The novel agonistic iNKT-cell antibody NKT14m induces a therapeutic antitumor response against B-cell lymphoma

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ABSTRACT

Invariant natural killer T (iNKT) cells are a small population of T lymphocytes that expresses an invariant T cell receptor with a unique specificity for glycolipid antigens. Their activation using the glycolipid α-galactosylceramide triggers innate and adaptive immune responses. The use of α-galactosylceramide in preclinical models as a single antitumor treatment showed moderate effect, but its efficacy in cancer patients was less effective. In addition, this glycolipid induces long-term iNKT-cell anergy precluding the possibility of retreatment. Recently, the first murine iNKT-cell agonistic antibody, NKT14m, has been developed. Here, we analyzed for the first time the antitumor efficacy of NKT14m in a B-cell lymphoma model. In a therapeutic setting, a single dose of NKT14m had a moderate antitumor efficacy that was associated with an increase of IFN-γ producing iNKT cells even after retreatment. Importantly, the combination of a single dose of NKT14m with cyclophosphamide had a potent antitumor efficacy and long-lasting immunity *in vivo*. Our findings provide the first evidence on the *in vivo* antitumor efficacy of NKT14m antibody, showing that either alone or in combination with chemotherapy induces an effective antitumor response. These results open new opportunities for iNKT-cell mediated immunotherapy to treat B-cell lymphoma.

INTRODUCTION

Invariant natural killer T (iNKT) cells represent a small T-cell subset that has an invariant T-cell receptor (iTCR), which recognizes glycolipid antigens presented by the non-polymorphic MHC class I-like molecule CD1d [1–3]. Preclinical studies have shown that iNKT cells can activate both innate and adaptive immune responses [1, 2, 4, 5]. In fact, activated iNKT cells can increase the activation and function of natural killer (NK) cells [6, 7], $CD4^+$ and $CD8^+$ T cells [6, 8–10], dendritic cells (DC) [9, 10] and B cells [11], coordinating a global immune response and promoting the killing of tumor cells. In addition, iNKT-cell activation also inhibits tumor-induced myeloid derived suppressor cells (MDSCs) [8] and tumor angiogenesis [12]. Increased numbers of circulating and intra-tumor iNKT cells have been associated with an improved prognosis in different cancers and hematological malignancies [13, 14], supporting the important role of iNKT cells in cancer immunosurveillance and its potential as a promising immunotherapeutic target.

The activation of these cells with the glycolipid α-galactosylceramide (α-GalCer) increases IFN-γ production and cytokine secretion (i.e., IL-12, IL-17, IL-21), which contributes to the enhancement of a T-cell mediated antitumor effect [5, 15, 16]. Different approaches using α-GalCer have been tested in several tumor models with success [15, 17–22], but their translation to the clinical setting proved to be less effective [23–29]. Moreover, administration of α-GalCer induces long-term iNKT-cell anergy, causing unresponsiveness to sequential stimulation with this glycolipid [18, 21, 30, 31], which

can explain partially the lack of clinical effect in patients with cancer.

Recently, the first iNKT-cell agonistic monoclonal antibody (mAb), the NKT14m that can activate murine iNKT cells, has been described [30]. This new murine IgG2a antibody activates iNKT cells *in vivo* by direct binding to the iTCR in fully immune-competent mice [30] and represents a surrogate antibody to the human specific iNKT cell activating antibody NKTT320 [32]. Direct acting antibodies would represent an alternative to α-GalCer in cancer treatment that is most effective by infusion of CD1d loaded dendritic cells for iNKT cell activation [20, 22–29]. Injection of NKT14m into naive Balb/c mice triggers the IFN-γ production by iNKT cells comparable to α-GalCer but, in contrast to α-GalCer, the NKT14m did not induced iNKT-cell long-term anergy, allowing the readministration of this antibody [30]. While the activation of murine iNKT cells by agonistic NKT14m antibody has been described, the *in vivo* antitumor effect of NKT14m has not been previously reported. Here, we describe for the first time the antitumor efficacy of NKT14m-mediated direct iNKT cell activation against B-cell lymphoma, as single agent or in combination with chemotherapy.

