

# Conditional Regulatory T-Cell Depletion Releases Adaptive Immunity Preventing Carcinogenesis and Suppressing Established Tumor Growth

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## Abstract

Foxp3 is a central control element in the development and function of regulatory T cells (Treg), and mice expressing a diphtheria toxin (DT) receptor–enhanced green fluorescent protein fusion protein under the control of the *foxp3* gene locus (DEREG mice) allow conditional and efficient depletion of Foxp3<sup>+</sup> Treg by DT injection. Herein, we use DEREG mice and a mouse model of carcinogenesis to show that conditional and effective Treg depletion can both protect mice from carcinogenesis by innate control, yet permanently eradicate a proportion of *de novo*–established tumors in mice in a largely CD8<sup>+</sup> T-cell– and IFN- $\gamma$ –dependent manner. Tumors displayed a heterogeneous response to Treg depletion, and suppression of established tumors was accompanied by an increase in the tumor-infiltrating CD8<sup>+</sup> T-cell/B-cell ratio. Tumor rejection occurred in the absence of overt autoimmunity, suggesting that effective transient Treg depletion strategies may be therapeutic in at least a proportion of spontaneous tumors developing in the host. *Cancer Res*; 70(20): 7800–9. ©2010 AACR.

## Introduction

Naturally occurring CD25<sup>+</sup>CD4<sup>+</sup> regulatory T cells (Treg) have a major importance in modulating host responses to tumors and infections, in preventing transplant rejection, and in inhibiting the development of autoimmunity and allergy (1–3). Originally, CD4<sup>+</sup> Treg were identified exclusively by the constitutive expression of CD25, and many *in vivo* experiments have been performed using depleting antibodies directed against CD25 (4, 5) or folate receptor 4 (FR4; ref. 6), which when highly expressed on CD4<sup>+</sup> T cells also defines Treg. However, the existence of Treg within peripheral tissues that lack CD25 (1, 7, 8), and the expression of CD25 on activated conventional T cells, which precludes discrimination between Treg and activated conventional T cells, limits the interpretation of data obtained by the use of anti-CD25–depleting antibodies (8–10). The most specific Treg marker currently known is the forkhead box transcription factor

Foxp3, which has been shown to be expressed specifically in mouse CD4<sup>+</sup> Treg and acts as a master switch in the regulation of their development and function (11). Bacterial artificial chromosome–transgenic mice, termed “depletion of regulatory T cell” (DEREG) mice, express a diphtheria toxin receptor (DTR)–enhanced green fluorescent protein (eGFP) fusion protein under the control of the *foxp3* locus, allowing both the detection and inducible depletion of Foxp3<sup>+</sup> Treg (12). DEREG mice have an eGFP expression pattern similar to that of previously published Foxp3 reporter mice (13, 14), and DT treatment, in contrast to conventional Treg depletion strategies, allows for efficient and selective depletion of Foxp3<sup>+</sup> cells without affecting CD25<sup>+</sup> effector T cells (12).

It has been previously shown, using anti-CD25 and anti-FR4 antibodies, that Treg depletion before, or at the time of, tumor inoculation can lead to an efficient antitumor effector response that suppresses and, in some cases, prevents tumor formation and growth (6, 9, 15–17). However, notably, these depletion strategies do not lead to effective tumor suppression when administered to established tumors. Because both anti-CD25 and anti-FR4 depleted only between 40% and 70% of Treg in peripheral blood and other tissues, it has remained unclear whether the antibody-resistant Treg remaining were preventing host immune–mediated rejection of established tumors or whether the depleted Treg were indeed superfluous to the microenvironment of established tumors. The inability of Treg depletion strategies to affect tumors established *de novo* and the prospect that more radical approaches were capable of triggering autoimmunity (18) in the host have tempered enthusiasm for this approach. Herein, using DEREG mice, and a model of *de novo* carcinogenesis, we show that conditional and effective Treg depletion

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**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi: 10.1158/0008-5472.CAN-10-1681

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can indeed permanently eradicate a proportion of established tumors in mice in a largely IFN- $\gamma$ -dependent manner, in the absence of detectable autoimmunity, by altering the leukocyte composition of the tumor microenvironment. This study illustrates the therapeutic potential of temporarily but efficiently depleting Treg in established tumors.

