The legend for Fig. 7 contained errors regarding the schedule of treatments administered. Fig. 7 and the corrected legend appear below.

Figure 7. α-GalCer/DC/IL-21 combination mediates extremely potent suppression of tumor metastases. Groups of five C57BL/6 WT mice were injected i.v. with 5 × 10⁵ B16F10 tumor cells. Mice were treated: (A and C) on day 4 i.v. with 5 × 10⁵ α-GalCer–pulsed DC (+) or vehicle-pulsed DC (−) and on day 7 i.v. with 20 μg of either pORF or plL21 DNA plasmid as indicated; and (B and D) on day 8 i.v. with 5 × 10⁵ α-GalCer–pulsed DC (+) or vehicle-pulsed DC (−) and on day 11 i.v. with 20 μg of either pORF or plL21 DNA plasmid; and (E) on day 8 i.v. with 5 × 10⁵ α-GalCer–pulsed DC (+) or vehicle-pulsed DC (−) and on day 11, 12, and 13 with 50 μg of recombinant mouse IL-21 or PBS as indicated. In some experiments (A, B, and E) 14 d after tumor inoculation the lungs of these mice were harvested and tumor colonies counted and recorded as the mean number of colonies ± SEM. Asterisks indicate the groups where combined α-GalCer/DC/IL-21 treatment significantly reduced that group’s number of lung metastases compared with all other groups (Kruskal-Wallis: *P < 0.05). In other experiments (C and D) the survival of these mice was monitored and recorded as the mean survival time (days) ± SEM. Asterisks indicate the groups where combined α-GalCer/DC/pIL21 treatment significantly increased that group’s survival compared with all other groups (Kruskal-Wallis: *P < 0.05). Results are representative of two experiments performed.
Sequential activation of NKT cells and NK cells provides effective innate immunotherapy of cancer

Mark J. Smyth,¹ Morgan E. Wallace,¹ Stephen L. Nutt,² Hideo Yagita,³ Dale I. Godfrey,⁴ and Yoshihiro Hayakawa¹

¹Cancer Immunology Program, Trescowthick Laboratories, Peter MacCallum Cancer Centre, East Melbourne, Victoria, 3002, Australia
²The Walter and Eliza Hall Institute for Medical Research, Parkville Victoria, 3050, Australia
³Department of Immunology, Juntendo University School of Medicine, Tokyo 113-8421, Japan
⁴Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria 3010, Australia

The CD1d reactive glycolipid, α-galactosylceramide (α-GalCer), potently activates T cell receptor–α type I invariant NKT cells that secondarily stimulate the proliferation and activation of other leukocytes, including NK cells. Here we report a rational approach to improving the antitumor activity of α-GalCer by using delayed interleukin (IL)–21 treatment to mature the α-GalCer–expanded pool of NK cells into highly cytotoxic effector cells. In a series of experimental and spontaneous metastases models in mice, we demonstrate far superior antitumor activity of the α-GalCer/IL–21 combination above either agent alone. Superior antitumor activity was critically dependent upon the increased perforin-mediated cytolytic activity of NK cells. Transfer of α-GalCer–pulsed dendritic cells (DCs) followed by systemic IL–21 caused an even more significant reduction in established (day 8) metastatic burden and prolonged survival. In addition, this combination prevented chemical carcinogenesis more effectively. Combinations of IL–21 with other NK cell–activating cytokines, such as IL–2 and IL–12, were much less effective in the same experimental metastases models, and these cytokines did not substitute effectively for IL–21 in combination with α-GalCer. Overall, the data suggest that NK cell antitumor function can be enhanced greatly by strategies that are designed to expand and differentiate NK cells via DC activation of NKT cells.

NK cells are components of the innate immune system that play a protective role against some viral infections and tumors (1, 2). These functions are achieved by the ability to recognize and lyse target cells and the provision of cytokines, such as IFN-γ, that control tumor initiation and metastases (3, 4). We and others have established that the activation of invariant NKT (iNKT) cells via the CD1d glycolipid ligand, α-galactosylceramide, protects the host from tumor cell metastases in several experimental mouse models (5–12). iNKT cell activation by soluble α-GalCer, leads to rapid downstream activation of other immune cells, including NK cells, which express CD69, secrete IFN-γ, become more cytotoxic, and proliferate (13). The antimetastatic activity of soluble α-GalCer, depends exclusively upon iNKT cell and NK cell IFN-γ production (11). Even greater antimetastatic activity of α-GalCer has been achieved by ex vivo pulsing and transfer of syngeneic DCs with α-GalCer (6, 12). Soluble α-GalCer and α-GalCer–pulsed autologous DCs have been examined in phase I clinical trials in humans who have advanced cancer, and notably, NK cell activation has been detected (14, 15). The promising immunomodulatory activity of α-GalCer has stimulated us to evaluate novel means of further enhancing host antitumor activity.