RESULTS

NKT14m has antitumor activity against B-cell lymphoma

We first studied the *in vivo* antitumor efficacy of NKT14m antibody in a therapeutic setting. The treatment with a single dose of NKT14m two days after tumor injection induced a moderate antitumor response in comparison with tumor-bearing mice injected with control IgG (37% vs. 0% survival, respectively; $p = 0.03$) and with mice treated with a single dose of α-GalCer (37% vs. 10% survival, respectively; $p = 0.04$) (Figure 1A). However, a delayed treatment (i.e., 4 days after tumor challenge) resulted in complete loss of efficacy. Importantly, we could detect a significant increase of IFN-γ-producing iNKT cells after NKT14m treatment in comparison with mice treated with α-GalCer (36.28 ± 6.02% vs. 4.15 ± 0.73%, respectively; $p = 0.007$) (Figure 1B). Collectively, this data shows that the NKT14m efficiently activates iNKT cells *in vivo*, inducing an antitumor response able to eliminate tumor cells, and this antitumor activity is higher than the induced by the prototypic iNKT-cell agonist α-GalCer. Mice treated with NKT14m antibody that eliminated the tumor were sacrificed at the end of the experiment and a pathology analysis was done. No abnormalities in spleen, liver, bone marrow, lymph nodes, and lungs were observed (data not shown). In contrast, mice that were not able to eliminate the tumor (either control or treated) showed lymphoma dissemination in the spleen, bone marrow, lymph nodes and liver and, in some cases, the tumor could also be detected in the ovary and intestine (data not shown). Tumor progression also induced mobility difficulties in some mice.

Retreatment with NKT14m increases the antitumor efficacy

We next evaluated the possibility to administrate a second dose of NKT14m to improve the observed antitumor effect. Mice treated with a single dose of NKT14m two days after tumor challenge received a second dose of antibody 6 weeks after tumor injection. Retreatment with NKT14m showed an important antitumor effect (50% survival vs. 0% in control mice, $p < 0.0001$), and mice exhibited an increase in survival compared with those injected with a single dose of antibody (50% vs. 25%, respectively; $p = 0.1$) (Figure 2A). In addition, an increase of IFN-γ-producing iNKT cells was detected after a second dose of NKT14m (19 ± 0.95% vs. IgG control mice, $p = 0.05$) with no significant differences with the number of IFN-γ-producing iNKT cells after the first injection of the antibody (19 ± 0.95% after retreatment vs. 24.5 ± 2.2% after a single dose at day 2, $p = 0.5$) (Figure 2B), demonstrating that iNKT cells remained functional after antibody readministration.

The combination of NKT14m and cyclophosphamide induces a potent antitumor immune response in a B-cell lymphoma mice model

After showing that NKT14m induced an *in vivo* antitumor effect in a B-cell lymphoma mice model, we sought to improve the NKT14m efficacy using clinically relevant strategies. To this end, we evaluated the therapeutic efficacy of a combination of cyclophosphamide (Cy) with NKT14m. Mice were treated with Cy 10 days after tumor challenge followed by a single dose of NKT14m 24 hours after Cy administration. In this setting, we did not observed any reduction of iNKT, $CD4^+$ and $CD8^+$ T cells of treated mice, suggesting that Cy does not have any detrimental effect on these cell subsets (Figure 3A). In addition, in line with other studies, toxicity was not observed in mice receiving cyclophosphamide [33]. NKT14m combined with Cy significantly enhanced the antitumor effect observed with Cy alone (87% vs. 37% survival, respectively; $p = 0.03$) (Figure 3B), and promoted a higher tumor control than NKT14m alone. IFN-γ-producing iNKT cells were increased in mice treated with Cy and NKT14m (15.3 ± 1.2% vs. control mice, $p = 0.006$) (Figure 3C). In addition, a significant increase of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells were found in mice treated with the combination of Cy and NKT14m ($CD4^+$ T cells: 7.5 ± 0,05% vs. control mice, $p = 0.006$; $CD8^+$ T cells: 37.5 ± 1.7% vs. IgG control mice, $p = 0.002$).