## Materials and Methods

### Mice

Inbred wild-type (WT) C57BL/6 and C57BL/6 DERE (Foxp3-DTR-GFP) mice were bred and maintained at the Peter MacCallum Cancer Centre. Six- to 18-week-old mice were used in all experiments that were performed according to Peter MacCallum Cancer Centre animal experimental ethics committee guidelines.

### Tumor models

**Experimental.** C57BL/6-derived MC38 colon adenocarcinoma expressing OVA (MC38-OVA<sup>dim</sup>) and EL4-OVA (EG7) lymphoma cell lines were maintained, injected s.c., and monitored as previously described (19, 20).

**3-Methylcholanthrene carcinogenesis models—prevention model.** Groups of 10 to 24 male DERE mice were inoculated s.c. in the hind flank with 100 or 400  $\mu$ g of 3-methylcholanthrene (MCA; Sigma-Aldrich) in 0.1 mL of corn oil as described (21). Mice were treated with PBS or 1,000 ng of DT A chain (DTA) on day 0 relative to MCA inoculation as indicated. Some DERE mice received control immunoglobulin (Ig) or weekly depletion of CD8<sup>+</sup> T cells (53.6.7) or natural killer (NK) cells (anti-asialoGM1) on days -1 and 0 (from the time of MCA inoculation) and weekly to day 56 (100  $\mu$ g i.p.). Development of fibrosarcomas was monitored weekly over the course of 250 days. Tumors >3 mm in diameter and showing progressive growth were recorded as positive. Measurements were made with a caliper square as the product of two perpendicular diameters (cm<sup>2</sup>), and individual mice are represented.

**MCA carcinogenesis models—therapy model.** Groups of 15 male DERE mice were inoculated s.c. in the hind flank with 400  $\mu$ g of MCA in 0.1 mL of corn oil as described (21). Mice were treated with PBS or 1,000 ng DTA (Sigma-Aldrich) on days 77, 84, 98, and 105 or 112, 119, 133, and 140 relative to MCA inoculation as indicated. Some DERE mice received control Ig or weekly depletion of CD8<sup>+</sup> T cells (53.6.7) or NK cells (anti-asialoGM1) weekly from days 77 to 126 (relative to the time of MCA inoculation, 100  $\mu$ g i.p.). Other groups of DERE mice were treated with control Ig (Mac-4), anti-IFN- $\gamma$  (H22), or anti-IFNAR1 (MAR1) weekly from days 77 to 126 (relative to the time of MCA inoculation, 250  $\mu$ g i.p.) to neutralize IFN- $\gamma$  or IFN- $\alpha\beta$  as previously described (22, 23). Development of fibrosarcomas was monitored weekly over the course of 250 to 300 days. Tumors >3 mm in diameter and showing progressive growth were recorded as positive. Measurements were made with a caliper square as the product of two perpendicular diameters (cm<sup>2</sup>), and individual mice or survival of groups is represented.

### Flow cytometry

**Depletion.** Groups of DERE mice were treated with a single dose of DT (10–1,000 ng), and inguinal lymph nodes, spleen, and peripheral blood were harvested on days 3, 7, and 14 after DT.

**Tumor-infiltrating lymphocytes.** DERE mice bearing established MCA tumors (>5 mm in diameter) were divided into cohorts with similar-sized tumors. Each cohort was treated with either DT (1,000 ng) or PBS, and tumors were measured twice weekly. DT-treated mice were classified as responders if two consecutive measurements showed progressive decrease in tumor growth, and nonresponders if tumors grew progressively over three measurements. Tumors were excised from mice, minced finely, and digested for 45 minutes in incomplete RPMI 1640 containing collagenase type 4 (Worthington Biochemical Corp.) and DNase I (Roche) at 37°C. Following digestion, tumor suspensions were passed through a 70- $\mu$ m cell strainer and washed twice in complete RPMI 1640. Draining lymph node (DLN) and non-DLN (NDLN) were excised from each mouse where possible, single-cell suspensions were generated, and cells were then used for fluorescence-activated cell sorting (FACS) analysis. Tumors were resuspended in FACS buffer and passed through a 45- $\mu$ m cell strainer before antibody staining.

