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TITLE: ROLE OF NATURAL KILLER T CELLS IN IMMUNOGENIC CHEMOTHERAPY FOR BREAST CANCER

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14. ABSTRACT

During this first year of investigation, we have highlighted the important role of γδ T cells in the efficacy of immunogenic chemotherapy using anthracyclines. In the meantime, the NKT cells seem to be dispensable for this effect at least in this particular situation. Moreover, we have also identified a population of γδ T cells with a unique phenotype never described before ($CD3^{bright}$). Interestingly, this population has a clear intrinsic ability to produce IL-17 compared to any other γδ T cells and are represented at a high frequency in breast cancer tumor environment. Our future efforts will focus on this population and their putative role in immunogenic chemotherapy for breast cancer.

15. SUBJECT TERMS
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Introduction:

The role of immunity in controlling and regulating development of breast cancer is largely accepted. Recent studies have also demonstrated that some common chemotherapy (eg. Anthracyclines, trastuzumab) used for breast cancer treatment relies on a competent immune system of the host to achieve an optimal response[1-3]. Interestingly seminal findings emphasized on the importance of various components of the immune system in this phenomenon[3-6]. In this proposal, we have hypothesized that innate-like T cells, including Natural Killer T (NKT) cells, are well positioned and equipped to participate in this effect.

Body:

As assigned in the original proposal, we have performed series of experiment to assess the contribution of NKT cells and γδ T cells in immunogenic chemotherapies. Here we report the findings generated in this first year mainly for task 1 (***Evaluation of type I NKT cell activation/involvement in immunogenic chemotherapy for breast cancer***) approved in the statement of work:

An extensive series of *in vitro* assays have been tested to evaluate NKT cell activation after Dox-induced breast cancer cell death. Using various *in vitro* settings we failed to detect any immunogenic signals provided by the AT3 tumor cell line on immune cells. As expected, we observed that Dox treatment could induce the cell death of breast cancer lines by an apoptotic pathway based on Annexin V expression (**Figure 1A**). However using various co-culture models including BMDC, spleen cells, and hepatic cells pulsed with Dox-treated AT3 (using various dose, number, ratio, time-point) did not lead to relevant cytokine production (IL-12, IL-1β, IL-17, IFN-γ) or modulation of any immunogenic markers (**Figure 1B and not shown**).

The absence of any signals in all our *in vitro* assays suggest that immunogenic cell death observed *in vivo* is challenging to reproduce *in vitro* and may suggest that other populations of

cells including stromal or epithelial cells could be critical in the establishment of such an immune process.

As assigned in subtask 1b, we have investigated the frequency and activation status of NKT cells in breast cancer tumor-bearing mice after Dox treatment. We observed an increase frequency of NKT cells in tumors treated with Dox compared to untreated (**Figure 2A**). Interestingly the increased frequency was associated with a higher expression of CD69 suggesting that Dox treatment leads to NKT cell activation (**Figure 2B**).

As proof of concept of our hypothesis, we have studied the potential role of type I NKT cells in the therapeutic efficacy of Dox. WT and Jα18$^{-/-}$ AT3 tumor-bearing mice have received a single dose of Dox. As stated in the approved statement, $\gamma\delta$ T cells are another population of innate-like T cells able to produce IL-17[7]. Thus we have also tested the contribution of these cells in efficacy of Dox treatment. Despite the relative accumulation and activated phenotype of NKT cells in AT3 tumors after Dox treatment observed in subtask 1b, we observed that NKT cell deficiency does not impact the efficacy of the chemotherapy at least in this model. Strikingly, the absence of $\gamma\delta$ T cells largely reduced the efficacy of the treatment indicating that in this particular context, $\gamma\delta$ T but not NKT cells are the key cells to bridge innate and adaptive immune response induced by Dox (**Figure 3**). This observation is in line with a recent report demonstrating the critical role of $\gamma\delta$ T cells in immunogenic chemotherapy for various solid tumors[8] . However, the fact that NKT cells display a phenotype of activated cells in the tumor and draining lymph nodes of Dox-treated mice indicate that they may play a role but are potentially irrelevant in this organ due to their scarce number (**Figure 2**). As a proof of concept that NKT cells can play a role in immunogenic cell death, we will test the efficacy of Dox (using suboptimal dose (1 mg/kg)) in Vα14 transgenic mice (available in Prof. D. Godfrey's Lab). Moreover, because the first set of experiments, performed this year has mainly focused on the assessment of the primary tumor growth, future experiments will be conducted with a particular focus on associated pulmonary metastases after resection of the primary tumor.

In absence of convincing data demonstrating a critical role of type I NKT cells in our settings, the subtask 1d and 1e have not been investigated yet. However regarding the real importance of $\gamma\delta$

T cells in anti-tumor response to Dox (at least using AT3 cell line) (**Figure 3**), experiments to assess the role of γδ T cells in activation/regulation of other accessory immune cells will be conducted in the coming next months by comparing WT and TCRδ$^{-/-}$ mice using the same approaches than described in the original proposal.

No experiments to study the role of NKT cells in trastuzumab therapy for breast cancer have been conducted yet. The contribution of the immune system has recently been published by our Lab and others and requires activated T cells and type I and II IFNs[2,3]. This subtask will be studied in the next 6 months including the monitoring of NKT cell activation as well as their contribution as tested in subtask 1b and 1c using Jα18$^{-/-}$ mice.

After a year of investigation, it seems that, so far, γδ T cells are far more important in immunogenic cell death than NKT cells. For this reason we have focused our interest more on these cells. Interestingly, we have highlighted the role of γδ T cells in immune response elicited by alpha-galactosylceramide (α-GalCer), a potent type I NKT cell ligand. This observation has been published in *The Journal of Immunology*[9] (**attached in "Appendices" section**). In this model, γδ T cells significantly contribute to the anti-tumoral effect of this compound and the development of adaptive immune responses when combined with another Ag (ovalbumin).

Of more interest, the use of α-GalCer also revealed to us an unpreviously described subset of γδ T cells displaying a high level of CD3 in comparison to the remaining population and preferentially associated to the mucosae (**phenotype summarized in Table 1**). Based on the high importance of this discovery, we have undergone a series of basic experiments to investigate the phenotype, cytokine profile as well as the factors regulating the homeostasis of this population. A manuscript is currently in preparation and should be submitted soon to a premier immunology journal according to the originality of the observation (*Immunity* is the targeted journal). Of upmost interest for the current proposal (therapeutic effect of Dox is IL-17-dependent), this population has a strong T_H17 bias and promptly produces IL-17 upon stimulation (no IFN-γ nor IL-4) compared the conventional γδ T cell population (**Figure 4**). Moreover, we have observed that this population is highly represented in 4T1.2 breast cancer tumor (compared to any other peripheral tissue where they only represent around 1-10% of the

total γδ T cells) and associated with an activated phenotype based on CD69 expression (**Figure 5**). Because this population can be specifically investigated by flow cytometry analysis, the potential IL-17 production of this population will be investigated after Dox treatment in comparison to other γδ T cells as proposed in subtask 1b. Unfortunately, no tools (gene-targeted mice or depleting antibodies) are currently available to directly assess the contribution of this population in development of breast cancer or immunogenic chemotherapy for breast cancer. However, reconstitution of TCR$\delta^{-/-}$ mice with either subset (CD3^{bright} γδ T cells vs conventional γδ T cells) could allow us to investigate their differential contribution in these settings.

Referring to our first findings, we will focus on γδ T cells for work proposed in Task 2. No experiments covering this part have been performed so far. Since γδ T cells and NKT cells have a lot of similarities, in term of functions and activation mechanisms (at least those which are TCR-independent), the design of this task is almost completely transferable to the study of γδ T cells.

No experiments covering the Task 3 have been performed yet. The observation that NKT cells display a phenotype of activated cells after Dox administration (**Figure 2**) reinforces the possibility to boost these cells to optimize the efficacy of chemotherapy and also indicates that the experiments planned in Task 3 are likely to be fruitful.

Key Research Accomplishments :

- Demonstration that **γδ T cells are crucial in immunogenic chemotherapy** for breast cancer but not type I NKT cells so far.
- Discovery of a **population of γδ T cells with a clear signature of IL-17-producing cells** and potentially critical for immunogenic chemotherapy and development of breast cancer.
- **Original publication in *the Journal of Immunology*** describing for the first time the contribution of γδ T cells in the antitumoral effect of α-GalCer.

Reportable outcomes:

- Publications:

- **Paget, C**., M. T. Chow, H. Duret, S. R. Mattarollo, and M. J. Smyth. 2012. Role of gammadelta T cells in alpha-galactosylceramide-mediated immunity. *J Immunol* 188:3928-3939.

- **Paget, C.**, M. T. Chow, A. P. Uldrich, H. Duret, K. Steegh, G. T. Belz, D. I. Godfrey, and M. J. Smyth. CD3 expression reveals gamma/delta T cell functions. *Manuscript in preparation.*

- Communications:

- 2011: 6th International Symposium on CD1 and NKT cells. 23-27 Sept. Chicago, Illinois, USA: ***Poster****. "Role of gamma/delta T cells in alpha-galactosylceramide-mediated immune responses".*

- 6th Aug. 2012: Ludwig Institute for Cancer Research. Austin-Melbourne Branch, Australia: ***Invited speaker.****"alpha-galactosylceramide reveals a new way to define gamma/delta T cell functions"****.***

- 11th Sept. 2012: Peter MacCallum Cancer Centre. Cancer Immunology Program Hub. Melbourne, Australia: ***Oral communication****. "CD3 expression reveals gamma/delta T cell functions "****.***

Conclusions:

After a year of investigation, we have observed the critical role of γδ T cells in effect of immunogenic chemotherapy using anthracyclines. In the meantime, the NKT cells seem to be dispensable for this effect. However, during our investigations, we have identified a population of γδ T cells with a unique phenotype never described before ($CD3^{bright}$). Interestingly, this population has a potent intrinsic ability to produce IL-17 compared to the other γδ T cells and is enriched in breast cancer tumor environment. Our future efforts will focus on this population and their putative role in immunogenic chemotherapy for breast cancer and more generally in the development of breast cancer.

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Appendices:

Figures shown in this section are unpublished.