We did not observe any relevant toxic effect of NKT14m antibody or its combination with cyclophosphamide during the daily examination of mice. All mice with clearance of tumors looked healthy, without significant changes in body weight, motility, hair aspect and normal behaviour. Furthermore, necropsy of those animals rejecting the tumor also revealed no macroscopic abnormal changes in organ morphology (spleen, liver, lymph nodes, and lungs) (data not shown).

A long-lasting immunity is conferred by NKT14m in combination with cyclophosphamide

To test whether NKT14m with Cy pretreatment could induce a long-term immunity against B-cell lymphoma, mice that survived after the first tumor injection were challenged again with 4TOO tumor cells. 43% of immunized mice remained tumor-free after tumor rechallenge ($p = 0.01$), whereas Cy treated were not able to reject de tumor (Figure 4), suggesting the establishment of a long-lasting immunity.

DISCUSSION

iNKT cells can activate innate and adaptive immunity and also contribute to tumor immune-surveillance [1, 2, 4, 5], making its activation an attractive approach for cancer immunotherapy. A large number of studies in cancer focused on the activation of iNKT cells using α-GalCer approaches did not observe potent antitumor responses [23–29]. A novel agonistic antibody that activates iNKT cells, the NKT14m, has been developed [30] and it represents a promising tool to activate iNKT cells *in vivo*.

We demonstrated, for the first time, the antitumor efficacy of NKT14m antibody in the setting of cancer. An effective therapeutic response against B-cell lymphoma was obtained using a single dose of the novel antibody, while no significant antitumor effect was observed with a single α-GalCer administration, as shown by previous studies [19, 34–36]. In fact, the poor antitumor efficacy of α-GalCer alone in our tumor model mostly resembles the clinical scenario, where patients treated with this