Advances have been made in understanding the cytokines that stimulate NK cell proliferation; however, much less is known about the regulation of NK cell differentiation. IL–21 is related structurally to the lymphoid cytokines, IL–2, IL–4, and IL–15, and was demonstrated to be expressed by activated CD4+ T lymphocytes (16, 17). The IL–21R is expressed in...
lymphoid tissues and shows homology to the common β chain of the IL-2 and IL-15 receptors. Like these receptors, IL-21R forms a complex with γc and can transduce signals via the Jak/STAT pathway (18). IL-21 has pleiotropic roles in the lymphoid lineages, including the promotion of CD8+ effector cell function (19) and the inhibition of IgE production in B cells (20, 21). Within the NK cell lineage, IL-21 was reported to enhance NK cell maturation from human multipotent bone marrow progenitors and to activate peripheral NK cells in the absence of other stimuli (16). This is in contrast with mouse NK cells, where IL-21 was reported to have an inhibitory effect on the IL-15–promoted expansion of NK cells, and to have no function in the absence of activating signals, such as IL-15 or IL-12 (19). After mouse NK cells are stimulated, IL-21 increases cytotoxicity and IFN-γ production over that observed for IL-15 alone (19). The distinct differences between the two species have been hypothesized to be a result of the immunological naivety of mouse NK cells as compared with human cells (17). This late and largely additive function of IL-21 in the mouse NK cell lineage is compatible with the reported phenotype of IL-21R–deficient mice, whose in vivo resting NK cell compartment is normal (19).

Recently, we (22) and others (23) established that systemic IL-21 alone in vivo promotes NK cell–mediated antitumor activity. Given the NK cell proliferative effects of α-GalCer and the ability of IL-21 to differentiate further activated mouse NK cells, we hypothesized that the combination of α-GalCer and IL-21 might display synergistic activities. Herein, we demonstrate extremely potent innate antitumor activity and mechanism of the α-GalCer/IL-21 combination in a variety of tumor models.

RESULTS

α-GalCer expands and activates peripheral NK cells

Because we previously established that α-GalCer–mediated suppression of tumor metastases by activation of iNKT cells and secondary expansion and activation of NK cells (11), we reasoned that the ability of IL-21 to differentiate further such activated NK cells might enable a combined antitumor activity of α-GalCer and IL-21. We first used flow cytometry of populations of splenocytes harvested from B6 WT mice receiving α-GalCer to determine the kinetics of NK cell activation and expansion. NK cell numbers were enumerated and CD3− NK1.1+ cells were evaluated for CD69 expression. Naive WT control mice are indicated by the gray line and treated mice are indicated by the solid lines. Results are representative of two different experiments.

Prolonged and elevated NK cell cytotoxicity after α-GalCer/IL-21 combination

On this basis, 3 d after the last α-GalCer injection was determined to be a suitable time to administer IL-21. To express mouse IL-21, we used a hydrodynamics-based gene delivery technique and a single injection of 20 μg mouse plasmid (p) IL-21 DNA that previously had been shown to result in high levels of circulating IL-21 in vivo for up to 5 d after plasmid administration (22). α-GalCer–treated mice were injected with control plasmid open reading frame (pORF) vector or IL-21 pORF vector 3 d after the second α-GalCer injection.
The cytotoxicity of spleen NK cells toward Yac-1 target cells was enhanced within 1 d after the second \(\alpha\)-GalCer injection, peaked at 3 d, and diminished rapidly thereafter (days 4 and 6; Fig. 2 A and not depicted). After a single pIL-21 treatment on day 3, splenic NK cell cytotoxicity was elevated by day 6, and some mild level of enhanced cytotoxicity persisted for >1 wk (day 11). Most strikingly, the combination of \(\alpha\)-GalCer (days −4 and 0) and pIL-21 (day 3) resulted in a very rapid and dramatic increase (20− to 50-fold) in NK cell cytotoxicity, and these levels of cytotoxic function were maintained for up to 4 d after pIL-21 treatment (day 7; Fig. 2, A and B). The \(\alpha\)-GalCer/IL-21 combination demonstrated some level of enhancement of NK cell cytotoxicity above the control groups almost 2 wk after the pIL-21 inoculation (day 17; Fig. 2 A).

**The role of perforin (pfp) and IFN-\(\gamma\) in prolonged NK cell cytotoxicity by \(\alpha\)-GalCer/pIL-21**

\(\alpha\)-GalCer/pIL-21 or pIL-21 activation of NK cell–mediated cytotoxicity was pfp-dependent and reduced in IFN-\(\gamma\)−/− mice compared with WT mice (Fig. 3 A). As reported previously (7), early NK cell–mediated cytotoxicity (day 3) induced by \(\alpha\)-GalCer alone was IFN-\(\gamma\)–dependent. We showed previously that IL-21 markedly enhanced the granule formation and pfp expression in cultured mature mouse NK cells, but did not increase NK cell number (22). Consistent with a lack of effect of IL-21 on NK cell proliferation in vivo (22), a further expansion of NK1.1+ TCR\(\alpha\beta\)− NK cells was not detected after pIL-21, compared with pORF, treatment (not depicted). Therefore, we next assessed the expression of pfp in whole spleen cells harvested from all treated groups at day 6 (3 d after the pIL-21 treatment; Fig. 3 B). The \(\alpha\)-GalCer/pIL-21 combination significantly enhanced pfp expression above that observed for \(\alpha\)-GalCer/pORF or vehicle/pIL-21 alone. The data strongly suggested that sustained induction of peripheral NK cell cytotoxicity by the \(\alpha\)-GalCer/pIL-21 combination was due to significant and high levels of expression of the key cytotoxic molecule, pfp.

