

# A nonclassical non-V $\alpha$ 14J $\alpha$ 18 CD1d-restricted (type II) NKT cell is sufficient for down-regulation of tumor immunosurveillance

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**The importance of immunoregulatory T cells has become increasingly apparent. Both CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD1d-restricted NKT cells have been reported to down-regulate tumor immunity in mouse tumor models. However, the relative roles of both T cell populations have rarely been clearly distinguished in the same tumor models. In addition, CD1d-restricted NKT cells have been reported to play a critical role not only in the down-regulation of tumor immunity but also in the promotion of the immunity. However, the explanation for these apparently opposite roles in different tumor models remains unclear. We show that in four mouse tumor models in which CD1d-restricted NKT cells play a role in suppression of tumor immunity, depletion of CD4<sup>+</sup>CD25<sup>+</sup> T cells did not induce enhancement of immunosurveillance. Surprisingly, among the two subpopulations of CD1d-restricted NKT cells, V $\alpha$ 14J $\alpha$ 18<sup>+</sup> (type I) and V $\alpha$ 14J $\alpha$ 18<sup>-</sup> (type II) NKT cells, type I NKT cells were not necessary for the immune suppression. These unexpected results may now resolve the paradox in the role of CD1d-restricted NKT cells in the regulation of tumor immunity, in that type II NKT cells may be sufficient for negative regulation, whereas protection has been found to be mediated by  $\alpha$ -galactosylceramide-responsive type I NKT cells.**

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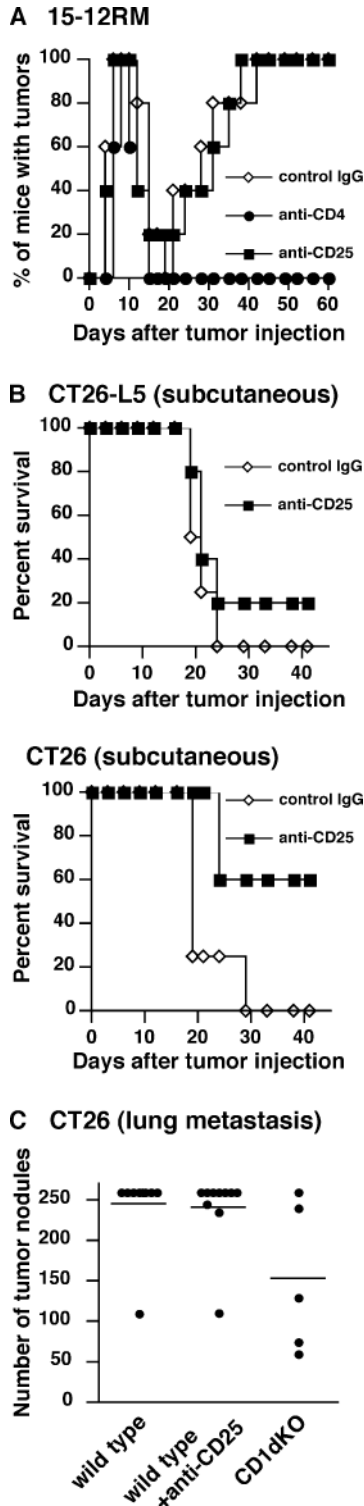
Although cancer vaccines and immunotherapy have become more successful at inducing CTL responses specific for the tumor, a remaining hurdle is to translate this success into actual tumor regression. It has come to light in recent years that a major reason that CTL activity measurable *in vitro* does not cause tumor regression *in vivo* is the existence of negative regulatory mechanisms inhibiting the CTL response. These include negative regulatory molecules on the T cells themselves, such as CTL antigen-4 (1) and program death-1 (2), as well as regulatory cells. In this context, there has been much interest in the CD4<sup>+</sup>CD25<sup>+</sup> T regulatory (T reg) cell (3, 4). However, there are other immunoregulatory cells, including CD1d-restricted NKT cells (5, 6), myeloid suppressor cells (7, 8), and M2 macrophages

(9, 10). Although all of these cells may contribute to the failure of vaccine-induced T cells to destroy tumors, little is understood about the relative importance of these different mechanisms.