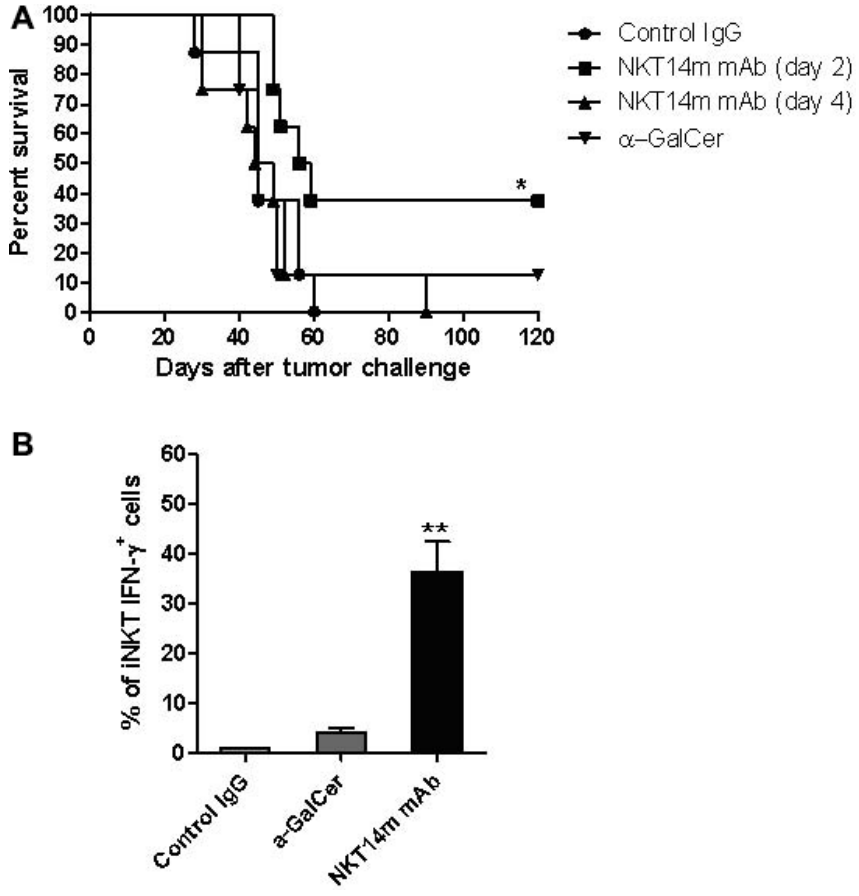

Figure 1: The agonistic NKT14m mAb induces an effective *in vivo* antitumor response against B-cell lymphoma. (**A**) Balb/c mice ($n = 8$) were injected with 4×10^5 4TOO tumor cells (iv) on day 0 and were treated 2 or 4 days later with a single dose of NKT14m mAb (100 μg/mice, iv) or IgG (100 μg/mice, iv). A group of mice received α-GalCer (2 μg/mice, iv) two days after tumor challenge. Mice were monitored daily for survival. Data represents survival from one of three independent experiments. (**B**) Splenocytes ($n = 4$) were analyzed by flow cytometry for IFN-γ producing iNKT cells 24 hours after NKT14m (day 2 infusion) and α-GalCer injection. Data are represented as mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.005$.

glycolipid did not experiment any response although they had a moderate iNKT-cell expansion and low IFN-γ production [23–26]. NKT14m antibody induced a potent iNKT-cell activation with a higher increase of IFN-γ production than α-GalCer, suggesting that this antibody could be a better option to improve responses in cancer patients. However, we also observed that mice treated with the NKT14m antibody 4 days after tumor injection were not able to eliminate the tumor in any case. This lack of tumor control was mostly due to the aggressiveness of the tumor model itself, and not to a defect in IFN-γ production by NKT cells, since we were able to detect IFN-γ in a number of time points after administration of NKT4m antibody (i.e., day 2, 11, and 42 after tumor challenge).

Since NKT14m did not induced long-term iNKT-cell anergy [30], we tried to improve its efficacy with the administration of a second dose of antibody, six weeks after tumor injection, which is the time previously shown to assure proper reactivation of iNKT cells [30]. In this situation, we could observe a reactivation of iNKT cells *in vivo* (i.e., increase of IFN-γ production) with an improved therapeutic efficacy. This is in line with previous data [30] and shows for the first time that readministration of NKT14m can induce iNKT cell reactivation *in vivo* and increases survival in a therapeutic setting of cancer. The possibility of retreatment with NKT14m makes it a promising therapeutic strategy and represents a critical advantage with important clinical implications over α-GalCer, which induces a potent iNKT-cell anergy that prevents further retreatment [18, 21, 30, 31]. In this regard, the use of NKT14m would allow to developing strategies of maintenance treatment like other antibodies, which hopefully would contribute to relapse prevention and increase survival of B-cell lymphoma patients.

In our study, the use of cyclophosphamide in the combined therapeutic approach did not affect iNKT and T cells *in vivo*, suggesting that the chemotherapeutic dose used in our experiments was not toxic. These observations are in line with previous studies, which have demonstrated that cyclophosphamide does not induce