The phenotype and cytokine secretion of spleen NK cells also were assessed in similarly treated mice 1 (not depicted) and 3 days after pORF/pIL-21 (Fig. 3 C). Significant alterations in NK cell maturity were detected. Splenic NK cells typically express Mac-1 (30% Mac-1lo and 70% Mac-1hi), and an immature NK cell developmental stage in the mouse has been defined by Mac-1lo expression (24, 25). Mice that were treated with \(\alpha\)-GalCer displayed a significantly greater frequency of Mac-1lo NK cells (Fig. 3 C); this suggested that although \(\alpha\)-GalCer expanded NK cell numbers, a large proportion of these were a Mac-1lo phenotype. Further treatment with the pORF vector did not alter this distribution; however, in mice receiving pIL-21, a proportion of NK cells was altered significantly as defined by Mac-1hi expression. The expression level of NKG2D, an activation receptor that was shown to regulate pfp-mediated cytotoxicity (26), was unchanged by any treatment combination (Fig. 3 C, not depicted). Collectively, these data suggested that immature NK cells that were generated after \(\alpha\)-GalCer injection were matured into highly cytotoxic pfp-expressing NK cells by IL-21. Although \(\alpha\)-GalCer injection was shown to stimulate early and prolonged (up to 3 d) NK cell IFN-\(\gamma\) production (7, 13), there was no detectable level of NK cell IFN-\(\gamma\) production at 1 (not depicted) and 3 d (Fig. 3 C) after pORF or pIL-21 treatment.

**Figure 2.** Prolonged and elevated NK cell pfp-mediated cytotoxicity after \(\alpha\)-GalCer/IL-21 combination. C57Bl/6 WT or gene-targeted mice were treated on days −4 and 0 i.p. with 1 \(\mu\)g \(\alpha\)-GalCer (200 \(\mu\)l) or 200 \(\mu\)l of vehicle, and on day 3 i.v. with 20 \(\mu\)g of pORF or pIL-21 DNA plasmid. Mice were harvested at various time points, and spleen cells were examined in a 4-h \(^{51}\)Cr release assay at several effector/target (E:T) ratios using Yac-1 target cells. (A) Cytotoxicity of WT spleen cells from treated mice at 25:1 on various days of harvest and (B) cytotoxicity of WT spleen cells on day 6 at various E:T ratios. Results were expressed as the mean percentage lysis ± SEM of triplicate samples. N.T., not tested.
Concurrent assessment of NKT cell and DC populations in these treated mice revealed that there were no changes in DC subsets or their activation status after pIL-21 alone (including CD40, CD80, CD86, and MHC class II expression), whereas DC activation by α-GalCer alone was evident 24 h after the second α-GalCer treatment (unpublished data). There was no further change to DC subsets or DC activation at 24 h, 48 h, 4 d, or 7 d after the pIL-21 treatment that followed α-GalCer. In addition, iNKT cell number and activation was not further enhanced above α-GalCer treatment by pIL-21 (unpublished data).

α-GalCer/IL-21 combination suppresses tumor metastases and prolongs survival by pfp- and IFN-γ-dependent mechanisms.

Given the potent antimetastatic activity of early α-GalCer treatment alone, we examined the α-GalCer/pIL-21 combination in two (early and late) different treatment regimes against B16F10 experimental metastases. Mice that received α-GalCer (days 0 and 4) alone demonstrated a significant reduction in B16F10 lung metastases compared with vehicle/pORF control-treated mice (Fig. 4 A). By contrast, when α-GalCer treatment was delayed (days 4 and 8), this therapy only caused a minor reduction in control lung metastases (Fig. 4 B). Mice receiving pIL-21 alone on days 7 (early) or 11 (late) demonstrated similar numbers of metastases as control-treated mice (Fig. 4, A and B). The early α-GalCer combination eliminated B16F10 lung metastases almost completely (Fig. 4 A), and even late treatment enabled a ≥60% reduction of established (day 8) metastases (Fig. 4 B). Suppression of B16F10 tumor metastases by the α-GalCer combination correlated with a significant increase in the mean survival time of these cohorts of mice (Fig. 4, C and D). The α-GalCer/pIL-21 combination was only more effective than α-GalCer alone when IL-21 was administered on day 7 (3 d after the second α-GalCer treatment; Fig. 4 E). Although α-GalCer alone has limited activity against established subcutaneous B16F10, the α-GalCer/pIL-21 combination suppressed B16F10 tumor growth almost completely (Fig. S1, A and B, available at http://www.jem.org/cgi/content/full/jem.20042280/DC1).

We (11) and others (7) showed that the antimetastatic activity of α-GalCer was completely IFN-γ-dependent, whereas pIL-21 suppressed B16F10 lung metastases by a pfp-dependent mechanism (22). Therefore, the effectiveness of the late α-GalCer/pIL-21 combination was examined in
several gene-targeted mice or WT mice depleted of NK cells (Fig. 5 A). The combination therapy was completely ineffective in RAG-1/H11002/H11002/H11002 mice (that lack iNKT cells) or WT mice depleted of NK cells. The combination also was without synergistic activity in mice that were deficient for pfp, IFN-γ/H9253/H9253, or IL-12, whereas no activity was retained in mice that were deficient in pfp and IFN-γ/H9253/H9253 (Fig. 5 A).