NKT cells are a unique T cell subset expressing both TCR and NK cell receptors. Most NKT cells are restricted by the MHC class I-like molecule CD1d. These CD1d-restricted NKT cells are known to include two subsets: the better characterized, more widely studied one expressing the canonical TCR, V $\alpha$ 14J $\alpha$ 18 (type I) NKT cells, and a less well characterized one of non-V $\alpha$ 14J $\alpha$ 18 (type II) NKT cells (11). In the mouse, most CD1d-restricted NKT cells are CD4<sup>+</sup> and the rest are CD4<sup>-</sup>CD8<sup>-</sup>. CD1d-restricted NKT cell function has been implicated in several tumor models (12). Activation of the type I NKT cells by administration of its strong agonist  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer) is beneficial in several

M. Terabe and J. Swann contributed equally to this work.

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**Figure 1. T reg cells are not necessary for down-regulation of tumor immunosurveillance.** (A)  $10^6$  15-12RM tumor cells were injected subcutaneously into BALB/c mice on day 0. 1 mg anti-CD25 (PC61; closed squares) or control rat IgG (open diamonds) was injected i.v. on days -4 and -2. 500  $\mu$ g anti-CD4 (GK1.5; closed circles) was injected i.p. on days 0, 1, 2, 6, and 10. The results are representative of two experiments ( $n = 5$ ). (B) T reg cell depletion is effective against subcutaneous CT26 but not

experimental tumor models, especially in preventing metastatic growth (13, 14).

Besides their ability to mediate antitumor effects, type I NKT cells enhance natural immunosurveillance in the absence of exogenously added ligands. In the TC-1 tumor model, type I NKT cells were beneficial in stimulating early adaptive immunity to tumor cells (15). In a methylcholanthrene-induced sarcoma model, type I NKT cells play a role in tumor immunosurveillance, as  $J\alpha 18$  KO mice—which specifically lack type I NKT cells—develop sarcomas faster than their wild-type counterparts. Tumors arising in methylcholanthrene-treated  $J\alpha 18$  KO mice were rejected in a type I NKT cell-dependent manner when transplanted into wild-type hosts (16), and adoptive transfer of type I NKT cells into  $J\alpha 18$  KO mice provided potent protection against these tumors (17).

In contrast, it has also been reported that CD1d KO mice—which lack CD1d-restricted NKT cells—have heightened immunity to tumors (18). The fibrosarcoma 15-12RM, which shows spontaneous regression, after initial growth of the tumor followed by recurrence in wild-type mice, failed to recur in CD1d KO mice (5). CD1d KO mice also developed fewer tumor nodules in the lungs than wild-type mice when the syngeneic tumor CT26 was injected i.v. (19). In these tumor models, a negative regulatory NKT cell was found to initiate a novel regulatory circuit involving IL-13, myeloid cells, and TGF- $\beta$  (6). Likewise, CD1d KO mice were considerably more resistant to spontaneous 4T1 tumor metastases than wild-type mice in a post-surgery setting (20). These contrasting results define a paradox in which NKT cells appear to both protect against tumors and inhibit tumor immunosurveillance.

We found that depletion of CD4<sup>+</sup> T cells in several of these tumor models removed a regulatory cell and thereby allowed CD8-mediated immunosurveillance to prevent tumor recurrence or metastases. We identified one such regulatory cell as a CD4<sup>+</sup> CD1d-restricted NKT cell that was absent in CD1d KO mice. However, those studies did not exclude the possible additional role of the T reg cell that has also been shown to suppress tumor immunity (3, 4). We also did not examine the role of CD1d in this process and could not further characterize the nature of the regulatory NKT cell because of the lack of tools, such as  $J\alpha 18$  KO mice on the BALB/c background, and little appreciation of the existence or function of type II NKT cells in normal mice at the time