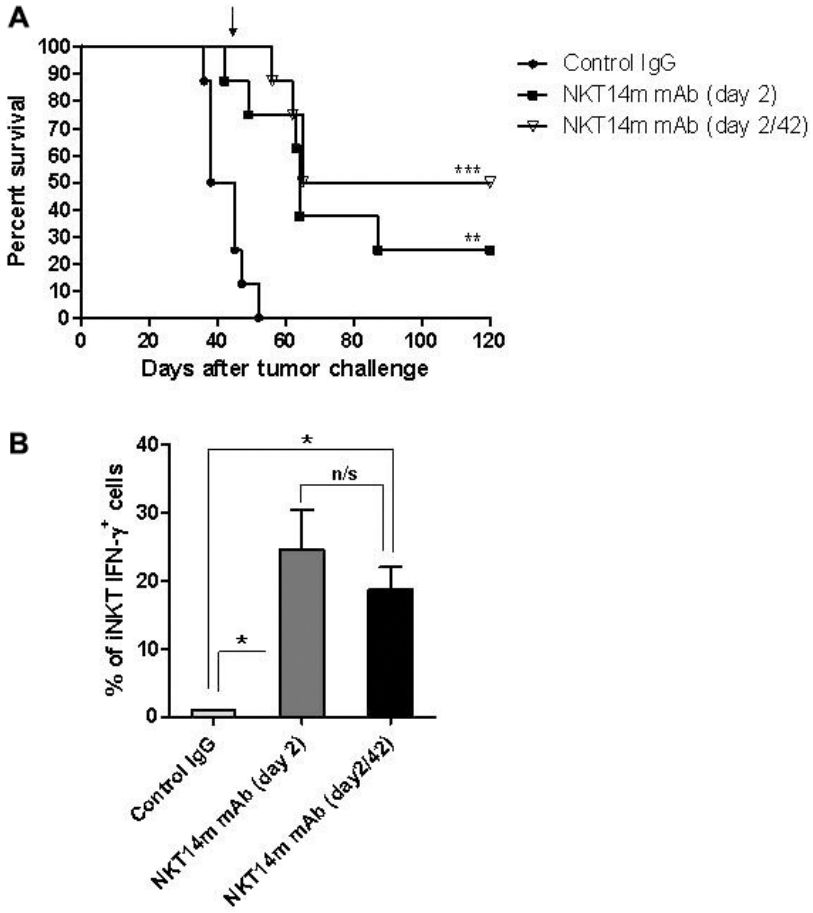

Figure 2: Retreatment with NKT14m mAb enhances the antitumor efficacy. (**A**) Balb/c mice ($n = 8$) were injected with 4×10^5 4TOO tumor cells (iv) and, 2 days later, treated with a single dose of NKT14m mAb or control IgG (iv). One group of treated mice ($n = 8$) was injected again with a single dose of NKT14m mAb 42 days after tumor inoculation (NKT14m mAb day2/42). Arrow indicates the day of retreatment. Mice were daily followed for survival. Data represents survival from one of three independent experiments. (**B**) Splenocytes ($n = 4$) from mice treated with NKT14m at day 2 and mice that received a second dose of the antibody 42 days after tumor challenge were analyzed by flow cytometry for IFN-γ producing iNKT cells (24 hours after treatment in all cases). Data are represented as mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$; n/s: no significance.

any significant changes in the total numbers of these different cell subsets [33]. Although it was reported that cyclophosphamide has immunostimulatory activities [37, 38] and can induce the activation of $CD4^+$ and $CD8^+$ T cells, including iNKT cells [33], its administration alone in our B-cell lymphoma model did not have any antitumor effect, in line with previous data [33]. The combination treatment with NKT14m antibody and cyclophosphamide resulted in a greater antitumor effect compared to either treatment alone. In contrast with previous studies using α-GalCer [33], iNKT-cell activation with NKT14m antibody seems to be more potent and its combination with a chemotherapeutic agent such as cyclophosphamide has a synergistic activity increasing IFN-γ-producing iNKT and T cells, improving mice survival and establishing a long-lasting memory. These data may have relevant clinical consequences since cyclophosphamide is a chemotherapeutic agent largely used to treat B-cell lymphoma and this therapeutic strategy offers a promising treatment that can be rapidly translated to the clinical scenario. In addition, we did not observed any toxic effect of cyclophosphamide or NKT14m antibody during the study; mice presented normal body weight, motility, behaviour and organ morphology, demonstrating that the therapy with NKT14m antibody and its combination with cyclophosphamide is safe. Furthermore, our data showing absence of toxicity of NKT14m antibody is in line with previous data regarding the use of this antibody in tumor-free mice [30].

In summary, our findings demonstrate that the novel NKT14m antibody can induce an important iNKT-cell mediated antitumor response associated with potent iNKT-cell activation in a therapeutic setting of B-cell lymphoma. More importantly, NKT14m combined with a standard chemotherapy, such as cyclophosphamide, shows an enhanced antitumor efficacy. The availability of a humanized agonistic iNKT-cell antibody for clinical use, the NKTT320