Host IL-18 activity was not required for the synergistic activity of ifn/H9251/ifn/H9251-IL21. Thus, a combination of IFN-γ/H9253/H9253– and pfp-dependent mechanisms mediates the antimetastatic function of ifn/H9251/ifn/H9251-IL21. In a similar fashion in BALB/c WT mice, the combination of ifn/H9251/ifn/H9251-IL21 synergistically suppressed Renca lung metastases, and suppression was critically dependent upon pfp and IFN-γ (Fig. 5 B).

α-GalCer/IL-21 combination suppresses spontaneous tumor metastases

To examine the effects of α-GalCer/pIL-21 in a model of spontaneous tumor metastases, the combination was assessed in the orthotopic 4T1 mammary tumor model in BALB/c WT mice. Mice were inoculated in the mammary gland and 8 d later the primary tumor (~10 mm²) was resected, leaving micrometastases several organs, including lung, liver, lymph node, and bone. Mice received α-GalCer (1 µg) on days 10 and 14 and pIL-21 (20 µg) on day 17 after tumor inocula-
tion and were killed on day 25 and lung and liver metastases quantitated, or were left to monitor survival (Fig. 6, A and B). The combination of α-GalCer/pIL-21 had a dramatic effect on lung and liver metastases (Fig. 6, A and B); this suppression resulted in a significant prolongation in lifespan of this treated group (Fig. 6 C). By contrast, α-GalCer or pIL-21 treatment alone basically was without effect.

α-GalCer/DC/pIL-21 combination mediates extremely potent suppression of tumor metastases

Because it was shown previously that α-GalCer was far more effective in suppressing tumor metastases when administered pulsed on DCs (6), we next assessed the efficacy of a combination of α-GalCer/DC and pIL-21 in the B16F10 model. In concert with the published data, α-GalCer–pulsed BM-DCs (GM-CSF and IL-4 cultured) were effective in suppressing B16F10 lung metastases, particularly when treatment commenced early (day 4; Fig. 7 A). Some discernable reduction in lung metastases was observed, even when α-GalCer–pulsed DCs were transferred 8 d after tumor inoculation (day 8; Fig. 7 B). As shown previously, pIL-21 alone (days 7 or 11) had little or no effect on B16F10 lung metastases (Fig. 7, A and B). By contrast, the combination of α-GalCer/DC and pIL-21 reduced B16F10 lung metastases

**Figure 5.** α-GalCer/IL-21 combination suppresses tumor metastases by pfp and IFN-γ-dependent mechanisms. Groups of (A) five C57BL/6 WT and C57BL/6 gene-targeted mice were injected i.v. with 5 × 10⁶ B16F10 tumor cells and (B) five BALB/c WT and BALB/c gene-targeted mice were injected with 2.5 × 10⁶ Renca tumor cells. Some groups of mice were depleted of NK cells by treatment with rabbit anti-asGM1 antibody on days −1, 0 (the day of tumor inoculation), and 7. Mice were treated: (A) on days 4 and 8 i.p. with 1 μg α-GalCer (200 μl) or 200 μl of vehicle and on day 11 i.v. with 20 μg of pORF or pIL21 DNA plasmid; and (B) on days 0 (the day of tumor inoculation) and 4 i.p. with 1 μg α-GalCer (200 μl) or 200 μl of vehicle and on day 7 i.v. with 20 μg of pORF or pIL21 DNA plasmid. In both experiments (A and B), 14 d after tumor inoculation the lungs of these mice were harvested, and tumor colonies were counted and recorded as the mean number of colonies ± SEM. Asterisks indicate the groups in which combined α-GalCer/pIL-21 treatment did not reduce lung metastases significantly compared with WT mice (Kruskal-Wallis: * P < 0.05).
dramatically, even when treatment commenced as late as day 8 after tumor inoculation (Fig. 7 B). The striking reduction in B16F10 lung metastases correlated tightly with a greatly enhanced survival of mice that received \( \alpha \)-GalCer/DC and pIL-21 combination (Fig. 7, C and D). Recently, we obtained access to mouse recombinant IL-21. To illustrate that recombinant mouse IL-21 protein could be as effective as pIL-21 and that the vector DNA sequence itself was not contributing to the antitumor activity, we examined the effectiveness of late \( \alpha \)-GalCer/DC (day 8) and IL-21 (50 \( \mu \)g on days 11, 12, and 13) treatment on B16F10 lung metastases (Fig. 7 E). Clearly, similar results were obtained with recombinant mIL-21, which indicates that it was a practical strategy to proliferate and mature NK cells sequentially using a surrogate iNKT cell ligand and IL-21 cytokine.

