areas of metastases; in wild-type mice treated with α-GalCer or rIL-12, infiltration is more relevant and dispersed in the hepatic parenchima. In IFN-γ-KO mice injected with α-GalCer, livers are fully metastatized and infiltration is less abundant, while mice treated with rIL-12 show heavy infiltration both in the hepatic parenchyma and in the portal spaces.

2.4 Effect of specific cell type depletions on α-GalCer and rIL-12 antitumor activity

To assess the role of different cell types in the antitumor activity of the two molecules, experiments have been performed in CD8 T cell- and in NK1.1-depleted mice. While CD8^{+} T cell depletion worsened tumor outgrowth in the untreated control group (mice showed very enlarged livers), it had limited impairment of α-GalCer and rIL-12 antitumor activity since treated mice did not show increased liver weight (Fig. 6A). Ab depletion of NK1.1^{+} cells completely abolished the efficacy of α-GalCer and severely compromised that of rIL-12 (Fig. 6B and Table 1) as expected by the expression of the NK1.1 receptor on both NKT and NK cells. Depletion of NK1.1[+]

cells confirms the role of NKT cells in mediating α-GalCer effect, and, together with the experiments performed in CD8-depleted mice and β2m-KO mice, it indicates that NK cells are the population mainly responsible for IL-12 activity in the treatment of C-26 hepatic metastases.

3 Discussion

Systemic and peritumoral injection of recombinant IL-12 markedly inhibits the growth of several tumors of different histotypes, and leads to the development of systemic immunity in some cases [15–19]. In humans, to obtain an efficient antitumor activity high doses of rIL-12
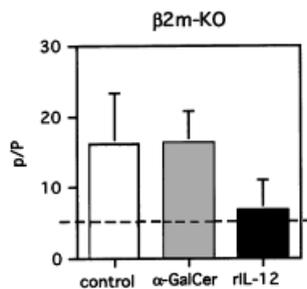
Fig. 5. α-GalCer, but not rIL-12, looses antitumor activity in β2m-KO mice. Liver weigh over total body weigh is shown (p/P).

Fig. 7. α-GalCer mechanism of action. α-GalCer binds to CD1d MHC class I-like molecule on DC and is presented to the invariant TCR on NKT cells (1). The encounter of NKT cells with DC causes the up-regulation of CD40L on NKT cells, and the subsequent triggering of CD40 on DC (2), which induces secretion of IL-12 by DC (3); IL-12, in turn, acts on NKT cells stimulating the production of IFN-γ (4) that initiates a cascade of events involving NK cells, and, with some delay, B and T cells.

given for an extended period of time seem necessary, with the price being severe side effects [12]. Particularly attractive is the possibility to combine IL-12 with other agents that, working in synergy, may reduce the amount of IL-12 needed to obtain a therapeutic effect. Alternatively, the use of other compounds able to induce endogenous production of IL-12 may be feasible, thus providing a non-toxic, and physiological cytokine's concentration.
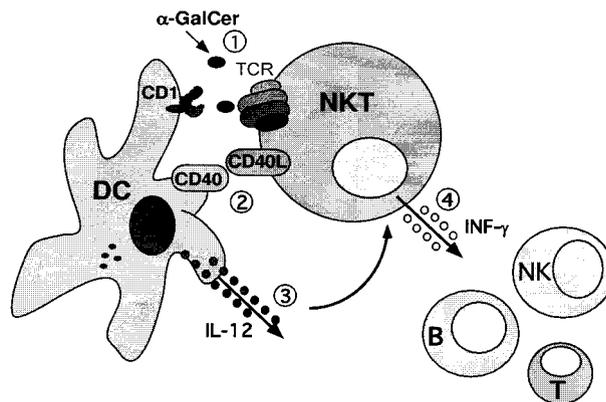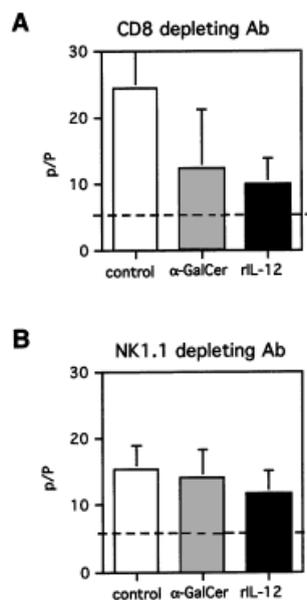
Among these compounds a lot of attention has been directed toward the glycolipid α-galactosylceramide (α-GalCer), and its synthetic homologue, KRN7000, which have been shown to induce antitumor immunity in mice [5, 6]. Recent studies have demonstrated that, indeed, these compounds exert their action through the induction of endogenous IL-12 [10].