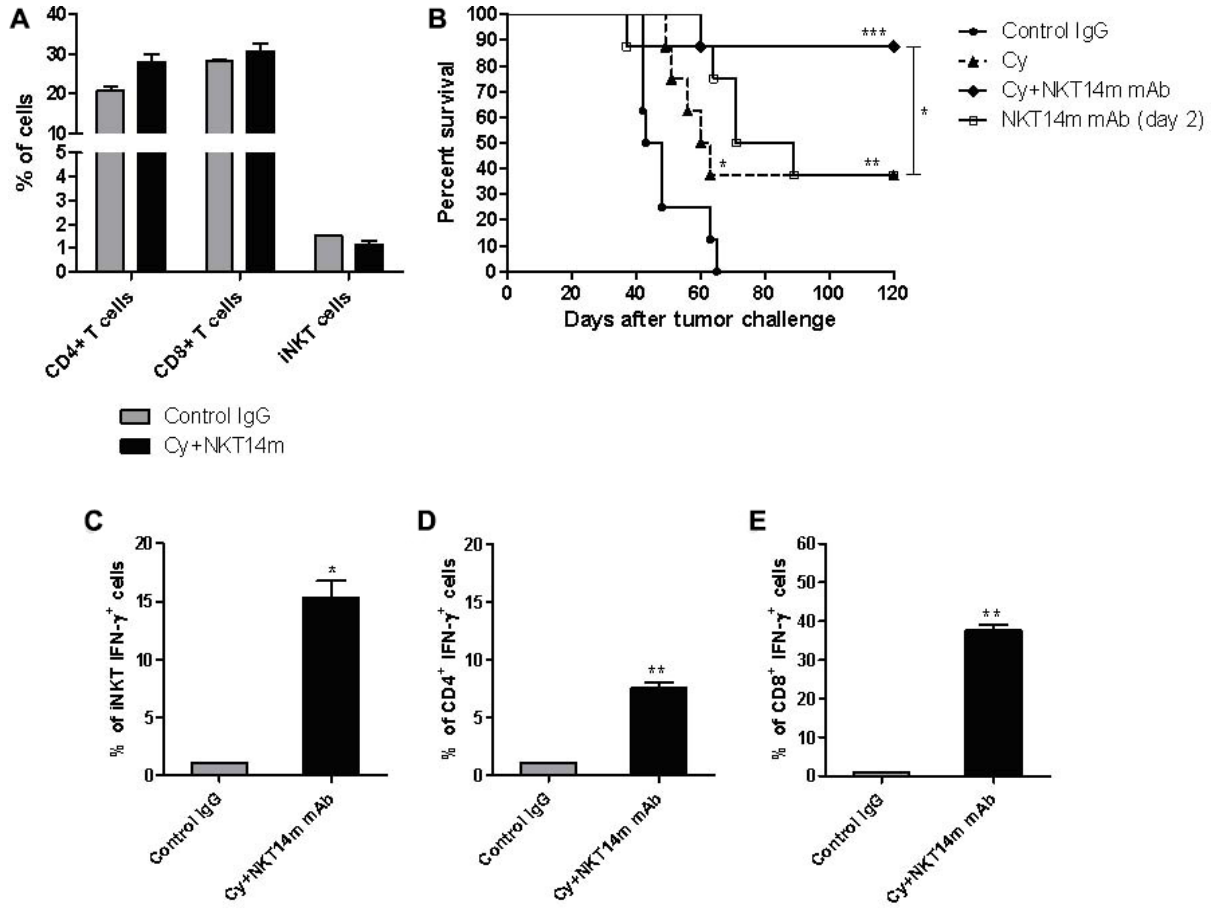

Figure 3: Treatment with cyclophosphamide following NKT14m mAb administration induces a potent antitumor response against B-cell lymphoma. (**A**) Percent of total iNKT, $CD4^+$ and $CD8^+$ T cells were analyzed from spleens (n = 3) of mice treated with Cy and NKT14m antibody (Cy+NKT14m mAb) and IgG control mice. (**B**) Balb/c mice (n = 8) were treated with cyclophosphamide (Cy) 10 days after tumor challenge. One group of Cy treated mice (n = 8) received a single dose of NKT14m mAb 24 hours after Cy injection (Cy+NKT14m mAb). Another group of mice (n = 8) received a single dose of NKT14m mAb 2 days after tumor challenge. Mice were monitored daily for survival. Data represents survival from one of three independent experiments. Splenocytes (n = 3) from Cy+NKT14m treated mice were analyzed by flow cytometry for IFN-γ producing (**C**) iNKT, (**D**) $CD4^+$ and (**E**) $CD8^+$ T cells, 24 hours after treatment. Data are represented as mean ± SEM. $^*p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$.