For surface staining, tumor cells were stained with A750-CD45, eF605-CD11b, PECy7-CD8, eF450-CD4, APC-TCR $\beta$ , PeCy7-NK1.1, PE-CD25, PE-CD19, and eF450 B220 (all from eBioscience) in the presence of 2.4G2 (anti-CD16/32, to block Fc receptors) on ice. 7-Amino-actinomycin D (7-AAD; BD Pharmingen) was added immediately before FACS analysis. Cells were acquired on the BD FACSCanto II (BD Biosciences). Analysis was performed using the software program FCS Express.

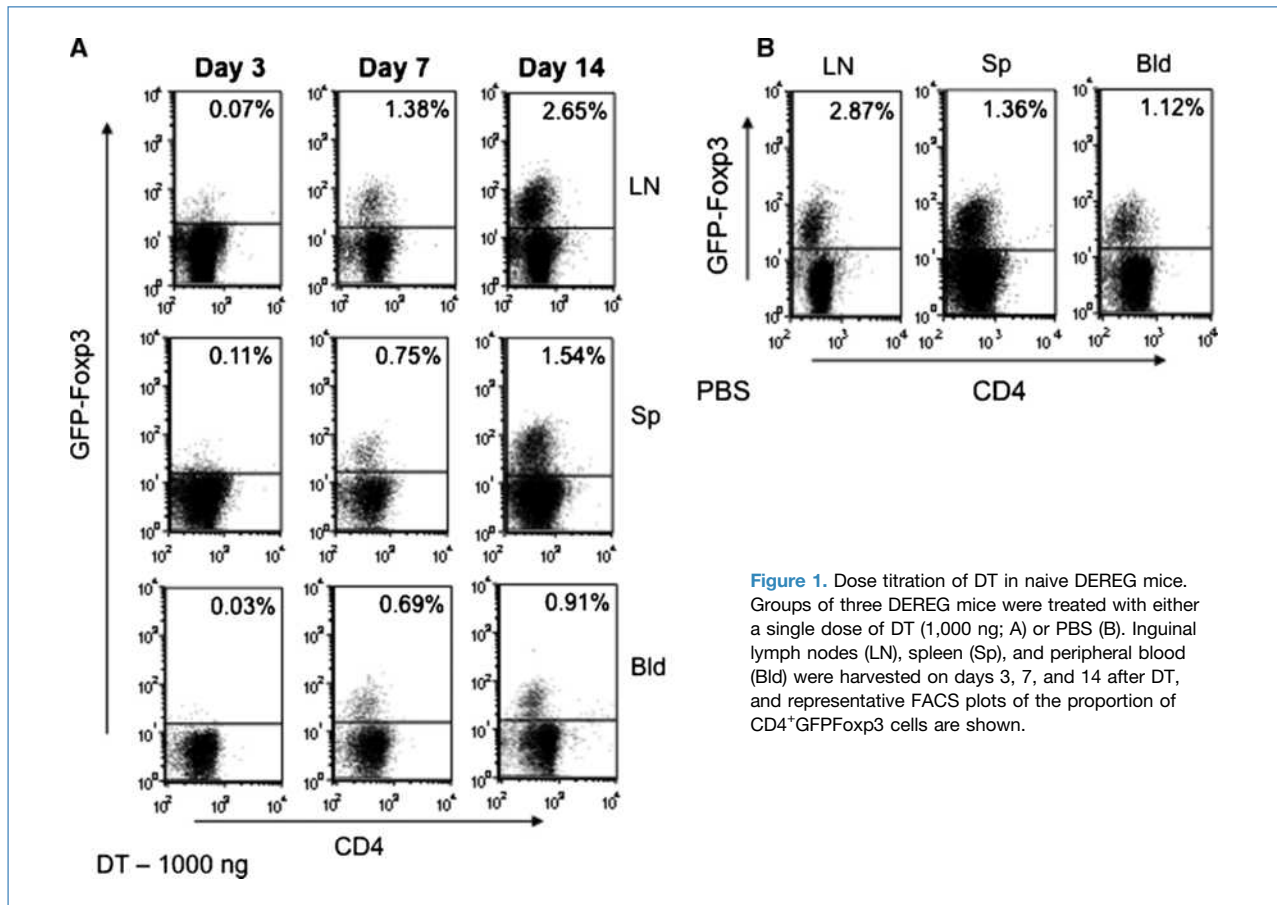
### Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Significant differences in metastases were determined by Kruskal-Wallis test, and differences in survival or proportion of tumor-free mice were determined by the log-rank Mantel-Cox test or Fisher's exact test, respectively. Values of  $P < 0.05$  were considered significant.