Figure 1

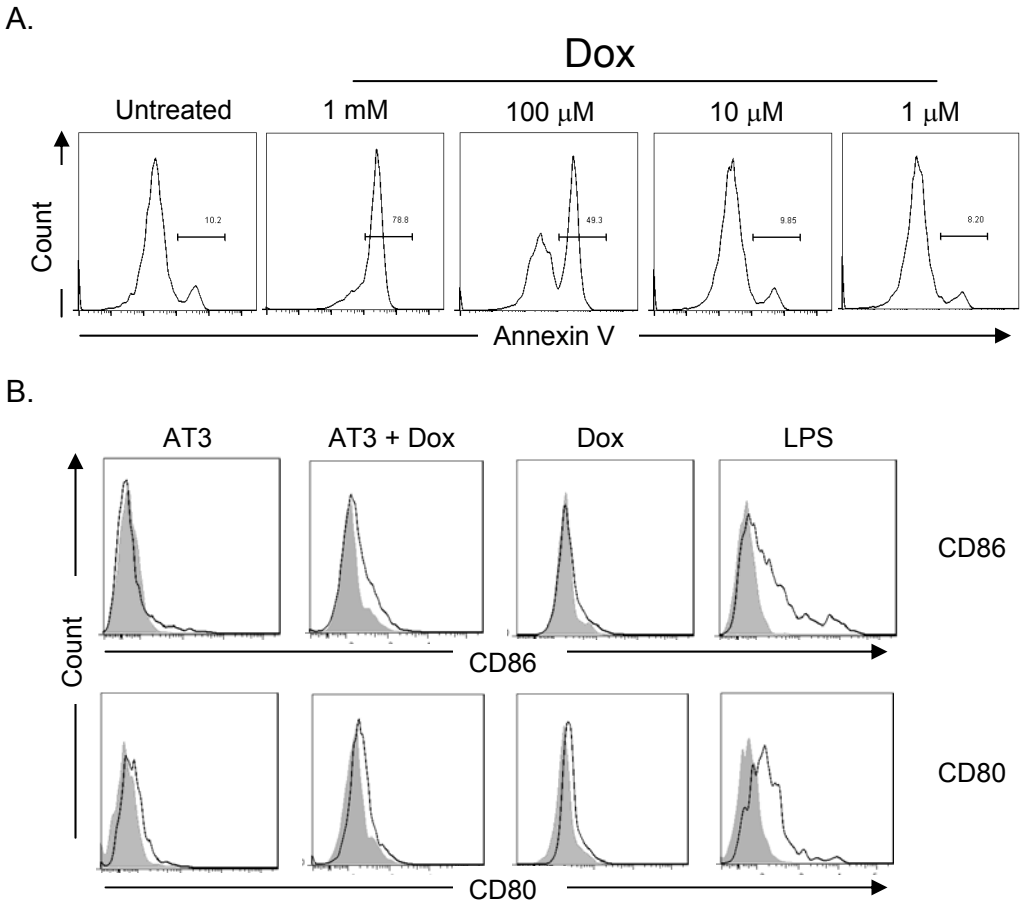

Figure 1: *In vitro* treatment of AT3 with Dox does not induce immune signals.
A, 50 000 AT3 cells have been seeded in a 48 well-plate and treated or not with Dox at various concentrations for 30 min. Then, cells have been extensively washed and subjected to flow cytometry for AnnexinV expression. *B*, 5×10^4 AT3 cells have been treated or not with Dox (100μM) for 30 min and then cocultured with BMDC (2×10^5). After 24 hrs, BMDC ($CD11c^+$ $MHC\ II^+$) have been stained for CD86 and CD80 expression. BMDC have been treated with LPS (10 ng/ml) as a positive control.

Figure 2

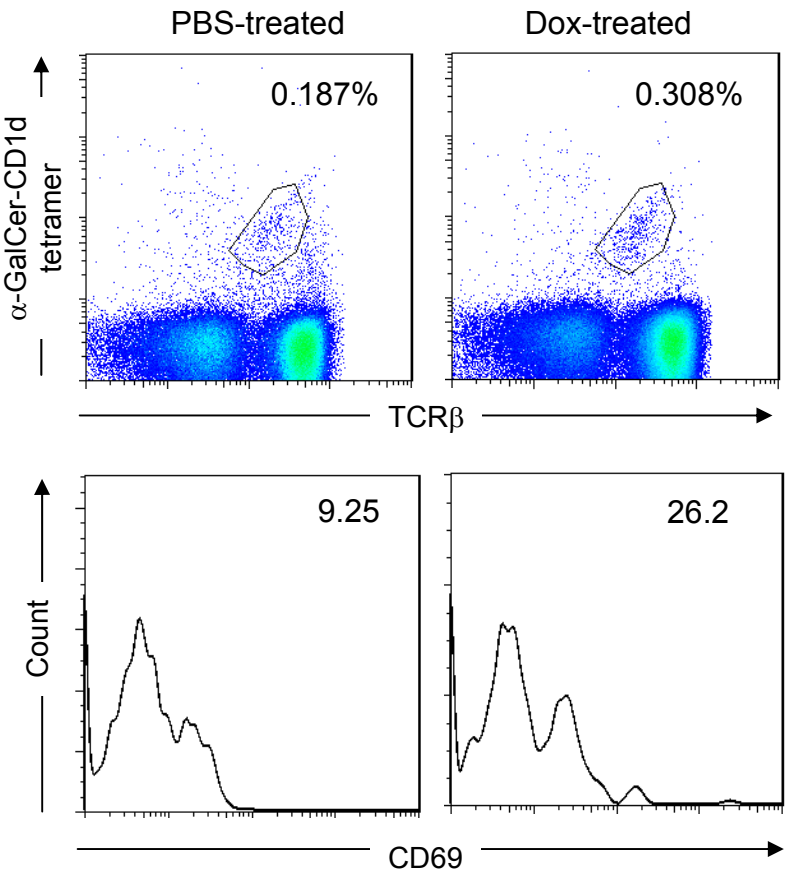

Figure 2: Frequency and activation status of tumor-associated type I NKT cells after Dox-treatment in breast cancer tumor.
Frequency (*upper panel*) and activation status (*lower panel*) of type I NKT cells (tetramer+ TCRβ+) in untreated (5 mice) or Dox-treated (5 mice) AT3 tumor-bearing mice. 5×10^5 AT3 cells have been injected s.c. and treated i.v. with PBS or Dox (2 mg/kg) after 20 days (tumor size around 15-20mm^2). 36 hrs after treatme nt, AT3 tumors have b een harvested and prepared for cell suspension. Frequency and activation status (CD69) of type I NK T cells have been investigated by flow cytometry on 7AAD$^-$ CD45.2$^+$ cells.

Figure 3

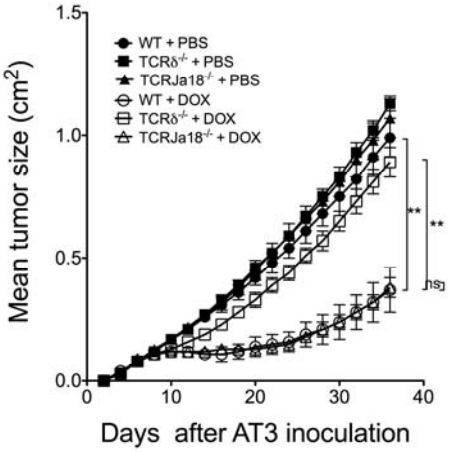

Figure 3: Dox therapy requires γδ T cells, but not type I NKT cells.
Groups of 5 WT or gene-targeted BL6 mice as indicated were injected subcutaneously with 5 x 10^5 AT3 mammary adenocarcinoma cells Mice then received either PBS or Dox (2 mg/kg) on day 7 after tumor inoculation. Tumor size was measured as indicated. Data shows means of 5 mice per group ± standard errors, representative of two independent experiments. Statistical analyses were performed at the time point indicated on the figure using Mann-Whitney test (**p<0.01; ns = not significant).

Figure 4

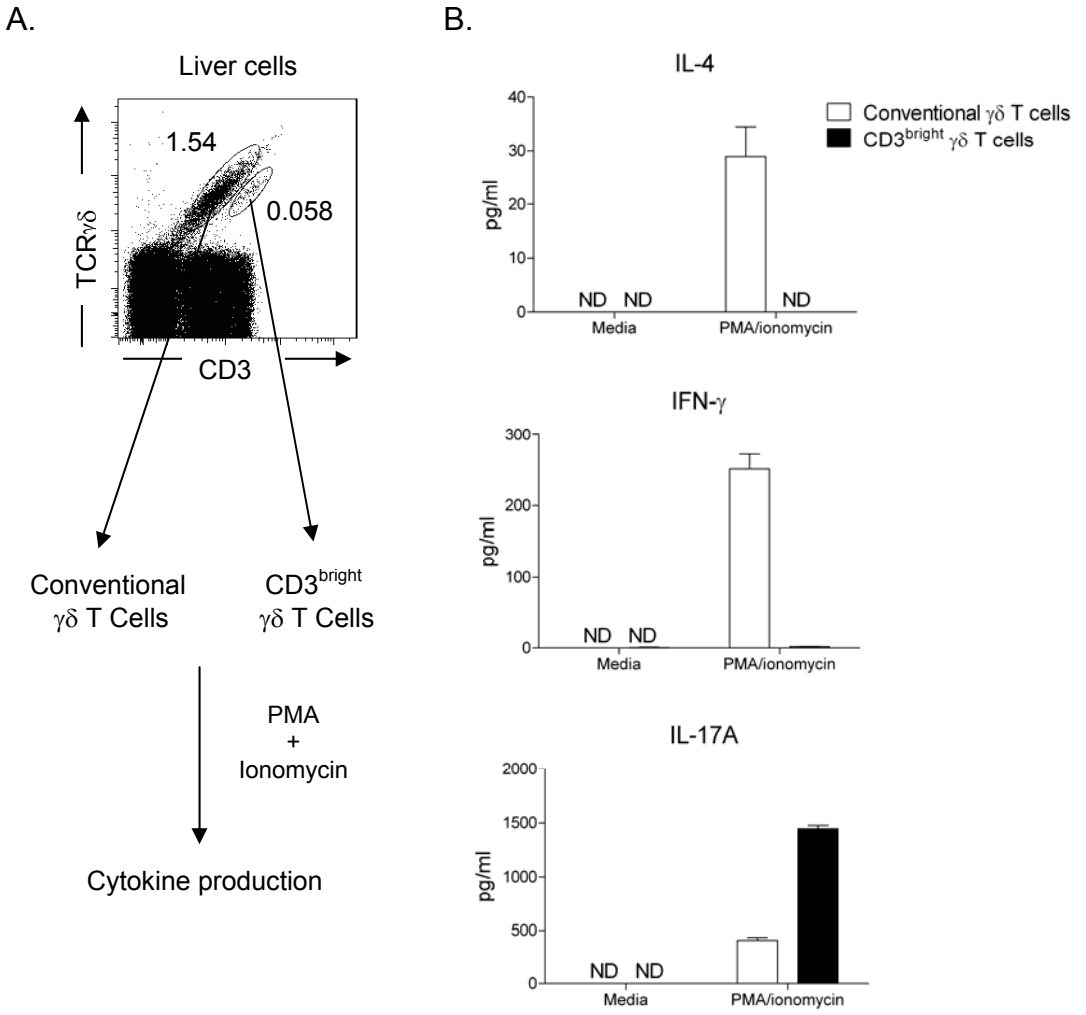

Figure 4: Cytokine production profile of CD3^{bright} γδ T cells.
A, γδ T cell subsets have been facs-sorted based on their CD3 expression. *B*, Purified cells (2000/well) have been stimulated for 20 hrs with PMA (100 ng/ml) and ionomycin (1 μg/ml). Supernatants have been harvested and tested for cytokine production using CBA system.