mAb [32], opens new possibilities for using iNKT-cell agonists for the treatment of patients with B-cell lymphoma in combination with current chemotherapy drugs, which warrants further testing in clinical trials.

MATERIALS AND METHODS

Mice

Female Balb/c mice (6–7 weeks of age; Charles River, France) were used for *in vivo* experiments. Animals were housed under specific pathogen-free conditions at the Laboratory Animal Facility at Hospital Sant Pau (Barcelona). All experiments and care of animals were conducted according to European Animal Care guidelines and approved by the Ethical Committee of Animal Experimentation at Hospital Sant Pau.

Tumor cell lines

4TOO is a Balb/c plasmacytoma cell line expressing MHC class I H-2d molecules gently provided by Dr. M. Khuel (NCI, Bethesda, MD). Tumor cells were maintained in complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µM β-2-mercaptoethanol (Life Technologies, Inc. Rockville, MD). Cells were grown in suspension culture at 37ºC in 5% CO_2. 4TOO tumor cells were thawed from a common frozen stock and grown *in vitro* in complete medium for 3 days before use. On the day of tumor injection, cells were washed with complete medium and diluted to the appropriate concentration in 0.1 ml of phosphate buffered saline (PBS) per mouse.

In vivo vaccination experiments with NKT14m

Mice (8 per group) were injected intravenously (iv) with 4TOO tumor cells (4×10^5 cells/mouse) and, two or four days later, were treated with the NKT14m (100 µg/mice, iv; NKT Therapeutics Inc.). In some cases, mice were retreated with 100 µg of NKT14m antibody 42 days after tumor challenge. Control mice received IgG (100 µg/mice, iv) or α-GalCer (2 µg/mice, iv), two days after tumor injection. Mice were followed daily for survival.

For combination studies, mice received a treatment that combined a single dose of cyclophosphamide (Cy; 70 mg/kg) intraperitoneally (ip) 10 days after tumor injection, and a single dose of NKT14m (iv) 24 hours after cyclophosphamide treatment. In some experiments, mice were rechallenged with a second dose of 4TOO tumor cells (4×10^5 cells/mouse, iv) 120 days after the first tumor injection. Animals were followed daily for survival.

Flow cytometry and intracellular IFN-γ detection

Spleens of treated and control mice were harvested 24 hours after treatment and were disaggregated in 5mL of complete medium by mechanical procedures. Splenocytes were collected, filtered through a 70 mm cell strainer (BC Falcon, Cultek S.L.U.) and centrifuged at 1500 rpm during 5 minutes. Erythocytes were lysed using an ammonium chloride solution (Pharmlyse Buffer, Pharmingen, BD Bioscience) during 3 minutes in agitation at room temperature. Finally, cells were centrifuged again during 5 minutes at 1500 rpm. Splenocytes were counted and maintained in 10 mL of complete medium until use.

Unloaded and α-GalCer analogue (PBS-57)-loaded CD1d tetramers were kindly provided by the NIH Tetramer Core Facility (Atlanta, GA) and were used for iNKT cell