\( \alpha \)-GalCer/DC/pORF combination suppresses tumor initiation

To assess the ability of the combination of \( \alpha \)-GalCer/DC and pIL-21 to prevent primary tumor initiation, we evaluated methylcholanthrene (MCA)-induced sarcoma formation. We had demonstrated previously that early treatment with soluble \( \alpha \)-GalCer alone could prevent a proportion of mice from succumbing to MCA-induced sarcoma (27). Consistent with those previous findings, we observed that three early \( \alpha \)-Gal-Cer/DC/pORF treatments given every 2 wk could prevent sarcoma formation in approximately half of the cohort of MCA-inoculated mice (Fig. 8). Strikingly, similar early treatment with \( \alpha \)-GalCer/DC/pIL-21 prevented tumor formation in all mice. Enhancement in protection also was observed when treatment commenced at day 70 (at a time when control mice were first developing palpable sarcomas); 50\% of the mice that received \( \alpha \)-GalCer/DC/pIL-21 were protected from sarcoma (Fig. 8). Delayed \( \alpha \)-GalCer/DC/pORF treatment was comparatively ineffective as was early or late treatment with vehicle-loaded DCs and IL-21 (Fig. 8). These results illustrated that the synergistic activity of \( \alpha \)-GalCer/IL-21 was not limited to suppression of experimental metastases, although clearly this is a model system in which host NK cells are important natural effectors.

**IL-2 and IL-12 do not mimic the activity of \( \alpha \)-GalCer or IL-21 in combination**

The success of the \( \alpha \)-GalCer/pIL-21 combination raised questions as to whether a similar efficacy could be obtained by expanding NK cells by a different means, or further activating NK cells by cytokines other than IL-21. Having previously established the activity of single early IL-2 and IL-12 cytokine therapy against Renca lung metastases (28), we
used this tumor model to examine whether these cytokines might synergize with IL-21 against more established metastases. Using an analogous treatment protocol, BALB/c WT mice received 5 d of sequential IL-2 or IL-12 treatment before commencing IL-21 on the last day of IL-2 or IL-12 treatment. In contrast to α-GalCer-pulsed DCs, IL-12 did not significantly synergize with IL-21 to reduce B16F10 metastases (Fig. 9 A). IL-2 had only a minor significant effect in combination with IL-21, which suggested that simply expanding NK cells before their differentiation with IL-21 may not be sufficient (Fig. 9 A). The same cytokines also were examined for their ability to activate NK cells 2 d after iNKT cell activation by α-GalCer/DC. IL-2 and IL-12 were unable to reduce Renca lung metastases further after α-GalCer/DC treatment, but the activity of IL-21 was superior in combination with α-GalCer (Fig. 9 B).

DISCUSSION

iNKT cells are particularly potent immunoregulatory T cells that can be activated after interaction with DCs to stimulate
innate and adaptive tumor immunity. Herein, we have shown that a DC–iNKT cell interaction prompted by the CD1d-reactive ligand, α-H9251-GalCer, can create a pool of pre-stimulated NK cells that responds strongly to systemic IL-21 treatment. In a series of tumor models in mice, we demonstrate the far superior antitumor activity of the α-H9251-GalCer/IL-21 combination above either agent alone. Transfer of α-H9251-GalCer–pulsed DCs followed by systemic IL-21 caused a particularly significant reduction in established (day 8) metastatic burden and prolonged survival. The combination also was effective in the early treatment of subcutaneous disease and in preventing MCA-induced sarcoma. Combinations of IL-21 with other NK cell–activating cytokines, such as IL-2 and IL-12, were not effective in the same experimental models, and these cytokines did not substitute effectively for IL-21 in combination with α-GalCer. However, the production of both of these cytokines by DCs was an important component in the effectiveness of the α-GalCer/IL-21 combination. The superior antitumor activity of α-GalCer/IL-21 also was critically dependent upon increased and sustained pfp-mediated cytotoxicity by NK cells and host IFN-γ activity. Overall, the data suggest that NK cell antitumor function can be enhanced greatly by strategies that are designed to expand and differentiate NK cells via DC activation of NKT cells.

In two different experimental tumor metastases models, the combination of α-GalCer and IL-21 required pfp- and IFN-γ-dependent mechanisms for optimal antitumor activity. IL-21 alone previously was shown to differentiate NK cells terminally and greatly enhance their pfp-mediated cytolytic activity and cytokine secretion (IFN-γ and IL-10; reference 22). In several different experimental models, the in vivo antitumor activity of IL-21 also seemed to be strictly dependent upon pfp-mediated cytotoxicity (22, 29). Herein, we have shown enhanced pfp expression and sustained NK cell pfp-dependent cytotoxicity when IL-21 treatment followed α-GalCer priming. More NK cells also were mature as defined by Mac-1hi expression after the addition of pORF, whereas NK cells from pORF-treated mice contained a greater proportion of Mac-1lo NK cells that have been shown to be immature and display lower cytotoxic activity compared with Mac-1hi NK cells (24). Significant NK cell IFN-γ production was not detected after the α-GalCer/IL-21 combination. Therefore, we contend that the synergistic antitumor activity of the α-GalCer/IL-21 combination is

Figure 8. α-GalCer/DC/IL-21 combination suppresses MCA initiation of sarcoma. Groups of 10 C57BL/6 WT mice were injected s.c. into the hind flank with 400 μg MCA as described (27). Mice were treated early on days 0 (the day of MCA inoculation), 14, and 28 i.v. with 5 × 10⁶ α-GalCer-pulsed DCs (+) or vehicle-pulsed DCs (−) and days 3, 17, and 31 i.v. with 20 μg of pORF or pL21 DNA plasmid. Alternatively, mice were treated late on days 70, 84, and 98 i.v. with 5 × 10⁶ α-GalCer–pulsed DCs (+) or vehicle-pulsed DCs (−) and on days 73, 87, and 101 i.v. with 20 μg of pORF or pL21 DNA plasmid. Palpable sarcomas (>15 mm²) were recorded and tumor-free mice were monitored for 250 d.