## Results

### Complete Treg depletion enables cell-mediated control of experimental tumors and lung metastases

C57BL/6 DERE mice carry a DTR-eGFP transgene under the control of an additional Foxp3 promoter, whereby DT application leads to Treg depletion at any desired time point during an ongoing immune response (12). In contrast to mouse models targeting other cell types such as dendritic cells, depletion of Treg requires more DT. In various modifications of the original depletion protocol (12), we first titrated DT to determine the minimum amount required for Treg depletion and a schedule based on their recovery over time. From these data, it seemed that Treg were maximally depleted from lymph nodes, spleen, and peripheral blood 3 days after DT injection, with partial recovery by 7 days and full recovery by 14 days after DT injection (Fig. 1; Supplementary Fig. S1).



**Figure 1.** Dose titration of DT in naive DERE mice. Groups of three DERE mice were treated with either a single dose of DT (1,000 ng; A) or PBS (B). Inguinal lymph nodes (LN), spleen (Sp), and peripheral blood (Bld) were harvested on days 3, 7, and 14 after DT, and representative FACS plots of the proportion of CD4<sup>+</sup>GFPFoxp3 cells are shown.

Overall maximal depletion of Treg at 3 days was between 90% and 95%. At lower doses of DT, recovery of Treg was more obvious by 7 days. We then tested the ability of various prophylactic DT doses to functionally compromise tumor growth using two different experimental tumor models: the immunogenic EG7 lymphoma and less immunogenic MC38-OVA<sup>dim</sup> colon adenocarcinoma. We have previously described the ability of OVA-specific CD8<sup>+</sup> T cells to control the growth of these tumors (19, 20). By using a high dose of each tumor, the majority of mice treated with PBS showed progressive tumor growth (Fig. 2). By contrast, a dose titration of DT treatment illustrated that all mice depleted with 1,000 ng DT on day 0 (= day of tumor inoculation) rejected their EG7 tumors by 15 to 20 days, whereas the effects at lower DT doses were attenuated and barely detectable at 1 ng DT (Fig. 2). A reduced effect of Treg depletion was noted for MC38-OVA<sup>dim</sup> tumors, highlighting that some tumors were less susceptible to Treg inhibition, but overall, these data also illustrated that 1,000 ng DTA was an optimal depletion dose (Fig. 2). Mice remained tumor-free for at least 100 days and showed no overt signs of toxicity or autoimmunity. Higher, and more than weekly, doses of DT were not routinely administered because these schedules caused DT toxic effects such as wasting and visceral organ damage.

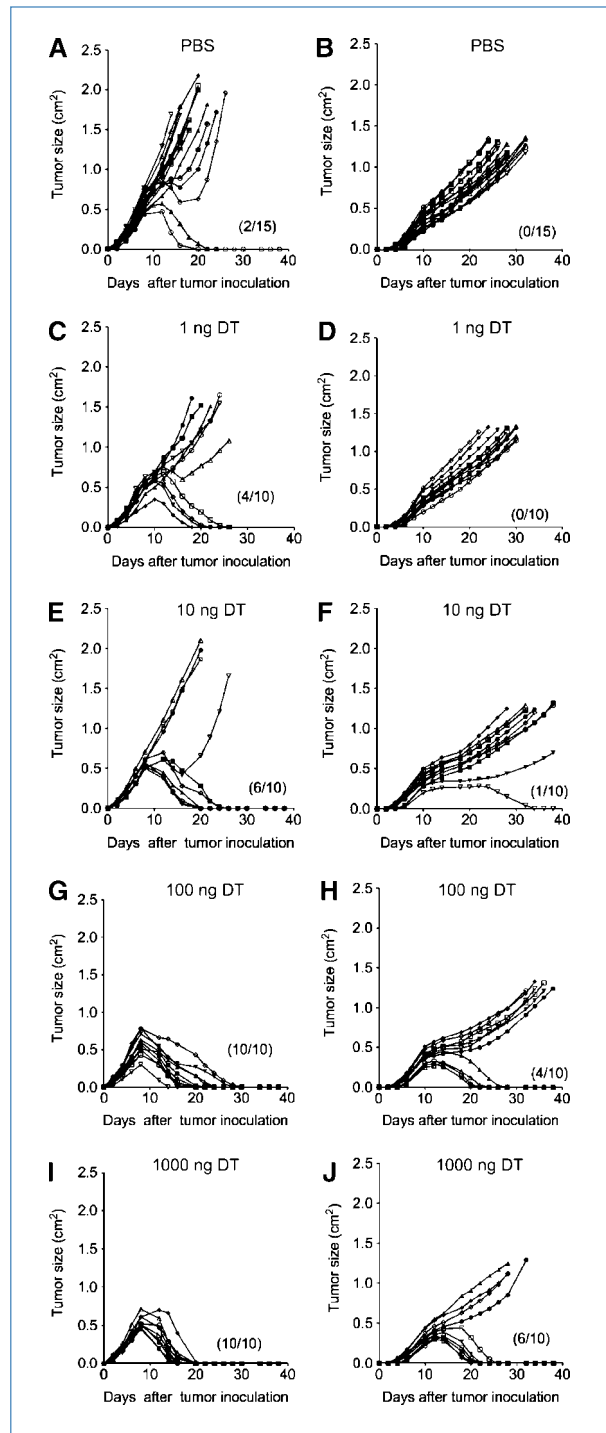
### Complete Treg depletion at the time of MCA inoculation protects mice from fibrosarcoma in a largely NK cell-dependent manner