Figure 5

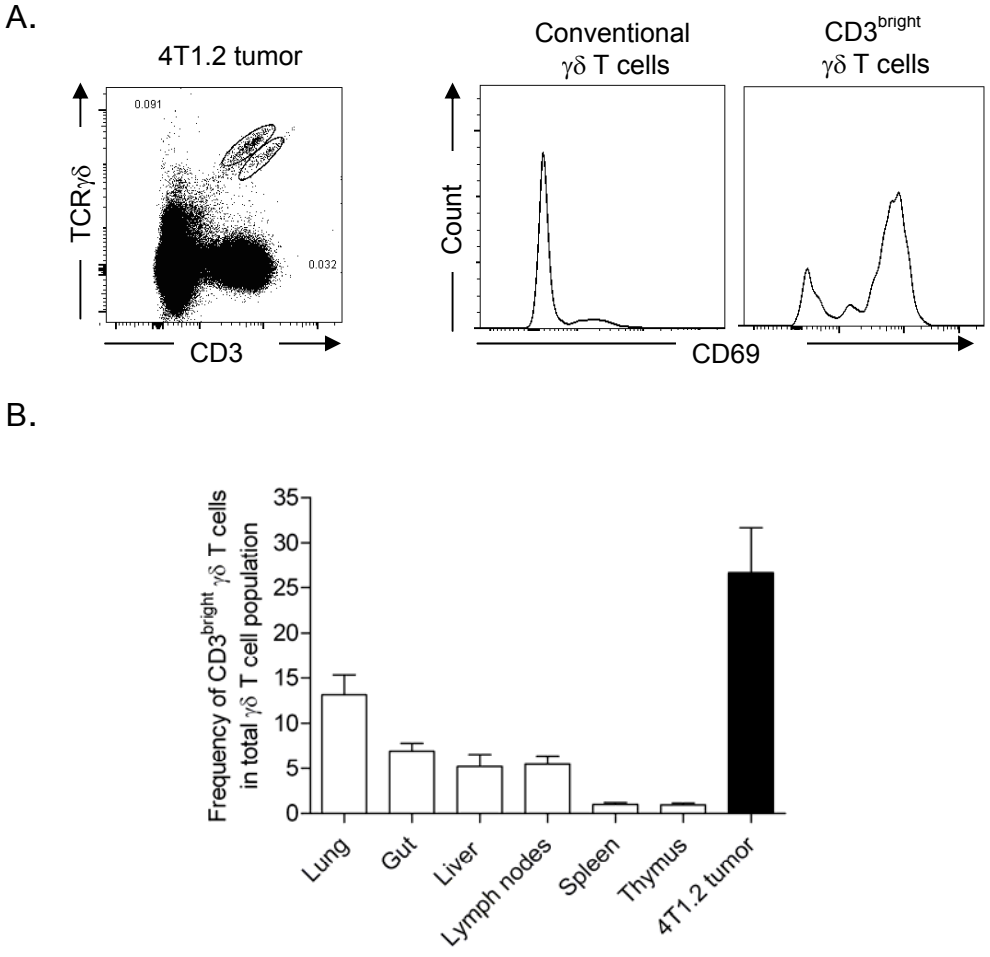

Figure 5: CD3^{bright} γδ T cells are enriched in breast cancer tumor and displayed a phenotype of activated cells.
A, 4T1.2 cells (5 x 10 ^{4}) have been injected in the mammary fat pad. At day 30, tumors have been harvested and prepared for cell suspension. Frequency and activation status (CD69) of γδ T cell subsets have been assessed by flow cytometry on 7AAD^{-} CD45.2^{+} cells. *B*, Distribution of CD3^{bright} γδ T cells in various tissues compared to 4T1.2 tumors.

Table 1

	Conventional γδ T cells *(MFI)*	***CD3^{bright} γδ T cells*** *(MFI)*
CD27	+/-	-
NK1.1	+/-	-
CD4	-	-
CD8	+/-	-
RORγt	*(419 ± 23)*	*(873 ± 37)*
IL-23R	+/- *(91 ± 23)*	+ *(852 ± 160)*
IL-1R1 (CD121a)	- *(14 ± 5)*	+ *(200 ± 19)*
IL-2R (CD25)	+ *(134 ± 24)*	+ *(301 ± 24.5)*
IL-15R (CD122)	+ *(926 ± 129)*	+ *(739 ± 101)*
IL-7R (CD127)	+ *(238 ± 43)*	+ *(1357 ± 176)*
CD69	*(263 ± 84)*	*(143 ± 26)*
CD62L	+/-	-
CD44	+/-	+
NKG2D	+ *(173.5 ± 10)*	+ *(129.5 ± 10.3)*

Table 1: Phenotype of γδ T cell subsets in pulmonary tissue.

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Role of γδ T Cells in α-Galactosylceramide–Mediated Immunity

Christophe Paget, Melvyn T. Chow, Helene Duret, Stephen R. Mattarollo and Mark J. Smyth

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Role of γδ T Cells in α-Galactosylceramide–Mediated Immunity

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Attempts to harness mouse type I NKT cells in different therapeutic settings including cancer, infection, and autoimmunity have proven fruitful using the CD1d-binding glycolipid α-galactosylceramide (α-GalCer). In these different models, the effects of α-GalCer mainly relied on the establishment of a type I NKT cell-dependent immune cascade involving dendritic cell, NK cell, B cell, or conventional CD4$^+$ and CD8$^+$ T cell activation/regulation as well as immunomodulatory cytokine production. In this study, we showed that γδ T cells, another population of innate-like T lymphocytes, displayed a phenotype of activated cells (cytokine production and cytotoxic properties) and were required to achieve an optimal α-GalCer–induced immune response. Using gene-targeted mice and recombinant cytokines, a critical need for IL-12 and IL-18 has been shown in the α-GalCer–induced IFN-γ production by γδ T cells. Moreover, this cytokine production occurred downstream of type I NKT cell response, suggesting their bystander effect on γδ T cells. In line with this, γδ T cells failed to directly recognize the CD1d/α-GalCer complex. We also provided evidence that γδ T cells increase their cytotoxic properties after α-GalCer injection, resulting in an increase in killing of tumor cell targets. Moreover, using cancer models, we demonstrated that γδ T cells were required for an optimal α-GalCer–mediated anti-tumor activity. Finally, we reported that immunization of wild-type mice with α-GalCer enhanced the adaptive immune response elicited by OVA, and this effect was strongly mediated by γδ T cells. We conclude that γδ T cells amplify the innate and acquired response to α-GalCer, with possibly important outcomes for the therapeutic effects of this compound. ***The Journal of Immunology*****, 2012, 188: 3928–3939.**

α-Galactosylceramide (α-GalCer) is a marine sponge-derived glycolipid Ag (1) that binds CD1d, a MHC class I-like molecule, expressed by APCs to specifically activate type I NKT cells through TCR ligation (2). Type I NKT cells (hereafter referred to as NKT cells) are T lymphocytes carrying a semi-invariant TCR composed by the canonical Vα14-Jα18 TCRα-chain (Vα24-Jα18 in humans) combined with a limited array of TCRβ-chains (Vβ8, Vβ7, or Vβ2 in mice, Vβ11 in humans). In response to α-GalCer, NKT cells rapidly and vigorously produce Th1, Th2, and Th17 cytokines (reviewed in Refs. 3–5), which in turn amplify or regulate innate/adaptive immune responses by inducing the maturation of dendritic cells (DC) (6, 7) and by influencing the functions of NK cells (8), macrophages (9), conventional CD4$^+$ and CD8$^+$ T lymphocytes (10, 11), and B lymphocytes (12). Consequently, α-GalCer exerts a potent adjuvant activity in vivo, rendering it a powerful candidate for clinical therapies (reviewed in Refs. 3, 4, 13, 14). Indeed, a large number of studies in mice confirmed the beneficial effect of α-GalCer in various mouse experimental models, predominantly in prophylactic settings including infectious diseases, autoimmunity, allergic reactions, and cancer. For example, the protective anti-tumor effect of α-GalCer has been shown against melanomas, carcinomas, hematopoietic malignancies, and their metastases. This effect is mainly due to the synthesis of IFN-γ by NKT cells and to the bystander activation of effector cells, including NK and CD8$^+$ CTL (15–18).

Similar to NKT cells, γδ T cells are unconventional T lymphocytes with innate-like cell hallmarks (reviewed in Refs. 19, 20). Their preactivated phenotype allows them to be one of the earliest responders during stress/inflammation, and so they can rapidly produce large amounts of cytokines to regulate immune responses (21). Cross-talk between NKT and γδ T cells has been recently reported. For example, activation/accumulation of γδ T cells during TLR3 agonist-induced liver inflammation can be resolved by NKT cells (22). Conversely, IL-17–producing (Vγ4$^+$) γδ T cells have been shown to negatively regulate NKT cell activation in a model of acute hepatitis (23). Finally, airway hyperresponsiveness can be enhanced through a synergistic activity of NKT and Vγ1$^+$ γδ T cells (24). Thus, γδ T cells are able to either positively or negatively regulate NKT cell response and vice versa according to the tissue studied and the subset of γδ T cells activated. However, surprisingly, no studies have yet investigated the potential contribution of γδ T cells in the immune responses triggered by α-GalCer. Additionally, in recent studies it was shown that γδ T cells directly responded to cytokines without any TCR engagement (25–28), suggesting that the cytokine cas-

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The online version of this article contains supplemental material.

Abbreviations used in this article: asGM1, asialo GM1; CBA, cytometric bead array; DC, dendritic cell; FasL, Fas ligand; α-GalCer, α-galactosylceramide; WT, wild-type.

cade elicited by α-GalCer could be strong enough to lead to γδ T cell activation. Moreover, α-GalCer treatment has been recently shown to alleviate murine listeriosis, an effect partially lost after γδ T cell depletion, suggesting a potential role of this population to achieve an optimal therapeutic effect of this lipid (29). Despite this finding, little is known about the precise functions of γδ T cells as well as the mechanisms leading to their activation in the context of α-GalCer. Our report demonstrates the novel finding that γδ T cells produce regulatory cytokines in α-GalCer–mediated immune responses that in turn amplify innate and acquired responses to this lipid.

Materials and Methods

Mice

C57BL/6J wild-type mice were purchased from the Walter and Eliza Hall Institute of Medical Research. C57BL/6 TCRδ cell-deficient (TCRδ$^{-/-}$) mice, C57BL/6 Jα18-deficient (Jα18$^{-/-}$) mice (30), C57BL/6 IL-12p35–deficient (IL-12p35$^{-/-}$) mice, and C57BL/6 IL-18–deficient (IL-18$^{-/-}$) mice (31) were bred in house at the Peter MacCallum Cancer Centre. All mice were backcrossed to C57BL/6J at least 10 times. Mice were used at the ages of 8–10 wk. All experiments were performed in accordance with the animal ethics guidelines ascribed by the National Health and Medical Research Council of Australia. All experiments were approved by the Peter MacCallum Cancer Centre Animal Ethics Committee.

Reagents and Abs

α-GalCer was from Alexis Biochemical (San Diego, CA). Anti-asialo GM1 (anti-asGM1) was purchased from Wako Chemicals (Richmond, VA). mAbs against mouse CD3 (17A2; Pacific Blue-conjugated), NK1.1 (PK136; PE-Cy7– or FITC-conjugated), TCRγδ (GL3; allophycocyanin-conjugated), CD69 (FN50; PE-conjugated), IFN-γ (XMG1.2; PE-conjugated), IL-17A (TC11-18H10; PE-conjugated), CD27 (LG.7F9; PE-Cy5–conjugated), TCRβ (H57-597; PE-conjugated), CD19 (1D3; Pacific Blue- or PE-conjugated), F4/80 (BM8; PE-conjugated), Vγ1 (2.11; FITC-conjugated), granzyme B (NGZB; PE-conjugated), NKG2D (C7; PE-conjugated), Fas ligand (FasL, MFL3; PE-conjugated), CD107a (1D4B; PE-conjugated), IL-12Rβ2 (305719; PE-conjugated), and IL-18Ra (112614; allophycocyanin-conjugated), as well as isotype controls, were purchased from BD Biosciences (San Diego, CA), BioLegend (San Diego, CA), R&D Systems (Minneapolis, MN), or eBioscience (San Diego, CA). Recombinant mouse IL-12p70 and IL-18 were from R&D Systems. PMA and ionomycin were from Sigma-Aldrich (St. Louis, MO).