Figure 9. IL-2 and IL-12 do not mimic the activity of α-GalCer or IL-21 in combination. All groups of five BALB/c WT mice were injected with 2.5 × 10⁵ Renca tumor cells. (A) Mice were treated with: (i) 5 × 10⁵ α-GalCer–pulsed DCs or vehicle-pulsed DCs i.v. on day 8; (ii) IL-2 (50,000 IU) i.p. on days 6, 7, 8, 9, and 10; or (iii) IL-12 (500 ng) i.p. on days 6, 7, 8, 9, and 10. These groups of mice received 50 μg IL-21 or PBS i.p. on days 11, 12, and 13. (B) Mice were treated with 5 × 10⁵ α-GalCer–pulsed DCs or vehicle-pulsed DCs i.v. on day 8. These groups of mice received 50 μg IL-21 or PBS i.p. on days 11, 12, and 13; IL-2 (50,000 IU) i.p. on days 10, 11, 12, 13, and 14; or IL-12 (500 ng) i.p. on days 10, 11, 12, 13, and 14. In both experiments (A and B), 14 d after tumor inoculation, the lungs of the mice were harvested, and tumor colonies were counted and recorded as the mean number of colonies ± SEM. Asterisks indicate the groups in which combined α-GalCer/DC/IL-21 treatment significantly reduced lung metastases compared with all other combinations (Kruskal-Wallis; *P < 0.05).
explained largely by the sustained increase in pfp-dependent cytotoxicity mediated by NK cells that have been expanded in number after α-GalCer activation of iNKT cells and fully matured by IL-21. The role that IFN-γ plays in the antitumor activity of the α-GalCer/IL-21 combination is less obvious. Clearly, α-GalCer alone demonstrates pfp-independent antitumor activity that requires IFN-γ secretion by NK cells and iNKT cells, and the enhanced cytotoxicity of NK cells after α-GalCer treatment is IFN-γ-dependent (7, 11). The retention of considerable antitumor activity of the α-GalCer/IL-21 combination in IFN-γ−/− mice was consistent with the ability of α-GalCer to expand NK cell numbers in IFN-γ−/− mice to a large extent (Table I). It remains possible that early IFN-γ secretion by iNKT cells or NK cells after α-GalCer may be required for additional downstream effects on NK cells or other cellular populations that may respond to IL-21.

Although soluble α-GalCer and DC–α-GalCer were reported to act primarily via DC IL-2 and IL-12 production (6, 11, 30), these cytokines could not substitute for α-GalCer when administered systemically before IL-21 injection. In part, this may be because α-GalCer additionally elicits IL-3, GM-CSF, and TNF from mouse and human NK T cells that may enable the peripheral mobilization of myeloid progenitors (15, 31, 32), and greater NK cell proliferation and NK cell cytotoxicity (33–35). Preliminary experiments suggest some enhanced antitumor activities. However, Flt3L in such a context.

The role that IFN-γ in the synergistic activity of DC/αGalCer/IL-21; however, our previous studies indicated that IL-15 and IL-21 together promote the terminal differentiation of NK cells (22), and others recently showed that DC IL-15 is not always the most important regulator of NK cell activity (36, 37). Nevertheless, a possible role of DC IL-15 in α-GalCer/IL-21 synergy remains to be tested formally. This study raises the possibility that IL-21 might be used successfully with other biological response modifiers to stimulate tumor immunity. Fms-like tyrosine kinase 3 ligand (Flt3L) administration inhibits experimental tumor metastases in an NK cell–dependent manner (38), and Flt3L in combination with IL-2 (39), IL-12, or CD40L (40) demonstrated some enhanced antitumor activities. However, Flt3L increases the relative number of functional NK cells in the spleen and blood of mice in vivo (38, 41, 42), and dose- and time-dependent increases in NK cell number up to >30-fold were reported in some organs (42). Therefore, we are examining the combined antitumor activity of Flt3L and IL-21. The capacity of IL-21 to enhance pfp-mediated cytotoxicity may be realized fully in stimulating the antibody-dependent cellular cytotoxicity of NK cells. The therapeutic effectiveness of several promising humanized mAbs that are reactive with tumor antigens (e.g., trastuzumab, rituximab, cetuximab) may be improved further in combination with IL-21. However, it is clear that human NK cells have distinct activation requirements with respect to IL-21 (17), and the efficacy of any of these strategies using IL-21 will require clinical assessment after IL-21 is deemed safe in early phase trials in humans.

According to a previous report, the DC–α-GalCer response, as assessed by the number of IFN-γ–secreting iNKT cells, was much stronger and prolonged than that obtained with soluble α-GalCer (6). Our experiments confirmed that the DC–α-GalCer response also was associated with a superior protection against experimental tumor metastases. Nevertheless, soluble and DC-pulsed α-GalCer displayed synergistic activity when combined with delayed treatment with systemic IL-21. The synergistic combination also seemed to be effective in suppressing the initiation and growth of subcutaneous tumors; however, it is now important to test further the ability of DC/αGalCer/IL-21 combinations to suppress chemical- and oncogene-driven carcinogenesis as well as established solid tumor deposits. Recently, it was shown that activation of iNKT cells by α-GalCer rapidly activates the maturation of DC, and thereby, acts as an adjuvant for T cell immunity to a coadministered protein (43). It is now of interest to evaluate the capacity of IL-21 to promote further T cell immunity and memory to foreign- and self-antigens in such a context.