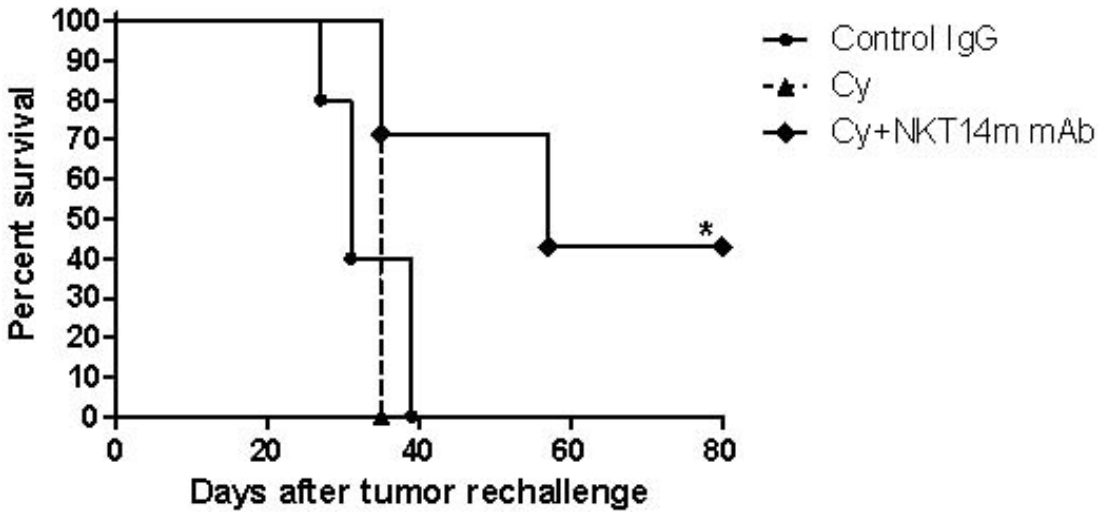

Figure 4: Long-term immunity is induced by the combination of cyclophosphamide and NKT14m mAb. Mice immunized with Cy+NKT14m treatment that survived the first 4TOO tumor injection ($n = 7$) were rechallenged with a second dose of tumor cells (4×10^5 cells/mouse, iv). Mice were followed daily for survival. Data represents one of three independent experiments. $^*p < 0.05$.

detection (1.2 μg for 10^6 cells in 100 μl), together with anti-mouse TCRβ antibody (REA318; Miltenyi Biotec). $CD4^+$ and $CD8^+$ T cells were identified using the anti-CD4 (GK1.5) and anti-CD8 (53–6.7) antibodies (both from Miltenyi Biotec). Splenocytes were stained for 30 minutes at 4ºC in PBS containing 1% bovine serum albumin (BSA) and 0.01% NaN3 (staining buffer).

For intracellular staining of IFN-γ, splenocytes were fixed and permeabilized following the manufacturer´s instructions (BD Cytoperm/Cytofix kit, BD Bioscience). Cells were stained using the anti-mouse IFN-γ mAb (AN.18.17.24; Miltenyi Biotec), for 30 minutes at 4ºC. All data was acquired on a MACSQuant Analyzer 10 (Miltenyi Biotec). Flow cytometer was automatically set-up daily accordingly to Miltenyi Biotec instructions. The compensation was performed automatically by the flow cytometer and was verified by our experimental controls (unstained cells, isotype antibodies and cells from control mice). Data from flow cytometry was analysed using the FlowJo Version 10 software (TreeStar), following the gating strategy showed in Supplementary Figures 1 and 2.

Statistical analysis

Results are expressed as the mean ± SEM. Kaplan-Meier plots were used to analyze mice survival and the significant differences between survival curves were assessed by the log- rank test. For all other data, *t* test was performed to analyze the differences between groups. All statistical analysis and graphics were performed using GraphPad Prism 6 (Graph Pad Software Inc.).

Abbreviations

iNKT: invariant natural killer T; iTCR: invariant T-cell receptor; α-GalCer: α-galactosylceramide; IFN: interferon; DC: dendritic cell; NK: natural killer; MHC: major histocompatibiliy complex; IL: interleukin; mAb: monoclonal antibody; IgG: immunoglobulin G; iv: intravenously; ip: intraperitoneally; Cy: cyclophosphamide; FBS: fetal bovine serum; PBS: phosphate buffer saline; BSA: bovine serum albumin.

Author contributions

LEG: design, experimental, analytical performance, and writing of the paper. CAF: experimental performance. AC: experimental performance. RS: contributed vital reagents. JS: analytical performance. JB: study´s conception, design, analytical performance, and writing of the paper. All the authors edited and approved the manuscript for submission.

CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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