Having established a depleting dose of DT, we next evaluated whether deep yet transient depletion of Treg could protect mice from carcinogen-induced tumor initiation. We have previously shown that host NK and NKT cells protect mice from the development of MCA-induced fibrosarcoma (24, 25), whereas several lymphocyte recognition (26) and effector molecules (21, 27, 28) have also been shown to be important in host resistance to this carcinogen. Previous studies have also illustrated the presence of Treg in established MCA-induced sarcomas (29) and the ability of prophylactic and continual anti-CD25 monoclonal antibody (mAb) to prevent fibrosarcoma formation (30, 31). More recently, we have replicated these latter studies using prophylactic anti-CD25, anti-CD4, or anti-FR4 mAbs (32). A single injection of DT (1 μg) on the day of MCA inoculation protected the majority of DERE mice from either 400 μg MCA (17 of 24 tumor-free) or 100 μg MCA (12 of 13 tumor-free) compared with those DERE mice treated with PBS (0 of 24 and 5 of 13 tumor-free, respectively; Fig. 3). Individual tumor growth curves revealed that fibrosarcomas that developed in Treg-depleted mice were also heterogeneous in their growth and tended

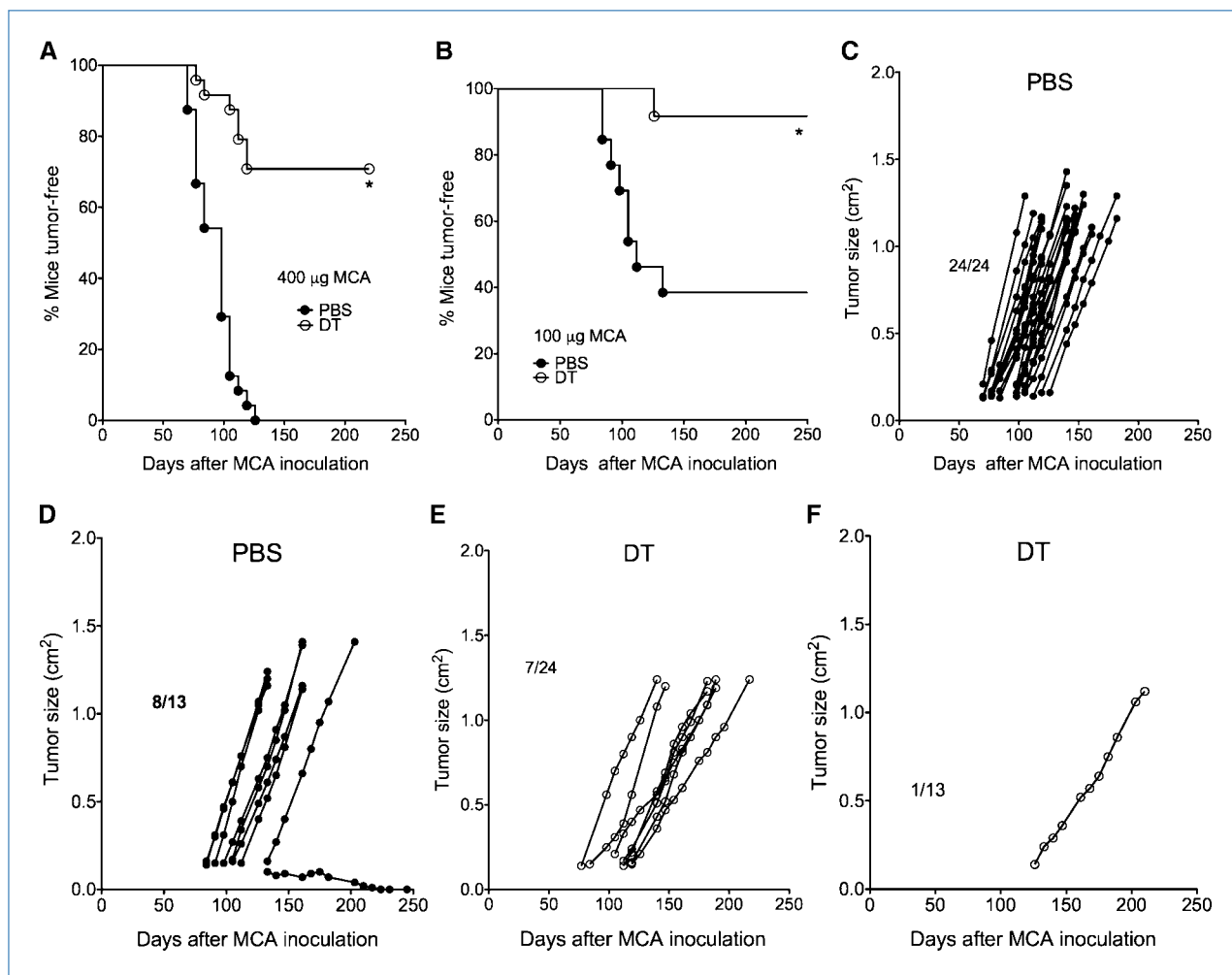
to develop later than in control-treated mice. This level of protection afforded by Treg depletion in DEREg mice was greater than that observed in WT C57BL/6 mice prophylactically treated with anti-CD25, anti-CD4, or anti-FR4 mAbs (up to 20–40% protection; ref. 32). Consistent with a known role for innate immunity in host protection from MCA-induced fibrosarcoma, Treg depletion continued to protect mice from tumor formation in mice additionally depleted of CD8<sup>+</sup> T cells, but survival was significantly reduced in mice that were depleted of either NK cells or both NK cells and CD8<sup>+</sup> T cells (Fig. 4). The data implicated a major role for NK cells, and a minor role for CD8<sup>+</sup> T cells, in host protection from sarcoma induction in mice transiently lacking Treg at the time of MCA inoculation.