CT26-L5 variant tumors. Mice challenged subcutaneously with  $10^4$  CT26 or CT26-L5 cells were given 1 mg PC61 i.p. on days -4 and -1 of tumor challenge.  $n = 5$  for all groups except those receiving control Ig, in which  $n = 4$ . Mice were culled when tumors exceeded 1 cm in diameter, and data are represented as percent survival. (C)  $5 \times 10^5$  CT26 tumor cells were injected i.v. into BALB/c mice on day 0. 1 mg anti-CD25 was injected i.v. on days -5 and -3. On day 14 or 15, mice were killed and pulmonary tumor metastases were enumerated. CD1d KO mice had a significantly smaller number of metastases ( $P < 0.002$  by one-way ANOVA). The results are pooled from two independent experiments, and additional results from these experiments are shown in Fig. 4 C. The horizontal lines indicate the means.

(21, 22). We have therefore now examined several tumor models in which this NKT regulatory cell plays a role to determine (a) whether the T reg cell also inhibits immunosurveillance in these systems, (b) whether the CD1d molecule must present antigen to activate the regulatory cell or is simply required for NKT cells to develop during ontogeny, and (c) whether the regulatory NKT cell is the same as the type I NKT cell ( $\alpha$ -GalCer responsive) that has also been shown to protect against tumors or is a different, type II NKT cell that does not respond to  $\alpha$ -GalCer.

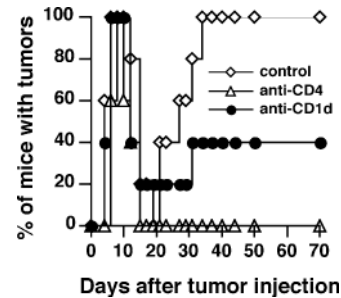
## RESULTS AND DISCUSSION

### T reg cells are not necessary for down-regulation of tumor immunosurveillance

We began by examining the role of T reg cells in down-regulation of immunosurveillance in tumor models in which CD1d-restricted NKT cells have been shown to play a critical role in immune suppression. Four different mouse tumor models—the subcutaneous 15-12RM fibrosarcoma, the subcutaneous CT26-L5 colon carcinoma, the 4T1 mammary carcinoma inoculated in the mammary gland, and the lung metastases of the CT26 colon carcinoma inoculated i.v.—were used in our several laboratories. Syngeneic BALB/c mice were treated with anti-CD25 antibody before tumor challenge (Fig. 1). In the subcutaneous tumor models, 15-12RM and CT26-L5, depletion of T reg cells had no impact on tumor growth, whereas CD4 depletion reduced tumor growth (Fig. 1, A and B). In contrast, as previously reported (23), anti-CD25 treatment was effective against subcutaneous parental CT26 in a side-by-side comparison with the CT26-L5 variant (which also serves as a positive control for the efficacy of anti-CD25 treatment; Fig. 1 B). The failure to observe a role for T reg cells in the CT26-L5 model may be caused by differences in the tumor lines. This idea is supported by the fact that the CT26 grows in CD1d KO mice in which the CT26-L5 does not grow (Fig. 4 B; unpublished data). In the CT26 lung metastasis model, we also could not see any effect of anti-CD25 treatment on the number of lung metastases (Fig. 1 C). We confirmed that these results were not caused by the failure of T reg cell depletion by observing that only  $<0.12\%$  of spleen cells were CD25<sup>+</sup> even 13 d after the final treatment with anti-CD25 (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20051381/DC1>). Although depletion of T reg cells had no effect on CT26 metastases, the absence of NKT cells in CD1d KO mice led to a reduced number of metastases ( $P < 0.002$ ) in this tumor model (Fig. 1 C), as we previously observed (19). Together with our previous observations that anti-CD25 treatment had no effect on primary growth or metastases of the 4T1 tumor (20), these results suggest that T reg cells are not necessary for down-regulation of immunosurveillance in any of those tumor models.