Nakagawa et al. [7] have recently shown that α-GalCer is efficient in the treatment of C-26 colon carcinoma hepatic metastases and that its action highly resembles that of rIL-12.

In the present study we further investigated α-GalCer activity, using the same tumor setting and schedule of treatment, and compared its efficacy with that of rIL-12, using mice knockout for some immunoregulatory genes. We confirm that in immunocompetent mice the two molecules display very similar antitumor activity, whereas the lack of β2m, IFN-γ or IL-12p40 produces a different efficacy of rIL-12 and α-GalCer, making them active and inactive, respectively, in the treatment of C-26 hepatic metastases.

Fig. 6. Effect of specific cell types depletion on α-GalCer and rIL-12 efficacy in the treatment of C-26 liver metastases. CD8+ T cell depletion worsened the outcome of untreated mice, while α-GalCer and rIL-12 treatments are not particularly affected (A). NK1.1+ cell depletion, which eliminates not only NK cells but also NKT cells, impairs the efficacy of both α-GalCer and rIL-12 (B). Liver weigh over total body weigh is shown (p/P).

According to a recent paper [20] multiple injections of α-GalCer might be detrimental to NKT cells function since NKT cells depletion occurs in liver 24 h after α-GalCer injection, but a full replenishment is obtained during the following 72 h. Since we injected α-GalCer on a 4-day interval such NKT cells depletion was not expected.

Accordingly, a single injection produced the same effect of multiple injections (not shown), and thus we used the multiple schedule of treatment described by Nagakawa et al. [7] to make the two studies comparable.

The strict dependence on NKT for α-GalCer activity was confirmed by injecting C-26 cells into the tail vein of mice to obtain lung metastases. α-GalCer was less effective in lung than in liver metastases, accordingly to the number of resident NKT cells in lungs and liver, respectively. Conversely, rIL-12 was equally effective in reducing the number of metastases in both liver and lungs.

Tumor microenvironment could also influence the efficacy of rIL-12; in fact C-26 injected subcutaneously is resistant to rIL-12 treatment, likely because of a lack of tumor infiltrating leukocytes that may sense and respond to rIL-12. Accordingly, C-26 cells transduced to secrete low amount of IL-2 remain fully tumorigenic, but are rejected by rIL-12 because IL-2 produced by the tumor cells attracts and activates leukocytes to become responsive to rIL-12 [18]. In the TSA mammary carcinoma, rIL-12 is able to cure a higher percentage of mice when administered from day 7 rather than from day 1 [19], suggesting that the effect of rIL-12 is more striking against established than incipient tumors, and that it depends on a balance between tumor size and the number and type of infiltrating leukocytes at the time rIL-12 is given. Like rIL-12, α-GalCer is inefficient against C-26 given s.c. (data not shown); again the lack of efficacy could be explained by the low number of NKT cells present in this tissue compartment.