### Complete Treg depletion cures a proportion of mice with established MCA-induced fibrosarcomas

To date, few studies have illustrated the ability of single immunotherapies to reject and cure mice of established tumors, particularly when that tumor is derived *de novo* in the host. We have recently shown that a combination of three immunomodulatory mAbs could cure mice of MCA-induced fibrosarcomas when treatment commenced at the time tumors were first palpable and determined to be in growth phase (33). However, single mAb treatments were largely ineffective and no mice survived. Similarly, Treg depletion using mAbs has been largely ineffective in treating established experimental and spontaneous tumors, with, at best, moderate tumor growth suppression. Herein, using the anti-FR4 mAb (Th6; which optimally depletes approximately 50–60% of host Foxp3<sup>+</sup> Treg) and commencing weekly treatment at day 77 after high-dose MCA inoculation (400  $\mu$ g and ~60% group with tumors 0.13–0.35 cm<sup>2</sup>), we saw typical moderate fibrosarcoma growth suppression when compared with control Ig treatment of mice (Supplementary Fig. S2). Some tumors were delayed, but eventually, all anti-FR4 mice developed fibrosarcoma and succumbed (Supplementary Fig. S2). Taking a similar approach to conditionally deplete Treg in DEREg mice, we performed two schedules of DT therapy (days 77, 84, 98, and 105, and days 112, 119, 133, and 140) in mice that had been inoculated with MCA. Remarkably, with earlier DT treatment, only 20 of 26 (77%) mice developed palpable fibrosarcomas, whereas PBS-treated mice all (13 of 13) developed rapidly growing tumors (Fig. 5A and B). Of those Treg-depleted mice that did develop fibrosarcomas, 6 of 20 (30%) were cured of their tumor. A tremendous heterogeneity in host response was illustrated, as among the other 14 tumors not rejected, 8 showed some significant period of retarded growth largely throughout the course of depletion, whereas 6 grew largely unaffected by Treg depletion (Fig. 5A and B). A very similar heterogeneous pattern of response was noted when all mice had established tumors at the time of first depletion (from day 112 after MCA inoculation; tumor size range, 0.19–1.04 cm<sup>2</sup>; Fig. 5C and D). In this instance, 9 of 26 tumors (~35%) were rejected in DT-treated DEREg mice, 6 of 26 responded, and 11 of 26 (mostly the largest at the time of depletion, 8 of 11 > 0.83 cm<sup>2</sup>) did not respond. Tumors rejected were predominantly smallest at the time of first treatment