### Activation of NKT cells through CD1d is necessary for down-regulation of tumor immunosurveillance

We and others have shown that NKT cell-deficient CD1d KO mice are highly resistant to tumor growth (Fig. 1 C) (5,



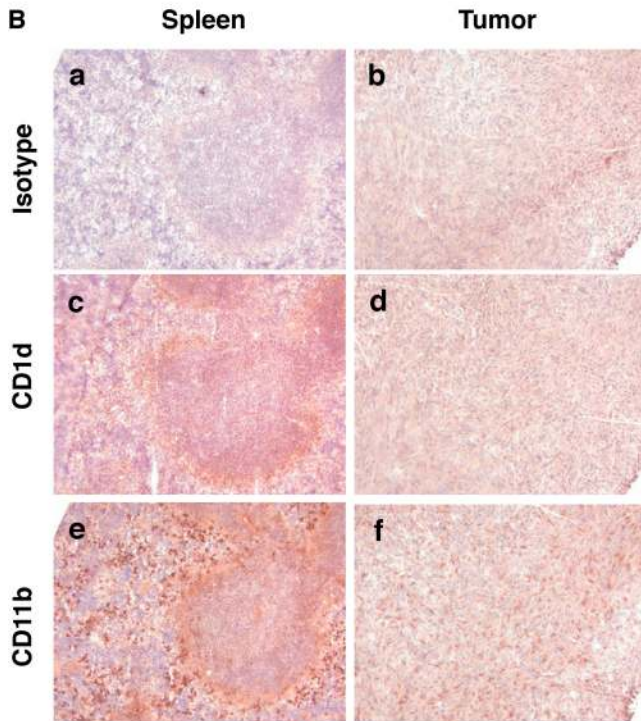
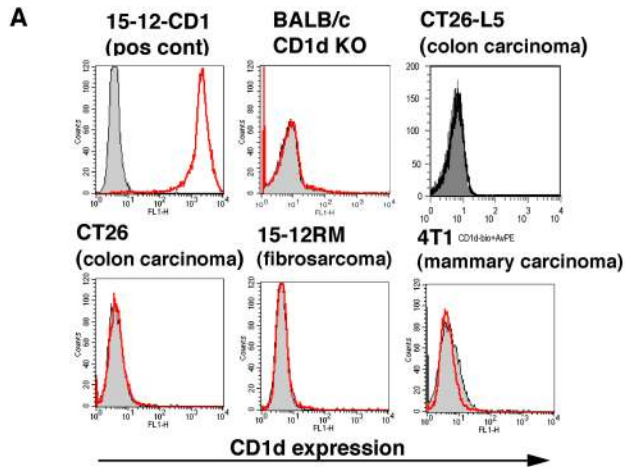
**Figure 2. Antigen presentation by CD1d is necessary for down-regulation of tumor immunosurveillance.**  $10^6$  15-12RM tumor cells were injected into BALB/c mice subcutaneously on day 0. 200  $\mu$ l anti-CD1d ascites (closed circles) was injected i.v. every other day for 1 wk from day 0. 500  $\mu$ g anti-CD4 (open triangles) was injected i.p. on days 0, 1, 2, 6, and 10.  $P < 0.05$  by log-rank test between control and anti-CD1d-treated or anti-CD4-treated groups. The results are representative of two experiments ( $n = 5$ ).

18–20). CD1d may simply be necessary during ontogeny for NKT cell development or may also be necessary to induce immunoregulatory activity of NKT cells. To confirm that the resistance observed in CD1d KO mice is not a result of any defects, besides the lack of NKT cells, caused by genetic disruption of the *cd1d* gene and to test whether CD1d is necessary to activate NKT cells to display regulatory activity, we blocked antigen presentation by a nondepleting anti-CD1d antibody *in vivo* in the 15-12RM tumor model (Fig. 2). Although the treatment did not completely protect mice from tumor recurrence, the mice were significantly protected ( $P < 0.05$ ). Thus, NKT cells need to be activated through CD1d to down-regulate tumor immunosurveillance.