MATERIALS AND METHODS

Mice. Inbred C57BL/6 and BALB/c WT mice were purchased from The Walter and Eliza Hall Institute of Medical Research. The following C57BL/6 gene-targeted mice were bred at the Peter MacCallum Cancer Centre: C57BL/6 pfp-deficient (B6 pfp−/−); C57BL/6 Fas ligand (Fasl) mutant (B6 gld); C57BL/6 RAG-1–deficient (B6 RAG−1−/−); from L. Corcoran, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia); C57BL/6 IFN-γ–deficient (B6 IFN−γ−/−); B6 pfp−/− × IFN-γ−/− mice (B6 pfp−/− IFN−γ−/−); C57BL/6 IL-12−deficient (B6 IL-12−/−); from Hoffmann-La Roche); C57BL/6 IL-18−deficient (B6 IL-18−/−); by S. Akira, Osaka University, Japan); and B6 IL-12−/− × IL-18−/− mice (B6 IL-12−/− IL-18−/−). The following BALB/c gene-targeted mice were bred at the Peter MacCallum Cancer Centre: BALB/c RAG-1−/−; BALB/c IFN−γ−/−; BALB/c pfp−/−; BALB/c IFN−γ−/−; BALB/c TNF-related apoptosis-inducing ligand (TRAIL)-deficient (TRAIL−/−; from J. Peschon, AMGEN; reference 44); and BALB/c pfp TRAIL−/− mice. All mice originally generated on a 129 background have been backcrossed between 10–12 times onto the C57BL/6 or BALB/c background. Mice of 6–14 wk of age were used in all experiments that were performed according to Animal Experimental Ethics Committee Guidelines.

Isolation of spleen NK cells and cytotoxicity assay. NK cells were prepared from the spleen of B6 WT or gene-targeted mice as described previously (45). The cytolytic activity of NK cells from various vehicle/α-GalCer– and pORF/pIL-21–treated mice was tested against Yac-1 target cells by a standard 6-h 51Cr release assay as described previously (46).

Flow cytometric analysis. Mononuclear cells from the spleen, liver, and thymus were isolated as described. For staining NK cells, mononuclear cells were preincubated with CD16/32 (2.4G2) mAb to avoid the nonspecific binding of antibodies to FcγR. Then the cells were incubated with a saturating amount of PE-Cy5.5–conjugated TCRB mAb, APC–NK1.1 mAb, and FITC-CD69 mAb or FITC-CD11b/Mac-1 mAb or PE-NKG2D mAb. To analyze intracellular levels of NK cell IFN-γ, cells were stained with PE-Cy5.5–conjugated TCR and FITC–CD69 mAb or FITC–CD11b/Mac-1 mAb or PE–NKG2D mAb. To analyze intracellular levels of NK cell IFN-γ, cells were stained with PE-Cy5.5–conjugated TCR and FITC–CD69 mAb or FITC–CD11b/Mac-1 mAb or PE–NKG2D mAb. For iNKT cells, cells were stained with APC–NK1.1 mAb, PE–NKG2D mAb, and either PE–CD3 or PE–CD4 mAb for surface staining. For staining of NK cells, mononuclear cells were preincubated with CD16/32 (2.4G2) mAb to avoid the nonspecific binding of antibodies to FcγR. Then the cells were incubated with a saturating amount of PE-Cy5.5–conjugated TCRB mAb, APC–NK1.1 mAb, and FITC-CD69 mAb or FITC-CD11b/Mac-1 mAb or PE-NKG2D mAb. To analyze intracellular levels of NK cell IFN-γ, spleen cells were harvested and incubated for 2 h in vitro with Golgistop (BD Bioscience). The cells were incubated with a saturating amount of PE-Cy5.5–conjugated TCRB mAb and PE-conjugated NK1.1 mAb. An APC-conjugated
IFN-γ mAb or isotype-matched control mAb was used after the cells were fixed and permeabilized with Cytofix/perm Buffer (BD Biosciences). For internal controls, mice were injected with poly I:C (200 μg, Sigma-Aldrich) and spleen cells were harvested 3 h later and subjected to intracellular IFN-γ staining. Flow cytometric analysis was performed with a LSR instrument using FCS Express software.

**Western analysis.** Western blotting was performed essentially as described (47). Spleen cells (10^7) were harvested and were lysed on ice for 30 min in NP-40 lysis buffer (0.5% NP-40, 5 mM MgCl2, 25 mM KCl, 10 mM TriHCL, pH 8.0), containing complete mini-protease inhibitor cocktail (Roche Molecular Biochemicals). Cell debris was removed by centrifugation at 13,000 rpm for 5 min, and the supernatant was retained. Equal amounts of protein (determined by means of a Bradford reaction) were separated on 12.5% or 15% SDS-polyacrylamide gels and electrophoresed onto nitrocellulose transfer membranes. Immunoblots were probed with rat mAb FL-8 to mouse pfp or mouse mAb to human α-tubulin (Sigma-Aldrich), and visualized by enhanced chemiluminescence (Amersham Biosciences).