Preparation of splenic and liver cells

Splenic and hepatic mononuclear cells from vehicle- or α-GalCer–treated mice were prepared as described previously (32). Briefly, livers were perfused with PBS, excised, and finely minced, followed by enzymatic digestion for 30 min at 37°C in PBS containing 1 mg/ml collagenase type IV and 1 μg/ml DNase type I (Roche). After washing, liver homogenates were resuspended in a 35% Percoll gradient, carefully layered onto 70% Percoll, and centrifuged at 2300 rpm at 22°C for 30 min. The layer at the interface between the two Percoll concentrations was carefully aspirated and washed in PBS containing 2% FCS. RBCs were removed with ACK lysis buffer.

Flow cytometry

Mice were injected i.p. with vehicle or α-GalCer (2 μg/mouse). In some cases, NK cells were specifically depleted using 200 μg i.p. rabbit anti-asGM1 Ab on days −2 and 0 prior to α-GalCer injection. Saline-perfused livers and spleens were harvested at different time points and mononuclear cells were prepared as described above. Then, GolgiPlug (for IFN-γ detection) or GolgiPlug plus GolgiStop (for IL-17A detection) (BD Biosciences) was added for 2 h. Cell suspensions were blocked in the presence of 2.4G2 prior to staining with appropriate dilutions of allophycocyanin-conjugated TCRγδ, Pacific Blue-labeled anti-CD3, and PE-Cy7–conjugated NK1.1 for 30 min in PBS containing 2% FCS and 0.01% NaN_3. Cells were then fixed and permeabilized using the BD Cytofix/Cytoperm fixation/permeabilization kit and incubated with PE-conjugated mAb against IFN-γ, IL-17A, or control isotype mAb in permeabilization buffer. Cells were acquired and analyzed on a LSR-II cytometer (BD Biosciences). FACS analysis was performed with FlowJo (Tree Star, Ashland, OR).

Detection of cytokines

Cytokines were detected using the BD cytometric bead array (CBA) system (BD Biosciences) according to the manufacturer's instructions. Acquisition was performed on an LSR-II (BD Biosciences). A total of 300 bead events for each cytokine were collected. Analysis of CBA data was performed using the FCAP array software (Soft Flow, St. Louis, MO). IL-18 quantification was determined using the ELISA kit from MBL International (Woburn, MA).

Isolation of γδ T and NK cells and cytotoxicity assays

Spleens were harvested from vehicle- or α-GalCer–treated mice. RBCs were lysed with ACK lysis buffer prior to γδ T and NK cell enrichment with autoMACS (depletion of TCR-β$^+$, CD19$^+$, and F4/80$^+$ cells). Then, γδ T- and NK-enriched splenic cells were sorted and purity was always >95%. For stimulation assays, purified cells were cultured for 20 h in complete RPMI 1640 (10% FCS, 10 U/ml penicillin/streptomycin, L-glutamine) containing recombinant mouse IL-12p70 (50 pg/ml) and/or IL-18 (1 ng/ml). For killing assays, cells were cocultured with targets labeled with [^{51}Cr] for a period of 20 h at an E:T ratio of 20:1. In some cases, cells were cultured with YAC-1 for 4 h and checked for cytotoxic marker expression using anti-NKG2D, anti-FasL, anti-granzyme B, or anti-CD107a and appropriated isotype controls.

Experimental lung metastasis and B cell lymphoma models

Lung metastasis model. B16F10 melanoma and 3LL Lewis lung carcinoma cells were maintained as described previously (33). Wild-type (WT) or TCRδ$^{-/-}$ mice received B16F10 or 3LL cells by i.v. injection. Three hours before, mice were injected i.p. with saline or α-GalCer (2 μg/mouse). Mice were killed on day 14, and surface lung metastases were counted with the aid of a dissecting microscope. NK cells were specifically depleted in mice using 200 μg i.p. rabbit anti-asGM1 Ab on days 0, 1, and 7 after tumor inoculation as described (34).

B cell lymphoma model. GFP$^+$ 299/3.2 cells (5×10^4) generated from Eμ−myc transgenic mice were i.v. injected into WT or TCRδ$^{-/-}$ mice. Three hours prior, mice were injected i.p. with saline or α-GalCer (2 μg/mouse). On day 11 after tumor inoculation, mice were bled and the tumor burden was assessed by flow cytometry. Concentration of WBCs in total blood was measured using the automated hematology analyzer Advia 120 (Siemens).

Immunization of mice and analysis of the CD8$^+$ T cell response

WT or TCRδ$^{-/-}$ mice were immunized with OVA (50 μg/mouse) by i.p. injection in the presence or absence of α-GalCer (2 μg/mouse). Spleens were harvested 10 d after, and cells were restimulated with OVA (100 μg/ml) at 37°C for 3 d. Cytokine production was measured by the CBA system.

Statistical analysis

Results are expressed as the means ± SD or means ± SEM. The statistical significance of differences between experimental groups was calculated by a one way-ANOVA with a Bonferroni post test or an unpaired Student *t* test (GraphPad Prism 5 Software, San Diego, CA). The possibility of using these parametric tests was assessed by checking whether the population was Gaussian and the variance was equal (Bartlett test). Results with a *p* value of <0.05 were considered significant.

Results

α-GalCer induces splenic and hepatic γδ T cell activation to produce IFN-γ

To investigate whether γδ T cells participate in the immune cascade elicited by α-GalCer, we investigated in a kinetic manner the activation status of splenic and hepatic γδ T cells. As shown in Fig. 1A, these cells expressed higher levels of CD69 as early as 4 h after α-GalCer injection. We next investigated by intracellular staining the IFN-γ production of γδ T cells. Interestingly, we observed that γδ T cells produced IFN-γ 8 h after α-GalCer administration and sustained this secretion at least for an extra 16 h (Fig. 1B). As a control, we observed that NK cells were activated with similar kinetics to those of γδ T cells. Investigation of other cytokines demonstrated that γδ T cells can also produce IL-17A, but not TNF (Fig. 1C and data not shown). A lack of IFN-γ

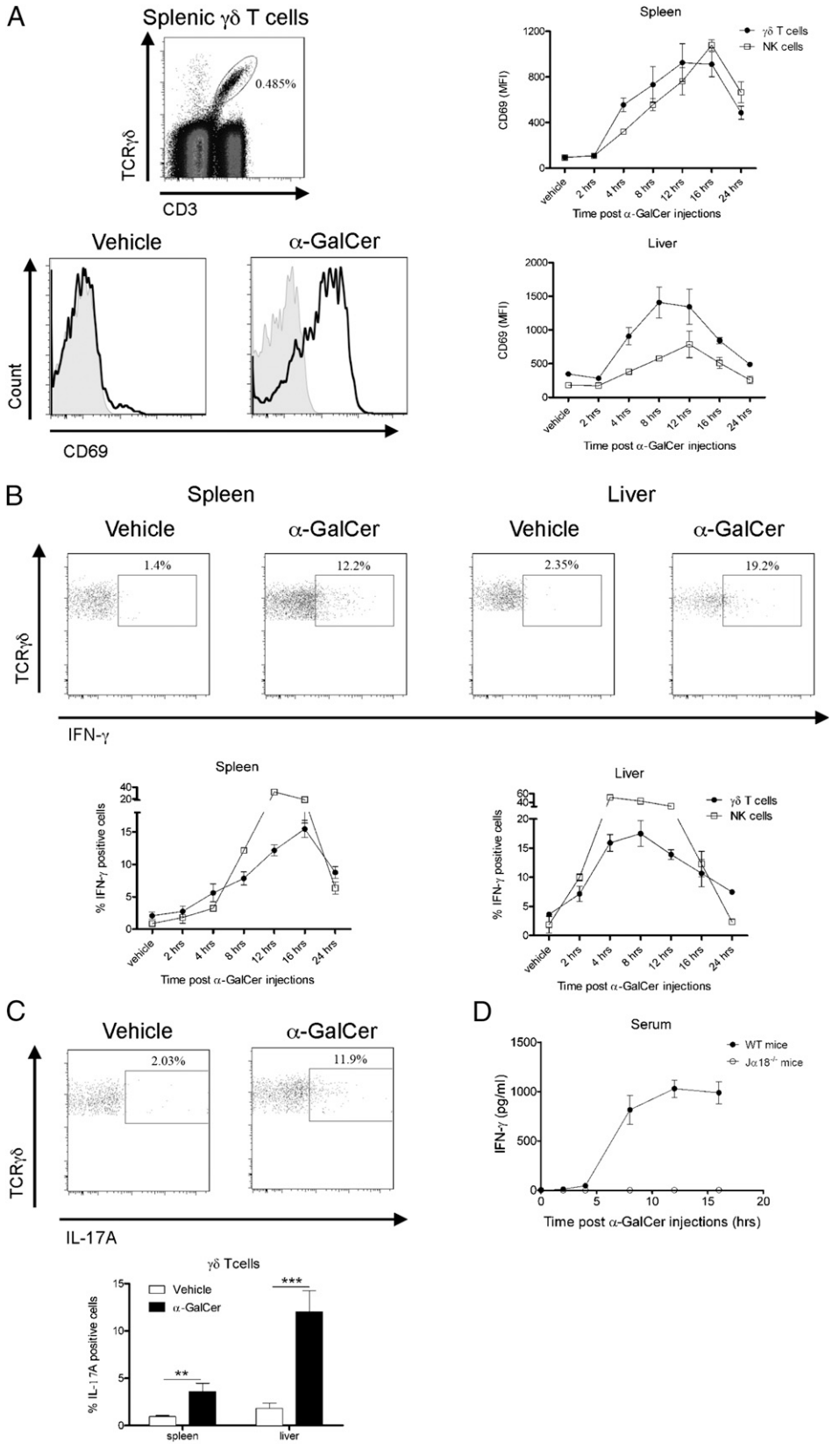

FIGURE 1. Hepatic and splenic γδ T cells produce IFN-γ and IL-17A in vivo after injection of α-GalCer. WT mice were injected i.p. with vehicle or α-GalCer (2 μg/mouse) and were sacrificed at different time points. (**A**) Cells were analyzed by flow cytometry and gated γδ T ($CD3^+$ $TCR\gamma\delta^+$) or NK ($CD3^-NK1.1^+$) cells were screened for surface CD69 expression. The mean fluorescence intensity is indicated. One representative experiment (liver) out of three is shown (*upper panel*). The average ± SEM of three independent experiments is shown in the *right panel* ($n = 9$ mice). (**B** and **C**) Spleen and liver cells were treated with GolgiPlug (or GolgiPlug plus GolgiStop for IL-17A detection) for another 2 h and were labeled with CD3, TCRγδ, and NK1.1 mAbs, fixed, and permeabilized for intracellular cytokine staining. Cells were analyzed by flow cytometry and gated $CD3^+TCR\gamma\delta^+$ cells were screened for intracellular IFN-γ and IL-17A production. Gates were set based on the isotype control. The percentages of cells positive for IFN-γ (B) or IL-17A (C) are represented. One representative experiment of three is shown. (B) The average ± SEM of three independent experiments is shown in the *lower panel* ($n = 9$ mice). (C) The average ± SEM of three independent experiments is shown in the *right panel* ($n = 9$). Differences in mean were analyzed using the two-tailed Student *t* test. $^{**}p < 0.01$, $^{***}p < 0.001$. (**D**) Sera of WT or $J\alpha18^{-/-}$ mice were collected at different time points after α-GalCer administration and levels of IFN-γ were quantified using CBA. Data represent the average ± SD of two independent experiments ($n = 8$ mice/group).

production by either γδ T or NK cells in α-GalCer–treated $J\alpha18^{-/-}$ mice revealed that these cells are likely to be activated in a response downstream of type I NKT cells (Fig. 1D, Supplemental Fig. 1). Additionally, when cocultured with α-GalCer–pulsed DC, sorted γδ T cells did not produce cytokines or enhanced CD69 expression (Supplemental Fig. 1). Similarly, use of α-GalCer/CD1d tetramer failed to stain γδ T cells (Supplemental Fig. 1). Overall, α-GalCer administration leads to IFN-γ production by γδ T cells probably through a bystander effect involving type I NKT cells.