To exclude the possibility that suppression of tumor growth in CD1d KO mice is simply the result of CD1d on tumor cells acting as a rejection antigen, we also examined the expression of CD1d. CD1d could not be detected on the surface of any of the tumors used by flow cytometry (Fig. 3 A), and immunohistochemical analysis of tissue sections from CT26-L5 tumors failed to detect CD1d expression *in vivo* (Fig. 3 B). Thus, inhibition of tumor growth in CD1d KO mice is unlikely to be caused by an immune response to the CD1d antigen. Although immunolabeling techniques might not detect extremely low levels of CD1d, the conclusion that CD1d is not simply acting as a rejection antigen is also supported by the protection afforded by anti-CD1d antibody in wild-type BALB/c mice (Fig. 2), which cannot be explained by lack of tolerance to CD1d, and by the enhancement of tumor rejection by CD4 depletion but not CD25<sup>+</sup> T reg cell depletion.

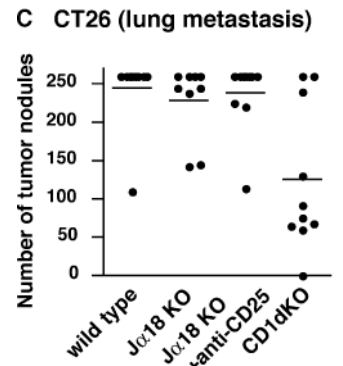
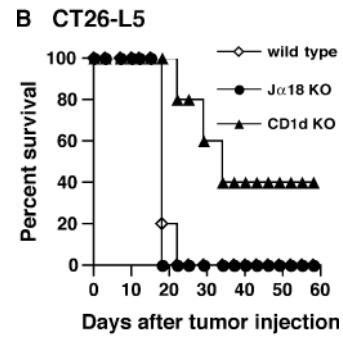
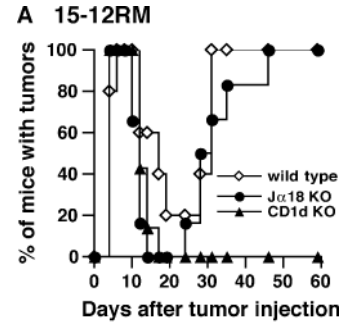
### Non- $V\alpha 14J\alpha 18^+$ (type II) NKT cells in the absence of $V\alpha 14J\alpha 18^+$ (type I) NKT cells are sufficient for down-regulation of tumor immunosurveillance

The ability of CD1d KO mice and of blockade of CD1d to spontaneously suppress tumor growth in multiple tumor



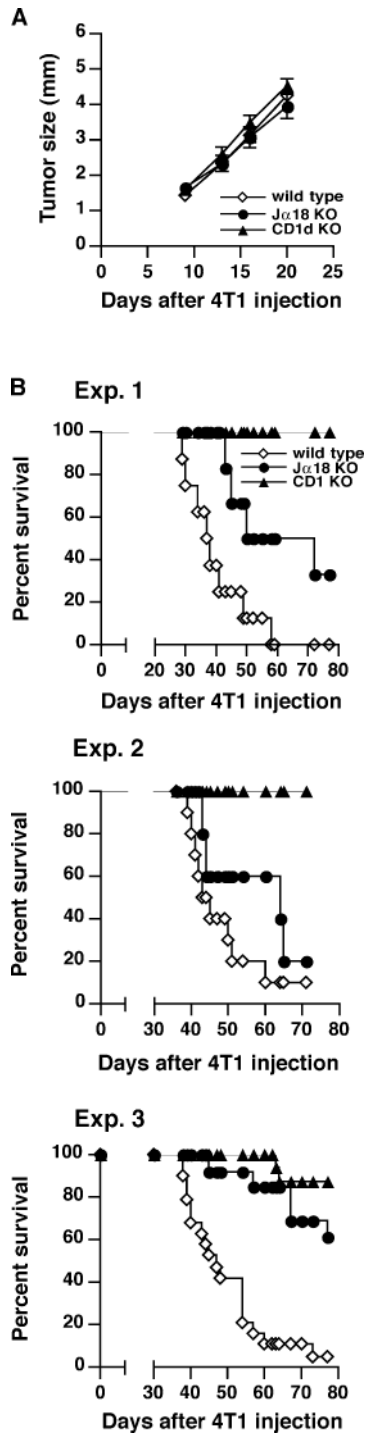
**Figure 3. Tumor cell lines do not express CD1d in vitro or in situ.** (A) 15-12-CD1d (CD1d-transfected positive control), 15-12RM, CT26, 4T1, and CT26-L5 tumor cell lines and spleen cells of CD1d KO mice (negative control) were stained with FITC-labeled anti-CD1d mAb and analyzed by flow cytometry. Shaded graphs indicate cells stained with isotype-matched control antibody; red and bold lines indicate cells stained with anti-CD1d antibody. (B) Frozen sections of subcutaneous CT26-L5 tumors (b, d, and f) or spleens (a, c, and e) were stained with control IgG (a and b), anti-CD1d (c and d), or anti-CD11b (e and f). The sections were counter stained with hemotoxylin ( $\times 100$ ).