**Figure 2.** Dose titration of DT in DEREg mice defines functional depletion of Treg. Groups of 10 to 15 DEREg mice were inoculated s.c. with either  $3 \times 10^6$  EG7 lymphoma (A, C, E, G, and I) or  $1 \times 10^6$  MC38-OVA<sup>dim</sup> colon adenocarcinoma (B, D, F, H, and J) cells. Mice were treated with either PBS (A and B) or DT (1 ng, C and D; 10 ng, E and F; 100 ng, G and H; 1,000 ng, I and J) on day 0 (the day of tumor inoculation). Mice were then monitored for tumor growth every second day as described, and results were recorded as the tumor growth curves (size in cm<sup>2</sup>) of individual mice in each group. The number of cured mice is indicated in parentheses in each panel.



**Figure 3.** Complete Treg depletion at the time of MCA inoculation protects mice from fibrosarcoma. Groups of 13 to 24 male DEREg mice were injected s.c. on the flank with either 400 (A, C, E) or 100 (B, D, F)  $\mu$ g MCA on day 0. DEREg mice were treated on day 0 with either PBS or DT (1,000 ng) as indicated. Mice were then monitored for tumor development over 250 d and recorded as either % tumor-free mice (A and B) or the growth curves (tumor size in  $\text{cm}^2$ ) of individual mice with tumor in each group (C–F). C to F, proportion of mice with developing tumors in each group. Statistical differences in % mice tumor-free were determined by log-rank Mantel-Cox test. \*,  $P < 0.05$ .

(5 of 9  $< 0.5 \text{ cm}^2$ ), but one lesion where treatment commenced at  $0.99 \text{ cm}^2$  was completely eradicated. Mice displaying complete tumor rejection remained tumor-free for at least 300 days, and none of these mice displayed any overt signs of autoimmunity over this entire period. Importantly, WT C57BL/6 mice treated with a similar schedule of DT did not show any detectable response compared with PBS-treated mice, indicating that the DT was not having any antitumor effect in its own right (Supplementary Fig. S3). The ability of Treg depletion to enable the suppression of established tumors was not restricted to tumors induced *de novo* by MCA. Delayed DT treatment of DEREg mice (day 7) injected to succumb to B16F10 lung metastases within 14 days significantly reduced metastatic burden and prolonged the survival of Treg-depleted mice (Supplementary Fig. S4).

#### Host $\text{CD8}^+$ T cells and $\text{IFN-}\gamma$ reject established fibrosarcomas in the complete absence of Treg

The rejection of established MCA-induced sarcomas following Treg depletion in DEREg mice offered for the very first time an opportunity to evaluate natural immune-mediated rejection of palpable and more clinically relevant growing tumor masses. Using a similar experimental setup to Fig. 5A and B where mice received DT on days 77, 84, 98, and 105 after MCA inoculation, mice were additionally conditionally depleted of  $\text{CD8}^+$  T cells, NK cells, or both or neutralized for type I or II IFN from days 77 to 126 (Fig. 6). As previously illustrated, the group of mice depleted of Treg and treated with control Ig developed sarcomas in 21 of 30 (70%) mice, and of these, 9 of 21 were cured (Fig. 6B). A similar proportion of DEREg mice treated with DT and depleted of NK cells (10 of 15; Fig. 6D) or neutralized for type I IFN (11 of 15; Fig. 6F) remained