α-GalCer–mediated IFN-γ production by γδ T cells is not restricted to a particular subset

Recent studies have demonstrated that the γδ T cell subsets have diverse functional specializations. This includes the spectrum of cytokines produced, which is regulated by TCR-dependent and -independent mechanisms and the organ studied (reviewed in Refs. 19, 35). For example, the CD27 molecule could be considered as a determinant of γδ T cell differentiation in which the $CD27^+$ subset is mainly associated with a Th1 profile (36). Based on this, we checked IFN-γ production by γδ T cell subsets distinguished by CD27 expression. Surprisingly, we observed that both subsets were able to produce IFN-γ (Fig. 2A), although more $CD27^+$ γδ T cells from spleen secreted IFN-γ compared with their $CD27^-$ counterparts. Similarly, the type of TCR expressed by γδ T cells appears to determine the properties of these cells (35). Thus $V\gamma1^+$ cells produce Th1- and Th2-type cytokines and $V\gamma4^+$

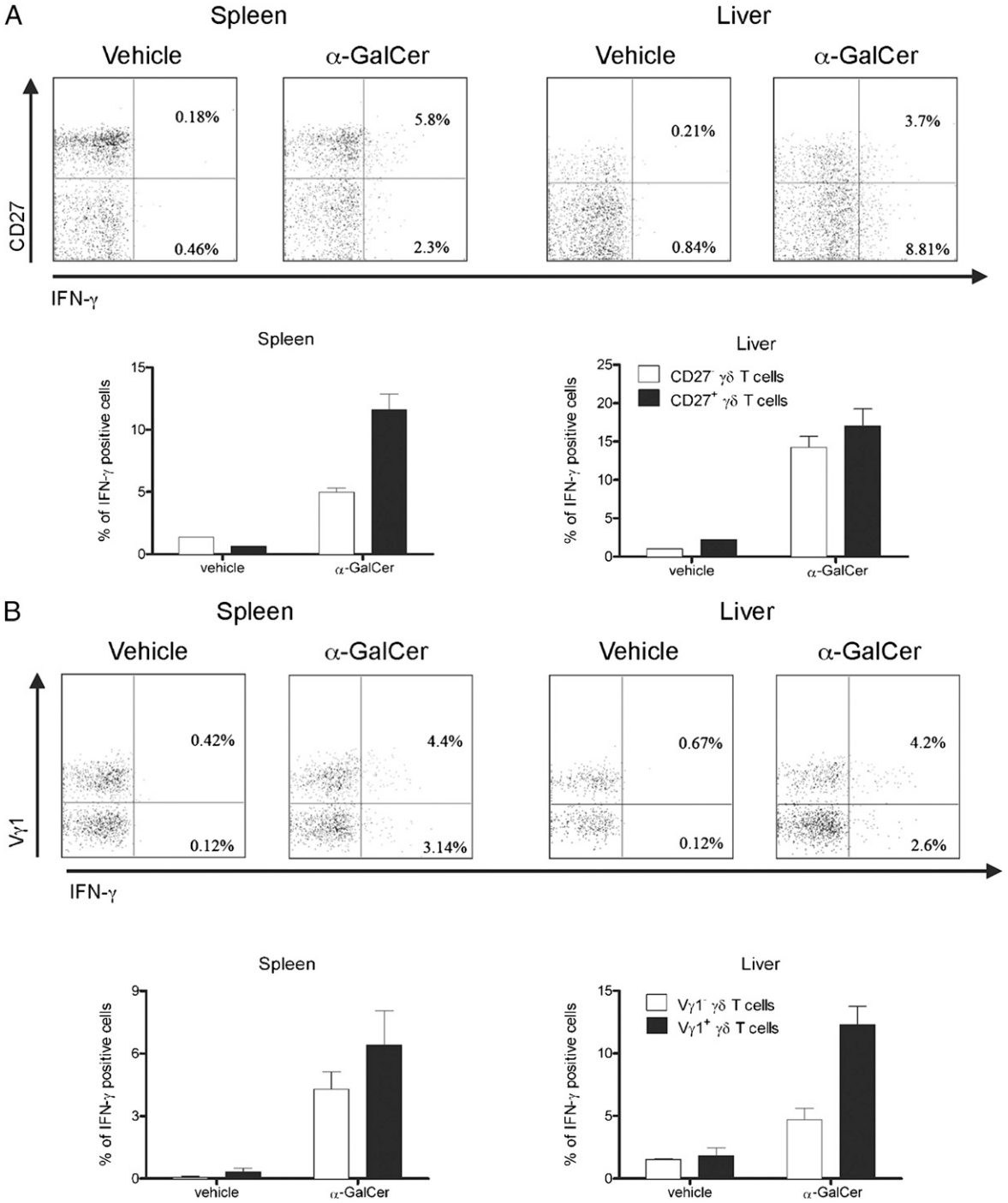

FIGURE 2. IFN-γ production by γδ T cell subsets. (**A** and **B**) WT mice were injected i.p. with α-GalCer (2 μg/mouse) and sacrificed 12 h later. Spleen and liver cells were treated with GolgiPlug for another 2 h and were labeled with CD3, TCRγδ, and CD27 (A) or Vγ1 (B) mAbs, fixed, and permeabilized. Gated $CD3^+TCR\gamma\delta^+CD27^{+/-}$ (A) or $CD3^+TCR\gamma\delta^+V\gamma1^{+/-}$ (B) cells were analyzed for intracellular IFN-γ production. (A) Percentages of either hepatic $CD27^+$ or $CD27^-$ γδ T cells positive for IFN-γ are represented (*upper panel*). The average ± SEM of three experiments is shown in the *lower panel* ($n = 9$ mice). (B) Percentages of hepatic $V\gamma1^+$ or $V\gamma1^-$ γδ T cells positive for IFN-γ are represented (*upper panel*). The average ± SEM of three independent experiments is shown in the *lower panel* ($n = 9$).

cells produce Th17-type cytokines. As shown in Fig. 2B, we observed a preference for IFN-γ production by $V\gamma1^+$ γδ T cells in response to α-GalCer in an organ-dependent manner with >80% of IFN-γ^+ γδ T cells in the liver are $V\gamma1^+$, whereas only 50% of $V\gamma1^+$ cells were the source of IFN-γ in the spleen. This suggests that α-GalCer–induced IFN-γ production by γδ T cells is not entirely restricted to a particular subset bearing a particular Vγ-chain and perhaps some γδ T cell activation is mediated through a TCR-independent mechanism.

IFN-γ production by γδ T cells is fully IL-12p35–dependent and partially IL-18–dependent

The kinetics of γδ T cell activation suggested that these cells are activated downstream of the type I NKT cells. It is well estab-

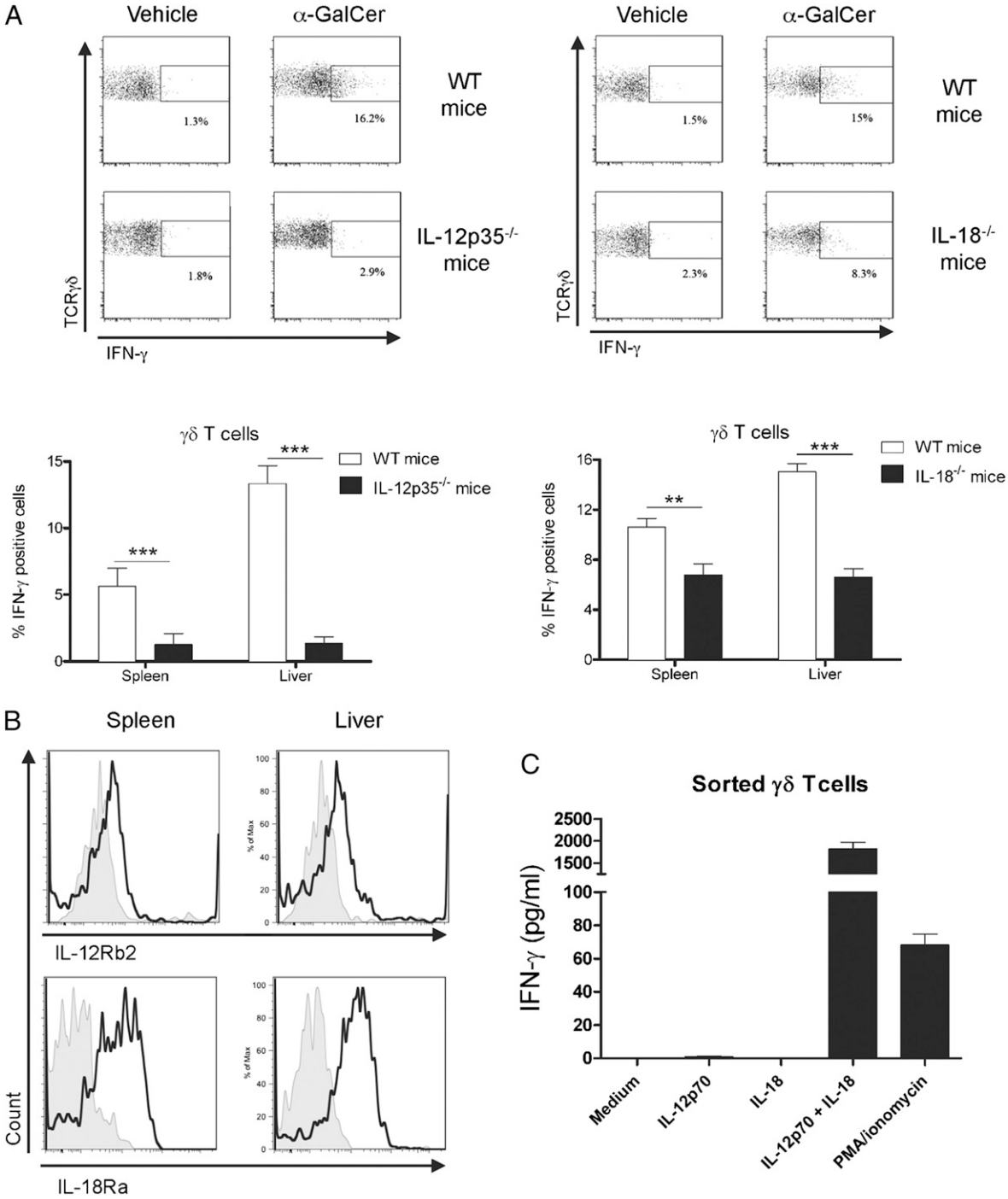

FIGURE 3. IFN-γ production by γδ T cells in response to α-GalCer requires IL-12p35 and IL-18, but not TCR engagement. (**A** and **B**) WT, IL-12p35$^{-/-}$, or IL-18$^{-/-}$ mice were injected i.p. with α-GalCer (2 μg/mouse) and sacrificed 12 h later. Spleen and liver cells were treated with GolgiPlug for another 2 h. Gated CD3$^+$TCRγδ$^+$ cells from vehicle- or α-GalCer–treated WT or IL-12p35$^{-/-}$ mice were analyzed for intracellular IFN-γ production. The percentages of hepatic γδ T cells positive for IFN-γ are represented (*upper panel*). (A) One experiment of two is shown. (B) One experiment of three is shown. The average ± SD of IFN-γ^+ splenic and hepatic CD3$^+$TCRγδ$^+$ cells is shown in the *lower panel* ($n = \geq 8$). Differences in mean were analyzed using the two-tailed Student *t* test. $^{**}p < 0.01$, $^{***}p < 0.001$. (**C**) Expression of IL-12Rβ2 and IL-18Ra has been assessed by flow cytometry on hepatic and splenic γδ T cells. (**D**) Cell-sorted splenic γδ T cells (CD3$^+$TCRγδ$^+$) were incubated with recombinant mouse IL-12 (50 pg/ml) and/or IL-18 (1 ng/ml) proteins. Twenty hours later, cytokine production was measured by CBA. Data represent the mean ± SEM of two independent experiments performed in triplicate.