models suggested that a CD1d-dependent mechanism suppressed the generation of effective antitumor immune responses to some tumors. As the most obvious defect in CD1d KO mice is the lack of  $V\alpha 14J\alpha 18^+$  (type I) NKT



**Figure 4.  $V\alpha 14J\alpha 18^+$  (type I) NKT cells are not necessary for down-regulation of tumor immunosurveillance.** (A) The wild-type (open diamonds), CD1d KO (closed triangles), and  $J\alpha 18$  KO (closed circles) BALB/c mice were injected subcutaneously with  $10^6$  15-12RM tumor cells on day 0, and tumor size was monitored over time.  $P < 0.05$  by log-rank test between CD1d KO and wild-type or  $J\alpha 18$  KO mice. The results are representative of two experiments ( $n = 5$ ). (B) The wild-type, CD1d KO, and  $J\alpha 18$  KO BALB/c mice were inoculated subcutaneously with  $10^4$  CT26-L5 cells and subsequently monitored for survival ( $n = 5$ ). Data are representative of two experiments. (C) The wild-type, CD1d KO, and  $J\alpha 18$  KO BALB/c mice were injected i.v. with  $5 \times 10^5$  CT26 cells. After 14 d, pulmonary metastases were enumerated. In one group, mice were treated with PC61 as in Fig. 1 C. CD1d KO mice had significantly fewer metastases ( $P < 0.002$  by one-way ANOVA). The results are pooled from two independent experiments. The horizontal lines indicate the means. The data in Fig. 4 C and in Fig. 1 C were all obtained within the same larger experiments.

cells, we investigated the role of these cells in down-regulation of tumor immunosurveillance by growing four different tumors—15-12RM, 4T1, CT26, and CT26-L5—in  $J\alpha 18$  KO mice, which specifically lack type I NKT cells but ex-



**Figure 5.**  $\nu\alpha 14\text{J}\alpha 18^+$  (type I) NKT cells are not necessary for down-regulation of tumor immunosurveillance against lung metastases of 4T1 breast tumors, and primary tumors are not affected by CD1d-restricted NKT cells. (A) The wild-type (open diamonds), J $\alpha$ 18 KO (closed circles), and CD1d KO (closed triangles) BALB/c mice were inoculated in the mammary gland on day 0 with 7,000 4T1 tumor cells, and mice were followed for primary tumor growth. Data are from seven J $\alpha$ 18 KO, seven wild-type, and three CD1d KO mice. (B) The wild-type (open diamonds), CD1d KO (closed triangles), and J $\alpha$ 18 KO (closed circles) BALB/c mice were injected with 7,000 4T1 cells in the mammary fat pad.