**Tumor cell lines.** The following standard experimental mouse tumor cell lines were used in vitro and in vivo: Yac-1 lymphoma (NK cell sensitive); B16F10 melanoma (pp-sensitive, FasL- and TRAIL-insensitive, H-2^d); 4T1 mammary carcinoma (pfp- and TRAIL-sensitive and FasL-insensitive, H-2^d); and Renca renal cell carcinoma (pfp- and TRAIL-sensitive and FasL-insensitive, H-2^d). The maintenance of all tumor cell lines and the sensitivities of lung metastases to various cytotoxic molecules in vitro and in vivo were described previously (11, 28).

**Tumor models in vivo.** B16F10 and Renca tumor cell lines were inoculated i.v. and 4T1 was inoculated into the mammary gland at the dose indicated in the figure legends. The α-GalCer/IL-21 treatment schedule was evaluated for its ability to reduce the expected metastatic tumor burden. For B16F10 and Renca experimental metastasis models, mice were injected i.v. with tumor cells and killed 14 d later, the lungs removed, and surface metastases counted with the aid of a dissecting microscope. In the 4T1 model, female mice were inoculated into the mammary gland with 2.5 × 10^6 cells on day 0; 8 d later the primary tumor (∼10 mm^2) was resected surgically. Mice were killed at 25 d and spontaneous metastasis was measured by harvesting lungs and livers as described (44). Clonogenic metastases were calculated on a per organ basis. In all metastasis models, the data were recorded as the mean number of metastases ± SE of the mean. Some groups of treated and control mice were monitored for survival and data are recorded as the mean survival time (± SE of the mean). Mice were killed at the first signs of labored breathing or loss of weight and ruffled fur, and lung tumor was confirmed by autopsy. Significance was determined by a Kruskal-Wallis test. Sarcomas were induced in male mice by 400 μg MCA in 0.1 ml maize oil in various α-GalCer/IL-21-treated C57BL/6 mice as described previously (27). Palpable sarcomas (>15 mm^2) were recorded and tumor-free mice were monitored for 250 d.

**Cytokine/α-GalCer treatment protocols.** Recombinant mouse IL-12 and IL-2 was provided by Genetics Institute and Chiron Corp., respectively. Recombinant mouse IL-21 was provided by Zymogenetics. The preparations of IL-2, IL-12, or IL-21 were diluted in PBS immediately before use. α-GalCer, a marine sponge glycolipid that activates CD1d-restricted NKT cells was provided by the Pharmaceutical Research Laboratories, Kirin Brewery, and prepared as described (11). α-GalCer and the control vehicle were reconstituted in saline supplemented with 0.5% polyborate-20 (wt/vol). Some groups of mice were treated with one or more of: (a) α-GalCer (1 μg i.p.) or vehicle (200 μl i.p.) and (b) IL-2 (100,000 IU i.p.), IL-12 (500 ng i.p.), or IL-21 (50 μg i.p.), as indicated. For hydrodynamic gene transfer, the expression pIL-21-pORF and the control vector pORF (provided by P. Hwu, National Cancer Institute, Bethesda, MD) were injected i.v. into mice as described previously (22). In brief, mice were injected with 20 μg of plasmid in a volume of 2 ml of saline over a 5- to 7-s period. The volume of saline was based on the age and weight of the mouse and did not exceed 10% of body weight.

**Isolation of bone marrow-derived DCs.** In some α-GalCer therapy experiments, α-GalCer was administered pulsed on DCs. Bone marrow-derived DCs were isolated from WT or gene-targeted mice and cultured in 10 ng/ml recombinant mouse GM-CSF and 5 ng/ml recombinant mouse IL-4 (R&D Systems) as described (6, 48). From days 6 to 8 of culture, DCs were supplemented with fresh media and additives, including 100 ng/ml α-GalCer (+) or vehicle (−). 500,000 pulsed DCs were injected i.v. per mouse on days 4 or 8 after tumor inoculation.

**NK cell depletion.** NK cells, but not iNKT cells, were depleted specifically in B6 and BALB/c mice using 100 μg i.p. rabbit anti-asialo GM1 antibody (Wako Chemicals) on days 0, 1, and 7 (after tumor inoculation) as described (49, 50).

**Statistical analysis.** Significant differences in metastases were determined by the Kruskal Wallis test. P < 0.05 were considered significant.

**Online supplemental material.** Fig. S1 shows the suppression of subcutaneous tumor growth by α-GalCer/IL-21 combination. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem. 20042280/DC1.

We thank M. Shannon for reagent acquisition, S. Mitchell for genotyping, and R. Cameron and S. Griffiths for maintaining the gene-targeted mice.

M.J. Smyth is supported by a National Health and Medical Research Council of Australia (NH&MRC) Principal Research Fellowship. Y. Hayakawa is supported by a Cancer Research Institute postdoctoral fellowship. M.E. Wallace is supported by a NH&MRC Doherty Fellowship. D.J. Godfrey is supported by a NH&MRC Research Fellowship. The project was supported by a Program Grant from the NH&MRC and by a R01 grant from the National Institutes of Health. The authors have no conflicting financial interests.

Submitted: 5 November 2004
Accepted: 22 April 2005

**REFERENCES**


induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a co-administered protein. J. Exp. Med. 198:267–279.