lished that APC maturation, including DC, is a critical determinant of the immune response elicited by α-GalCer (innate and acquired) (7, 37). Indeed, DC maturation leads to the release of an array of activating cytokines, including IL-12p70 and IL-18, two cytokines known to participate in γδ T cell activation in particular contexts (26, 38). Thus, we next investigated the potential requirement of these cytokines in α-GalCer–induced γδ T cell activation. To address this possibility, we assessed IFN-γ production by splenic and hepatic γδ T cells using IL-12p35$^{-/-}$ and IL-18$^{-/-}$ mice. As depicted in Fig. 3A (*left panel*), IL-12p35 deficiency results in a complete abrogation of γδ T cell activation. Moreover, the lack of IL-18 also significantly reduced splenic and hepatic γδ T cell activation by 38 and 55%, respectively (Fig. 3A, *right panel*). Of note, IFN-γ production by NK cells was also abrogated in IL-12p35$^{-/-}$ mice and reduced in IL-18$^{-/-}$ mice (not shown). Flow cytometric analysis of γδ T cells showed that both hepatic and splenic γδ T cells expressed IL-12Rβ2 and IL-18Ra (Fig. 3B). Finally, to further investigate the involvement of these two cytokines, sorted γδ T cells were treated with recombinant IL-12p70 and/or IL-18. As shown in Fig. 2C, IL-12p70 or IL-18 individually failed to promote IFN-γ synthesis by splenic γδ T cells. In contrast, combined addition of both cytokines induced IFN-γ production by γδ T cells. Taken together, these results indicated the critical role of IL-12p70 and IL-18 in α-GalCer–mediated γδ T cell activation, and that these cytokines were necessary and sufficient to activate γδ T cells.

α-GalCer enhanced γδ T cell cytotoxicity

We have previously shown that α-GalCer can increase cytotoxic properties of NK cells (17, 39), so we tested whether this effect can also be observed for γδ T cells by assessing the expression of different cytolytic effector markers. Interestingly, we observed that, along with NK cells, splenic γδ T cells from α-GalCer–treated mice triggered CD107a degranulation as well as FasL, NKG2D, and granzyme B upregulation when cultured with YAC-1, suggesting increased cytotoxic properties of these cells (Fig. 4A, Supplemental Fig. 2). Of note, in vitro priming of spleen cells with α-GalCer also resulted in an increase expression of cytotoxic

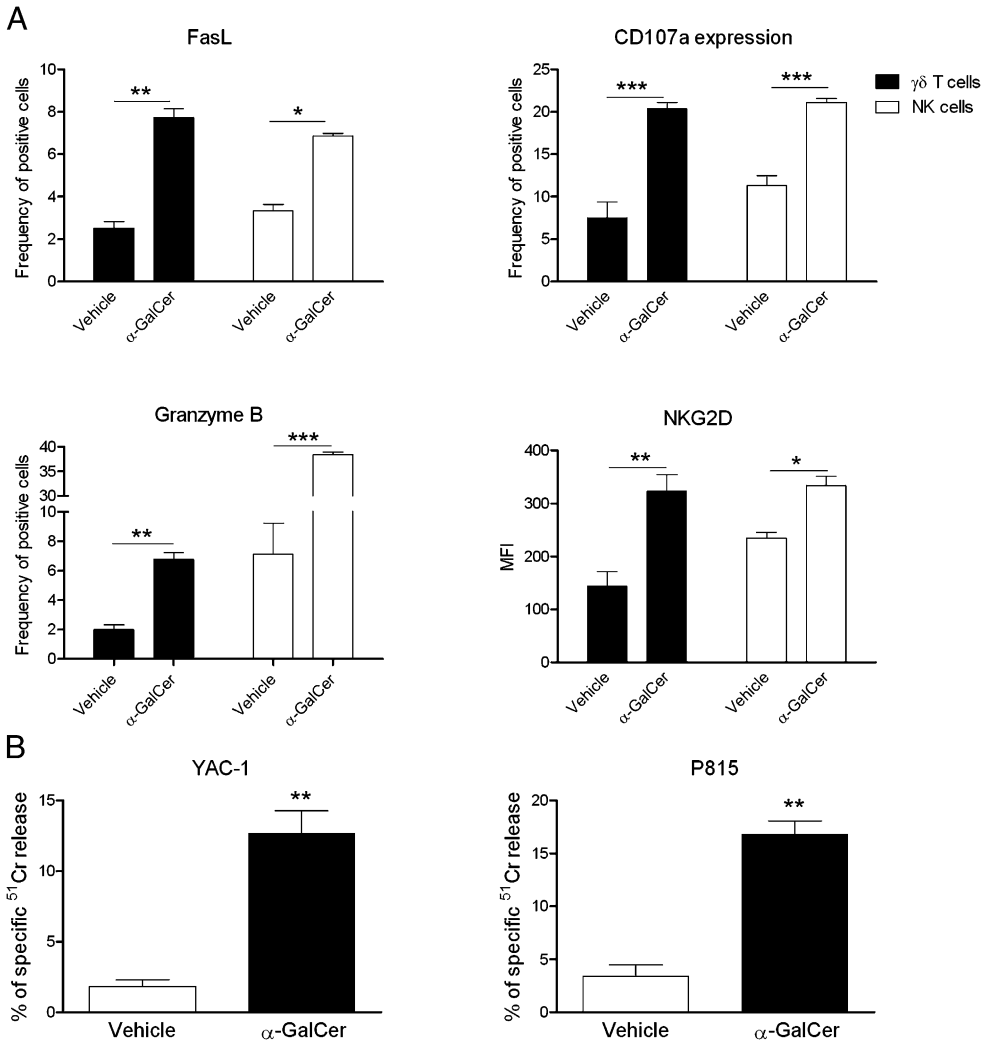

FIGURE 4. α-GalCer treatment in vivo increases cytotoxic properties of γδ T cells. (**A**) WT mice were injected i.p. with vehicle or α-GalCer (2 μg/mouse) and were sacrificed 24 h after. Spleen cells (E) from vehicle- or α-GalCer–treated mice were cocultured with YAC-1 cells (T) at an E:T ratio of 15:1 for 4 h. Surface (CD107a, FasL, and NKG2D) or intracellular (granzyme B) expression of cytotoxic markers was evaluated on/in gated γδ T (CD3$^+$TCRγδ$^+$) and NK (CD3$^-$NK1.1$^+$) cells. The average ± SEM of positive cells (FasL, CD107a, and granzyme B) or mean of fluorescence intensity (NKG2D) for the indicated markers is depicted ($n = 6$ mice). Differences in mean were analyzed using the two-tailed Student t test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (**B**) Purified splenic γδ T cells (CD3$^+$TCRγδ$^+$) from vehicle- or α-GalCer–treated mice were tested for ex vivo cytotoxicity against YAC-1 (*left panel*) or P815 (*right panel*) targets in a standard 200 h [^{51}Cr] release assay at an E:T ratio of 20:1. Each bar is the mean of triplicate wells ± SD of three independent experiments. Differences in mean were analyzed using the two-tailed Student t test. **$p < 0.01$.

markers in/on γδ T cells (Supplemental Fig. 3). To directly test this, we next employed a [^{51}Cr] release assay, using YAC-1 and P815 cells as target cells. We demonstrated that sorted splenic γδ T cells from α-GalCer–treated mice more effectively killed target cells compared with those sorted from control mice (Fig. 4B). A consistent increase in killing activity was observed against these two cell lines (from 2–3 to 12–18%). Taken together, these results demonstrate that α-GalCer induced overexpression of cytotoxic molecules on γδ T cells resulting in an increased ability to kill transformed cells.

Cross-talk between NK cells and γδ T cells

Because NK and γδ T cells displayed a similar behavior (IFN-γ production and cytotoxic properties) after α-GalCer injection, we investigated the potential cross-talk between these two cell populations. Examination of IFN-γ production by hepatic and splenic NK cells in TCRδ$^{-/-}$ mice demonstrated no alterations in the ability of these NK cells to produce cytokines compared with the WT mice (Fig. 5A). In contrast, NK cell depletion prior to α-GalCer treatment significantly reduced IFN-γ production by γδ T cells underlying a role of this population in the bystander activation of γδ T cells (Fig. 5B). Of note, NK cell depletion was consistently >95% (Fig. 5B) and did not affect NKT and γδ T cell compartments (Fig. 5B and data not shown). Because NK cells have been demonstrated to cross-talk with DC, we studied whether the decrease in IFN-γ production by γδ T cells could be due to a reduced DC activation in absence of NK cells. Consistent with this, we observed that levels of IL-12p70 (Fig. 5C, *left panel*) and IL-18 (Fig. 5C, *right panel*) in the sera of NK cell-depleted mice were significantly decreased compared with untreated mice.

γδ T cells partially contribute to the anti-tumoricidal activity of α-GalCer

α-GalCer induces a strong cytokine burst resulting in the secretion of an array of regulatory cytokines. To investigate the potential contribution of γδ T cells in this cytokine cascade, we quantified the level of different cytokines potentially produced by γδ T cells in the serum of α-GalCer–treated TCRδ$^{-/-}$ mice compared with their WT counterparts. As depicted in Fig. 6A, the absence of γδ T cells significantly affects the overall IFN-γ, but not IL-4, production elicited by α-GalCer. Of note, although we could not detect the presence of IL-17A in the serum of α-GalCer–treated mice, in vitro stimulation of spleen cells with α-GalCer led to IL-17A secretion in a γδ T cell-dependent manner (Supplemental Fig. 4). The protective anti-tumor effect of α-GalCer is mainly due to the rapid synthesis of IFN-γ by type I NKT cells and the bystander activation of both NK and CD8$^+$ CTL (15, 16). Thus, we addressed the possibility that the tumoricidal effect of α-GalCer could partially depend on γδ T cells. Using B16F10 melanoma and 3LL Lewis lung carcinoma, we compared the efficacy of prophylactic administration of α-GalCer on pulmonary metastases development in mice lacking γδ T cells compared with control mice. As depicted in Fig. 6B, the anti-metastatic effect of α-GalCer was significantly reduced in the absence of γδ T cells in both models. Of note, in concert with our previous study (17), NK cell depletion completely abrogated the anti-metastatic effect of the α-GalCer. In parallel, we have also addressed this question using a model of hematological malignancy by transplanting a GFP$^+$-B cell lymphoma cell line (299/3.2 clone) generated from Eμ-myc transgenic mice, a model mimicking human non-Hodgkin's lymphomas (40). Interestingly, pretreatment of mice with α-GalCer substantially delayed the development of B cell lymphoma in control mice (Fig. 6C). Once again, this effect was dependent on γδ T cells as the tumor burden was significantly less well controlled in α-GalCer-treated TCRδ$^{-/-}$ mice. Taken together these data demonstrate that γδ T cells are not critical but are required for an optimal tumoricidal effect of α-GalCer.