press CD1d (Fig. 4). Surprisingly, in contrast to CD1d KO mice, growth of 15-12RM (Fig. 4 A) and CT26-L5 (Fig. 4 B) tumors in J $\alpha$ 18 KO mice was similar to that in wild-type mice. J $\alpha$ 18 KO mice also developed >250 lung metastases in the CT26 lung metastasis model, which was comparable to levels seen in wild-type mice, whereas CD1d KO mice developed significantly fewer metastases ( $P < 0.002$ ; Fig. 4 C). In the 4T1 model, there was no apparent difference in the size of primary tumors among wild-type, CD1d KO, and J $\alpha$ 18 KO mice (Fig. 5 A). However, CD1d KO mice showed a significantly higher ( $P < 0.02$ ) survival rate after surgical removal of primary tumors compared with wild-type BALB/c mice. In this model, J $\alpha$ 18 KO mice showed significantly shorter ( $P < 0.02$ ) survival than the CD1d KO mice in all three experiments even though they also had greater survival than wild-type mice in two of three experiments, suggesting that in this model type I NKT cells also may play some role in the suppression (Fig. 5 B). These results demonstrated that type I NKT cells were not necessary for down-regulation of tumor immunosurveillance against these tumors and that type II NKT cells may be sufficient for complete or partial suppression, whereas type I NKT cells may play a minor role in the 4T1 mammary carcinoma. The difference in the role of type I NKT cells in the 4T1 model may be partly caused by the use of different cytokines and a different mechanism downstream of NKT cells to suppress tumor immunity compared with the 15-12RM and CT26 tumors (9, 20).

We further examined the role of T reg cells in the absence of type I NKT cells in the CT26 lung metastasis model. J $\alpha$ 18 KO mice were treated with PC61 mAb before the tumor challenge as in Fig. 1. The depletion of CD4<sup>+</sup> CD25<sup>+</sup> cells in J $\alpha$ 18 KO mice did not affect the development of pulmonary metastases (Fig. 4 C), just as we observed in wild-type mice. Thus, in the absence of both type I NKT cells and T reg cells, type II NKT cells are sufficient to suppress immunosurveillance.

Differences between the two types of CD1d-restricted NKT cells have been reported (21, 22, 24). In autoimmune disease, graft rejection, and allergy, type I NKT cells have been found to be immunosuppressive (25, 26), whereas in hepatitis (27) and colitis (28), type II NKT cells have been implicated in tissue destructive processes. However, their functional difference in regulation of tumor immunity was unknown. We showed that type II NKT cells were sufficient to suppress tumor immunosurveillance in multiple mouse tumor models. There are two possible explanations for these results. First, either type II or type I NKT cells are each sufficient for down-regulation of tumor immunosurveillance. Thus, eliminating either population alone will not

Primary 4T1 tumors were surgically removed 14 d after tumor injection, and mice were subsequently monitored for survival. In all three experiments, CD1d KO mice survived significantly longer than wild-type and J $\alpha$ 18 KO mice ( $P < 0.02$  by log-rank test). In experiments 1 and 3, J $\alpha$ 18 KO mice survived significantly longer than wild-type mice ( $P < 0.02$ ).

relieve the immune suppression. Second, type II NKT cells are the primary type of NKT cell that regulates immunosurveillance to induce immune suppression. Existing reagents do not allow these two possibilities to be distinguished because there are no unique markers identified for type II NKT cells. Also, recent findings in inflammatory bowel disease suggest that human type II NKT cells can make IL-13 (28). Thus, the findings we report in this paper reconcile previous observations ascribing opposing functions in tumor immunity to the same NKT cells and, thus, may resolve an apparent paradox. Furthermore, these experiments also identify an important regulatory cell that operates in tumor models in which the T reg cell plays little or no role.