To act α-GalCer requires the encounter of DC with NKT cells. α-GalCer binds to CD1d on DC and is presented to the invariant TCR on NKT cells. Downstream effects of such encounter are the up-regulation of CD40L on NKT cells, and the subsequent triggering of CD40 on DC which induces secretion of IL-12, that, in turn, causes the production of IFN-γ by NKT cells; IFN-γ then initiates a cascade of events involving NK cells, and, with some delay, B and T cells [21]. Therefore, α-GalCer activity requires the sequential activation of several molecules. Fig. 7 summarizes α-GalCer mechanism of action; blockade at any point causes inhibition of its antitumor activity. In CD40-KO mice α-GalCer looses completely efficacy (data not shown), likely because the lack of CD40 triggering on DC impairs IL-12 production, as seen by RNase protection experiments in liver of CD40-KO mice treated with α-GalCer (C. Chiodoni, unpublished results). Experiments performed in β2m-KO, which almost completely lack the NKT population, or IL-12p40-KO or IFN-γ-KO mice show that the antitumor activity of this glycolipid is completely abolished. In such KO mice, rIL-12 remains active. In β2m-KO mice, the NK popula-

tion is perfectly functional, and could account for rIL-12 activity. This result seems in contrast with the findings of Taniguchi and colleagues [8], showing the requirement of NKT cells for IL-12-mediated tumor rejection. Besides the use of different experimental tumor models, recent papers reported that the relative contribution of NK and NKT cells as mediators of IL-12 antitumor and antimetastatic activity, varies depending upon the dose and the time of IL-12 administration; NKT cells are described to be more sensitive to low doses of rIL-12 [22], while NK cells activation requires higher doses (>2,000 U). This difference is not surprising given that NKT cells express higher level of IL-12R than NK cells [23]. In our experiments we utilized a standard regimen of 1 μg/mouse of rIL-12 that, although repeated only three times, is likely to bypass NKT cells requirement seen in other tumor systems [8].

Different host response to IL-12 released by gene-transduced C-26 tumor cells has been described by our group to be dependent on the amount of IL-12 produced [18]. Low production (pg level) has minimal effect, while higher production (ng level) protects 50% of mice injected with C-26/IL-12 cells. Protection can also be obtained in case of low production of the cytokine if CD4+ cells are depleted [24].

CD4+ NKT cells have also been described to be immunosuppressive to both CTL-mediated antitumor immunity and DTH reaction [25, 26]. Therefore, the IL-12 locally produced by DC following interaction with NKT cells might have a late suppressive effect once the local killing activity of NKT cells is terminated. In contrast, systemic rIL-12 (μg level) might act directly on NK cells that subsequently migrate to the metastatic areas of the liver.

In IFN-γ-KO mice, rIL-12 retains its anti-tumor activity, suggesting the existence of an alternative, IFN-γ-independent, mechanism of action. This data supports our previous finding showing that C-26 tumor cells transduced to produce IL-12 were still able to induce a partial antitumor immune response in IFN-γ-KO mice [27]. We do not know which factor might substitute IFN-γ in mediating rejection of C-26 hepatic metastases following rIL-12 treatment in IFN-γ-KO mice. However, experiments in IFN-γ- and IL-12p40-double-KO mice indicate that endogenous production of IL-12 is required when IFN-γ is lacking, and *in situ* hybridization experiments showed increased expression of IL-12p40 in response to rIL-12 in IFN-γ-KO in comparison to BALB/c wild-type mice (S. Sangaletti, unpublished results). Recently a new cytokine, IL-23, has been identified that utilizes the p40 subunit of IL-12 to form a heterodimer with biological activities similar as well as distinct from IL-12 [28]. Since *in situ* hybridization has been performed with a probe for

p40, until now uniquely identifying IL-12, the expressed mRNA could likely account for IL-12, but also for IL-23, or for a mixture of the two cytokines. IL-23 might act as a substitute for IL-12 in absence of IFN-γ; to clarify this point we have initiated a breeding between IL-12p35-KO mice (lacking only IL-12 but not IL-23) and IFN-γ-KO mice to obtain double-KO mice to be treated with rIL-12.