γδ T cells are required in the promotion of the CD8$^+$ T cell response triggered by α-GalCer

α-GalCer has been demonstrated to promote the development of strong Ag-specific responses by enhancing CD4$^+$ and CD8$^+$ T cell functions as well as B cell maturation in the context of a coadministered protein (12, 41, 42). In this study, we investigated whether γδ T cells could play a part in the α-GalCer–specific enhancement of adaptive immune responses. For that purpose, we immunized WT or TCRδ$^{-/-}$ mice with OVA in presence or absence of α-GalCer. After 10 d, spleen cells from immunized mice were restimulated in an Ag-specific manner and cytokine production was assessed. As expected, OVA restimulation of spleen cells from α-GalCer–treated mice resulted in an enhanced level of both Th1 (IFN-γ) and Th2 (IL-4, IL-5, and IL-13) cytokines compared with mice immunized with OVA alone, even if the pro-Th1 was far more pronounced than the pro-Th2 effect (Fig. 7 and data not shown). Of note, we were unable to detect IL-17A after antigenic restimulation. Interestingly, the Th1-, but not Th2-, promoting effect of α-GalCer was partially decreased in TCRδ$^{-/-}$ mice. Thus, these results indicate that γδ T cells are mandatory in the optimal Th1-promoting effect of α-GalCer on the development of an adaptive immune response.

Discussion

α-GalCer exerts powerful type I NKT cell-dependent immunomodulatory activities that are currently being tested in therapy against different pathologies such as cancer, infections, autoimmunity, or allergy (reviewed in Refs. 3, 43–45). Our data demonstrate the functional importance of γδ T cells in α-GalCer–mediated immune responses and by extension of its protective effect.

First, we show that i.p. administration of α-GalCer leads to splenic and hepatic γδ T cell activation (CD69 overexpression), resulting in IFN-γ production by these cells. Kinetic analysis demonstrates that this cytokine production starts only 4 h after α-GalCer injection and peaks around 8–12 h. Numerous studies have highlighted that individual subsets within the γδ T cell population have more specialized effector functions (reviewed in Refts. 19, 35). However, our analysis failed to precisely identify a specific subset of γδ T cells involved in IFN-γ production. For example, the nature of the TCR expressed, especially the Vγ-chain, is responsible for the functional properties of the γδ T cells in mice (35). We observed that both Vγ1$^+$ and Vγ1$^-$ (including a large proportion of Vγ2$^+$ γδ T cells; data not shown) produced IFN-γ, although Vγ1$^+$ preferentially produced the cytokine in the liver. Similarly, a new classification of thymic and peripheral γδ T cell subsets has been recently proposed (36) in which the TNFR family member CD27 molecule could be considered as a marker in the Th1/Th17 balance. In this study, the CD27$^+$ subset is mainly associated with a Th1 profile, and CD27$^-$ has a Th17 profile. In concert, a rough analysis of IFN-γ production by γδ T cells regarding the expression of CD27 indicates that ~75% of this cytokine was produced by the CD27$^+$ subset. A differential analysis of each subset demonstrates that both hepatic CD27$^+$ and CD27$^-$ γδ T cells produced IFN-γ, indicating no intrinsic properties of the CD27$^+$ subset to secrete IFN-γ in this setting. However, results from the spleen indicated that CD27$^+$ γδ T cells are more capable producers of IFN-γ compared with their CD27$^-$ counterpart. Overall, the absence of a clear preference for CD27$^+$ γδ T cells after α-GalCer could be explained by the fact that we failed to

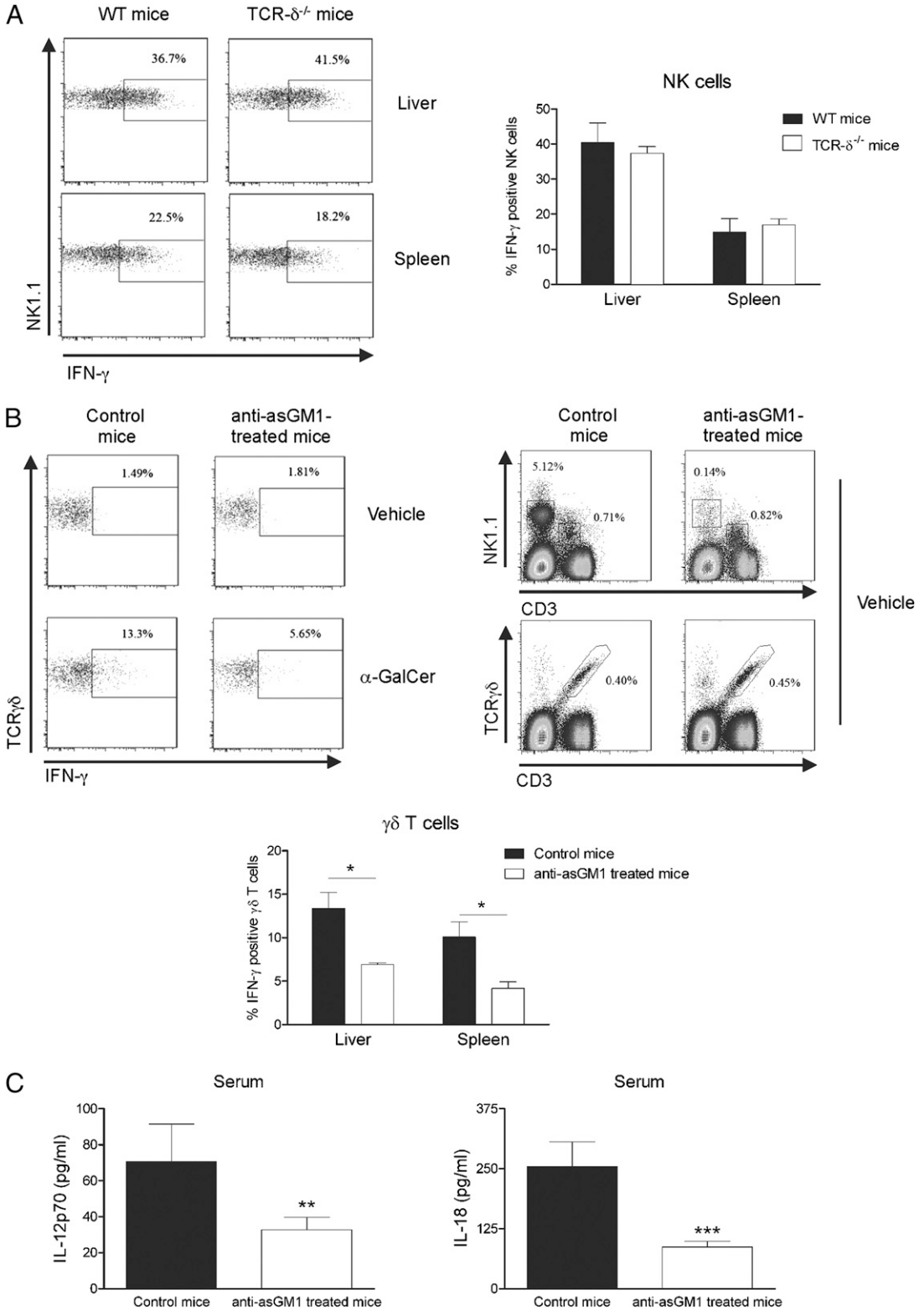

FIGURE 5. NK cells are required for α-GalCer–induced γδ T cell activation. (**A**) WT or TCRδ$^{-/-}$ mice were injected i.p. with vehicle or α-GalCer (2 μg/mouse) and culled 12 h later. Liver and splenic cells were treated for another 2 h with GolgiPlug and gated NK (CD3$^-$NK1.1$^+$) cells were fixed and permeabilized for intracellular IFN-γ staining. Plots represent the percentage of IFN-γ$^+$ NK cells in liver and spleen of α-GalCer–treated animals (*upper panel*) of one representative experiment out of three. The average ± SEM of IFN-γ$^+$ NK cells is shown in the *lower panel* ($n = 9$). (**B**) Control or NK cell-depleted mice were treated as in (A). Plots represent the percentage of IFN-γ$^+$ γδ T (CD3$^+$TCRγδ$^+$) cells in liver and spleen of α-GalCer–treated animals (*upper panel*) of one representative experiment out of two. The average ± SD of two independent experiments is shown in the *lower panel* (n = 6–8). Differences in mean were analyzed using the two-tailed Student t test. *$p < 0.05$. Of note, anti-asGM1 treatment depleted efficiently liver and splenic NK cells (*upper right panel* and data not shown). (**C**) Sera of control or NK cell-depleted mice were collected 12 h after α-GalCer administration and levels of IL-12p70 and IL-18 were quantified using CBA and ELISA kits, respectively. Data represent the average ± SD of two independent experiments (n = 6–8). Differences in mean were analyzed using the two-tailed Student t test. **$p < 0.01$, ***$p < 0.001$.