## MATERIALS AND METHODS

**Mice.** Inbred BALB/c mice were purchased from the Frederick Cancer Research Facility or the Walter and Eliza Hall Institute of Medical Research or bred in the University of Maryland, Baltimore County animal facility from stock mice obtained from the Jackson Laboratory. BALB/c CD1d-deficient mice (CD1d KO mice; provided by M. Grusby, Harvard University, Boston, MA or by L. van Kaer, Vanderbilt University, Nashville, TN) and BALB/c  $\alpha$ 18-deficient mice ( $\alpha$ 18 KO mice; provided by M. Taniguchi and T. Nakayama, Chiba University, Chiba, Japan or by D. Umetsu, Stanford University, Stanford, CA) were bred at the National Cancer Institute, the Peter MacCallum Cancer Centre, and the University of Maryland. All gene-targeted mice were backcrossed onto the BALB/c background for 10 generations and genotyped by both PCR and flow cytometry. Mice >6 wk old were used in all experiments. Female mice were used for experiments with the 4T1 tumor. All animal experiments were approved by the Animal Care and Use Committee of the National Cancer Institute, the Peter MacCallum Cancer Centre, or the University of Maryland, Baltimore County.

**Antibodies.** Purified mAbs reactive with mouse CD4 (GK1.5; American Type Culture Collection) and CD25 (PC61; American Type Culture Collection) were purified from ascites by Harlan Bioproducts for Science, Inc. Ascites of anti-mouse CD1d (3C11) was provided by J. Yewdell (National Institute of Allergy and Infectious Diseases, Bethesda, MD).

**Tumor cell lines.** The fibrosarcoma line 15-12RM (29) was maintained in RPMI 1640 with 10% FCS, penicillin/streptomycin, nonessential amino acids, L-glutamine, and 200  $\mu$ g/ml of G418. The colon carcinoma line CT26 was maintained in RPMI 1640 with 10% FCS, penicillin/streptomycin, nonessential amino acids, and L-glutamine. The mammary carcinoma line 4T1 was maintained in IMDM with 10% FCS, penicillin/streptomycin, and L-glutamine. The carcinogen-induced colon carcinoma line Colon-26 (CT26-L5) was a gift from I. Saiki (Toyama Medical and Pharmaceutical University, Toyama, Japan) and was maintained in RPMI 1640 with 5% FCS, penicillin/streptomycin, and L-glutamine.

**In vivo tumor assays.** Single cell suspensions of tumor cells were prepared in PBS and injected either in the mammary fat pad, subcutaneously or i.v., as indicated in the figures. In various groups of mice, CD4<sup>+</sup> cells were depleted by injecting 500  $\mu$ g anti-CD4 mAb (clone GK1.5). The antibody was administered i.p. as indicated in the figures. CD25<sup>+</sup> cells were depleted by i.p. administration of anti-CD25 mAb (500  $\mu$ g or 1 mg; clone PC61) as indicated in the figures, which was previously shown to be sufficient to deplete all CD4<sup>+</sup>CD25<sup>+</sup> cells in the spleen (as determined by flow cytometry). Staining of lungs to enumerate CT26 tumor nodules and surgical removal of primary 4T1 tumors was performed as previously described (19, 30).

**In vivo staining of CD1d.** Tumor and spleen samples were harvested and snap frozen in OCT compound and stored at  $-80^{\circ}$ C until processing. 6- $\mu$ m tissue sections were cut on a cryostat (CM 3050 S; Leica), fixed for

10 min in ice-cold acetone, and rehydrated in PBS + 1% BSA for 20 min. Sections were washed twice, and Fc receptors were blocked by incubation with 2.4G2 for 30 min. Blocked sections were then stained with biotinylated anti-CD1d (clone 1B1), anti-CD11b (clone M1/70), or rat IgG isotype control (all purchased from BD Biosciences) for 2 h at 4°C. After incubation with primary antibodies, sections were washed three times and endogenous peroxidase activity was blocked by incubation in 0.3% H<sub>2</sub>O<sub>2</sub> in PBS. After washing, biotinylated antibody staining was visualized by the StreptABCComplex/HRP and Liquid DAB+ Substrate Chromogen System (both from DakoCytomation) according to the manufacturer's instructions. Sections were counterstained with hematoxylin.

**Statistical analysis.** The Mann-Whitney U rank sum test, one-way analysis of variance (ANOVA), or log-rank test were used for the statistical analysis of data. p-values <0.05 were considered significant.

**Online supplemental material.** Fig. S1 shows that the depletion effect of anti-CD25 persists for at least 13 d. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20051381/DC1>.

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