4 Materials and methods

4.1 Animals

Female BALB/c (H-2^d) and (BALB/c×C57BL/6)F1 (H-2^{dxb}) hybrid mice, 8–10 weeks old, were purchased from Charles River (Calco, Italy) and maintained at the Istituto Nazionale Tumori under standard conditions according to institutional guidelines. IFN-γ-KO mice [29] on a BALB/c background (BALB/c-Ifng^{tm1Ts}) were purchased from the Jackson Laboratory (Bar Harbor, ME). IL-12p40-KO, IFN-γ/IL-12p40-double-KO, and β2m-KO mice, all on BALB/c background, were kindly provided by L. Adorini (Roche Milano Ricerche, Milano, Italy).

4.2 Compounds

α-GalCer [(2S, 3S, 4R)-1-O-(α-D-galactopyranosyl)-2-(N-hexacosanoylamino)-1, 3, 4-octadecanetriol] (Kirin Brewery Co., Gunma, Japan) was provided as solution, in 0.5% polysorbate 20 in 0.9% NaCl solution, and was further diluted in phosphate buffer before use at the final dose of 100 μg/kg.

Recombinant murine IL-12 (rIL-12) (kindly provided by Dr. M. Gately, Roche, NJ) was diluted in phosphate buffer and used at a dose of 1 μg /mouse.

4.3 C-26 hepatic metastasis model

C-26 is a murine colon adenocarcinoma cell line derived from BALB/c mice treated with N-nitroso-N-methylurethane. Tumor cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Paisley, GB) supplemented with 10% FCS (GIBCO).

Hepatic metastases of C-26 were induced according to the method of Kopper et al. [30]. Briefly, mice were anesthetized, and the spleen was exposed to allow direct injection of 5×10^5 C-26 tumor cells, in 0.1 ml of sodium chloride solution. The spleen was then removed after artery and vein were clamped; abdomen and skin were then surgically sutured.

Mice inoculated with C-26 by intrasplenic injection were divided into three groups (*n*=5–10 mice/group): control (no treatment or vehicle solution), α-GalCer-treatment and rIL-

12-treatment. The subsequent schedules have been followed: α-GalCer (100 μg/kg) on days 1, 5, and 9; rIL-12 (1 μg/mouse) on days 1, 3, 5, 7, and 9, by i.p. injection. At 12 days after tumor inoculum, mice were killed, and livers removed for weighing and detection of metastases. The liver weight was evaluated in relation to the total body weight (p/P). At the same time liver samples were embedded in OCT compound for immunohistochemical analysis.

For the induction of lung metastases, 10^4 C-26 tumor cells were injected i.v. into the tail vein of the animals; after 16 days some mice were killed and the number of superficial pulmonary metastases counted after lung insufflation with 15% India ink and bleaching in Fekete solution. In survival experiments, mice were killed when they displayed respiratory symptoms.

In depletion experiments mice were injected i.p. twice a week with 0.2 ml HBSS containing 300 μg anti-CD8 (53.6.72 hybridoma, Lyt2) or anti-NK1.1 (PK136 hybridoma) mAb obtained from ATCC (Rockville, MD).

4.4 Morphological analysis and immunocytochemistry

Liver lobes were embedded in OCT compound (Miles Lab., Elkert, IN), snap-frozen in liquid nitrogen and stored at –80°C. Immunochemical analysis using the peroxidase-anti-peroxidase (PAP) method was performed as described [31]. Briefly, 5-μm cryostat sections were fixed in acetone and immunostained with rat anti-mouse mAb against CD45 (M1/9.3.4.HL2 hybridoma, T200), and CD8 (53.6.72 hybridoma, Lyt2). Sections were preincubated with rabbit serum and sequentially incubated with optimal dilutions of primary antibodies, rabbit anti-rat IgG (Zymed Laboratories, San Francisco, CA) and rat PAP (Abbot Laboratories, North Chicago, IL).

Each incubation step lasted 30 min and was followed by a 10-min wash in TBS. Sections were then incubated with 0.03% H_2O_2 and 0.06% 3, 3'-diaminobenzidine (BDH Chemicals, Poole, GB) for 2–5 min, washed in tap water, and counterstained with hematoxylin.

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