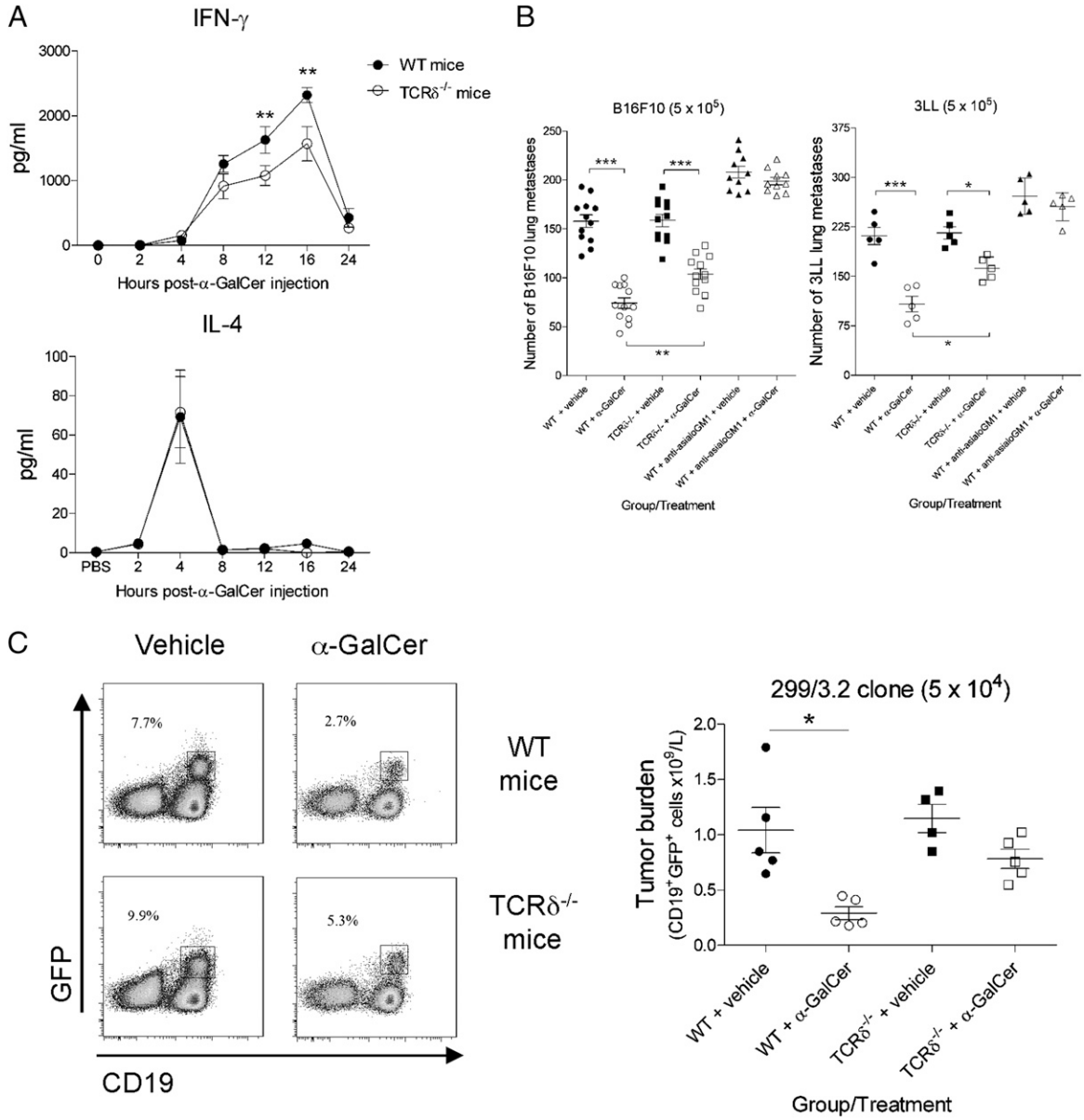

FIGURE 6. γδ T cells participate in α-GalCer–mediated anti-tumor responses. (**A**) Sera of WT or TCRδ$^{-/-}$ mice were collected in a kinetic manner after α-GalCer administration and levels of IFN-γ and IL-4 were evaluated by CBA. Data represent the average ± SEM of three independent experiments (n = 9). (**B**) WT or TCRδ$^{-/-}$ mice were treated i.p. with vehicle or α-GalCer at day 0. Three hours later, mice were inoculated i.v. with 5×10^5 B16F10 cells (*left panel*) or 5×10^5 3LL cells (*right panel*). Fourteen days after tumor inoculation, lungs were harvested and B16F10 or 3LL lung colonies were counted and recorded. Data represent the mean ± SEM of two independent experiments pooled (n = 12) (B16F10) or the mean ± SD of one representative experiment (3LL). Differences in mean were analyzed using a one-way ANOVA. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (**C**) WT or TCRδ$^{-/-}$ mice were treated i.p. with vehicle or α-GalCer at day 0. Three hours later, mice were inoculated i.v. with 5×10^4 B cell lymphoma cells (clone 299/3.2 GFP$^+$) generated from Eμ-myc Tg mice. Eleven days after inoculation, mice were bled and tumor burden was assessed by flow cytometry (GFP$^+$CD19$^+$) and are represented as number of tumor cells per volume of blood ($\times 10^9$/L). Data represent the mean ± SD of one representative experiment out of two (n = 5 mice). Differences in mean were analyzed using a one-way ANOVA. $*p < 0.05$.

detect any modulation of CD70 (CD27 ligand) by flow cytometry on both DC and macrophages after α-GalCer administration (data not shown). This contrasts with upregulation of CD70 mRNA transcripts in CD8α$^+$, but not CD8α$^-$, DC reported using the same model (13).

Using gene-targeted mice, we demonstrated that host IL-12 and IL-18 were important for an optimal IFN-γ production by γδ T cells. Similar to NK cells, it is probable that IL-12 induces IFN-γ production by γδ T cells and IL-18 potentiates the effect of IL-12 by upregulating expression of IL-12R on γδ T cells (46, 47). Moreover, given the observation that γδ T cells expressing diverse TCR produced IFN-γ, we suggest that these cells are probably not able to directly recognize the α-GalCer/CD1d complex. In line with this, we failed to stain γδ T cells with the α-GalCer/CD1d tetramer. When cocultured with α-GalCer–loaded DC, purified γδ T cells could not produce cytokine or enhance activation marker (CD69). IFN-γ production by γδ T cells approximately fits the kinetics of NK cell activation, suggesting that this cytokine production occurs downstream of type I NKT cells. In concert, administration of α-GalCer in Jα18$^{-/-}$ mice fully abrogated IFN-γ production by γδ T cells regardless of the time point analyzed. However, in the absence of commercially available neutralizing Ab against pan-γδ TCR, we cannot definitely rule out the possibility that TCR engagement is required in our model. Nonetheless,

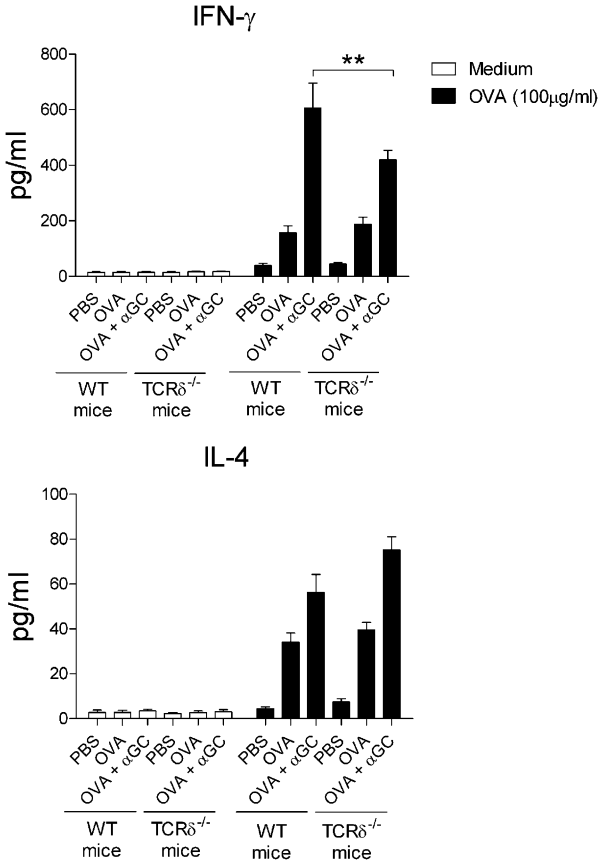

FIGURE 7. α-GalCer promotes OVA-specific Th1 response in a γδ T cell-dependent manner. Spleen cells from WT or TCRδ$^{-/-}$ mice immunized with OVA (50 μg/mouse) in presence or absence of α-GalCer (2 μg/mouse) were restimulated in an Ag-specific manner with OVA (100 μg/ml) for 72 h. Cytokine profile (Th1 versus Th2) was evaluated by CBA. One representative experiment of two is shown (n = 5 mice). A one-way ANOVA has been used to analyze the variance followed by a Bonferroni multiple comparison test to compare all groups of mice. $^{**}p < 0.01$.

our findings suggest that γδ T cells can secrete IFN-γ in response to IL-12 and IL-18 without any TCR signaling. Of note, recent studies have also demonstrated that IL-18 (or IL-1β) and IL-23 can lead to Th17 cytokine secretion by γδ T cells without TCR engagement (26, 28). Moreover, it is still possible that along with these two cytokines, other TCR-independent factors, including others cytokines, TLR agonists, Ig, or TNFR superfamily coreceptors (48, 49), could influence IFN-γ production by γδ T cells.

Interestingly, we have also highlighted the ability of γδ T cells to produce IL-17A in response to α-GalCer. The role of IL-17A in host defense against pathogens including bacteria, fungus, and parasites is well documented essentially through the ability of this cytokine to induce neutrophil recruitment (50, 51). However, the potential contribution of this cytokine in the beneficial role of α-GalCer is poorly understood. For instance, a protective role of host IL-17 has been shown in α-GalCer–induced acute hepatitis (52). As suggested by our in vitro results, it is still possible that along with NKT cells, IL-17A–producing γδ T cells significantly participate in this cytokine production and by extension in the beneficial effect of this cytokine in this setting. However, additional investigations will be required to address this point.

Our analysis of γδ T cell activation indicates these cells behave like NK cells (mechanisms of activation, IFN-γ production, and increase cytotoxicity) after α-GalCer administration. Nevertheless, we consistently observed that IFN-γ production by NK cells peaked slightly earlier than γδ T cells. To investigate whether this difference could be explained by an influence of NK cells on γδ T cell activation, we studied IFN-γ production of γδ T cells in absence of NK cells. Interestingly, we observed that both splenic and hepatic γδ T cells from anti–asGM1-treated mice produced far less IFN-γ compared with controls. Because NK cells have been proven to participate in DC maturation (46), the slightly earlier production of IFN-γ by these cells could amplify DC maturation, including cytokine production, initially engaged through their interaction with type I NKT cells and in turn be part of γδ T cell transactivation in our setting. In line with this, levels of IL-12p70 and IL-18 in sera of anti–asGM1-treated mice were significantly reduced compared with control mice. Nevertheless, NK cell depletion only partially reduced IFN-γ production by γδ T cells, indicating that DC maturation engaged in cross-talk with type I NKT cells was sufficient to activate γδ T cells, and subsequently NK cell-enhanced cytokine production (e.g., IL-12 and IL-18) by DC led to optimal IFN-γ secretion. In agreement with this proposed scenario of activation kinetics (type I NKT/NK/γδ T cells), the absence of γδ T cells did not modulate the ability of NK cells to produce IFN-γ, suggesting no feedback loop to NK cells.

We have already demonstrated the capacity of α-GalCer to increase NK cell cytotoxicity (17). In this study, we have also shown that α-GalCer treatment leads to an increase in the cytotoxicity mediated by splenic γδ T cells. Indeed, when cocultured with target cells, γδ T cells from α-GalCer–treated mice modulated their phenotype by increasing NKG2D, FasL, CD107a, and granzyme B, whereas those from vehicle-treated mice failed to do so. This is a feature also observed after in vitro γδ T cell priming with α-GalCer. Even if γδ T cells are less capable of exerting cytotoxicity compared with NK cells, this observation, combined with their ability to produce IFN-γ, led us to investigate the potential role of γδ T cells in the anti-tumor effect of α-GalCer. Using mouse models of lung metastases and B cell lymphoma, we show that γδ T cells are required for the full anti-tumor activity of α-GalCer.

Finally, while confirming that α-GalCer enhanced adaptive Th1 immunity to a coadministered protein (7, 17, 42), we have also highlighted the pivotal role of γδ T cells in the Th1 arming. Despite a key role for DC, CD40, IFN-γ, and TNF-α being proposed after α-GalCer (7, 41), the contribution of other cellular components of the immune system have never been studied. The reasons why γδ T cells can contribute to the development of a strong Th1 adaptive immune response remain elusive. Because IFN-γ can enhance MHC class I Ag processing and presentation via JAK/STAT1 signaling and so in turn potentiate APC functions (53), the early IFN-γ production by γδ T cells could, along with type I NKT and NK cells, potentially have an impact at this level. Furthermore, past studies have shown that activated human and mouse γδ T cells acquired Ag-presenting functions, including MHC class II and CD40 expression (25, 54). This conversion into APC could also partially explain our results. The potential involvement of γδ T cells in the Ab response downstream of α-GalCer has not been addressed in our study. Nevertheless, IFN-γ has been proposed to be a key factor for IgG2a, but not IgG1, production in mice immunized with α-GalCer and proteins (12). In this context, we can speculate that γδ T cells may play a role in B cell response triggered by α-GalCer and coinjected proteins. This work underlies the role of γδ T cells in α-GalCer–mediated immune responses and highlights how the NKT/NK/γδ T cell axis is important in the development and regulation of innate and acquired immune responses.

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Disclosures

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