tumor-free, whereas of the mice developing tumors, 3 of 10 or 6 of 11 were cured, respectively (Fig. 6D and F). By striking contrast, those groups of mice additionally depleted of CD8<sup>+</sup> T cells (Fig. 6C), CD8<sup>+</sup> T cells, and NK cells (Fig. 6E) or neutralized for IFN- $\gamma$  (Fig. 6G), all developed tumors in a similar manner to PBS-treated DEREg mice (Fig. 6A). These data indicated that CD8<sup>+</sup> T cells and IFN- $\gamma$ , and to a minor extent NK cells, were responsible for the protection afforded by conditional Treg depletion. Notably, however, some tumor development was comparatively delayed and, in some individual cases, obviously suppressed in mice depleted of CD8<sup>+</sup> T cells (Fig. 6C) or neutralized for IFN- $\gamma$  (Fig. 6G), suggesting that other minor mechanisms may also be able to play a more minor role.

### Regressing MCA tumors contain an elevated ratio of CD8<sup>+</sup> T cells to B cells

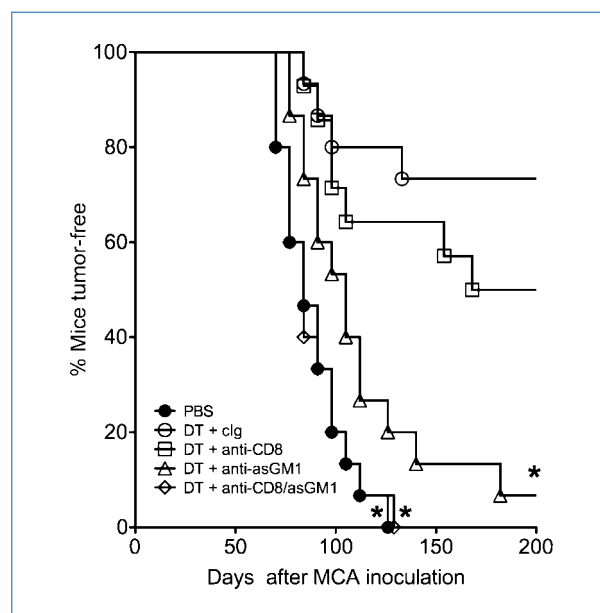
To further evaluate the mechanism by which established MCA-induced fibrosarcomas were rejected in some mice depleted of Treg and not others, we undertook an analysis of the tumor-infiltrating leukocytes (TIL) in MCA-induced fibrosarcomas responding to Treg depletion (Table 1). Tumors (PBS,  $n = 10$ ; nonresponders,  $n = 13$ ; responders,  $n = 6$ ) were gated for live cells and then for leukocytes infiltrating CD45<sup>+</sup>CD11b<sup>-</sup> and CD45<sup>+</sup>CD11b<sup>+</sup> populations as previously described (29). CD45<sup>+</sup> TILs (~34–94% of the tumor mass) were specifically increased in the tumors of mice depleted of Treg (~74  $\pm$  7% versus 50  $\pm$  10% live cells) without an increase in the proportion of CD45<sup>+</sup>CD11b<sup>+</sup> cells (28  $\pm$  2% versus 33  $\pm$  4% live cells). By contrast, the CD45<sup>+</sup>CD11b<sup>-</sup> population was increased in the TILs of Treg-depleted mice, particularly those with tumors regressing (PBS treated, 5.0  $\pm$  0.7%; DT-treated nonresponders, 14.2  $\pm$  2.4%; DT-treated responders, 20.0  $\pm$  2.9% live cells). Within the CD45<sup>+</sup>CD11b<sup>-</sup> population, there was an elevation in the proportion CD8<sup>+</sup> T cells among CD11b<sup>-</sup> leukocytes in DEREg mice depleted of Treg compared with PBS-treated mice. CD4<sup>+</sup> T-cell proportions were comparatively unchanged. There was also reduction in B-cell (CD19<sup>+</sup>) proportions, particularly in the DT-treated responding group. Coupled with a greater lymphocyte infiltrate in the responding group, the CD8<sup>+</sup> T-cell/B-cell ratio seems a possible predictor of tumor regression. Coincident analysis of tumor DLN and NDLN revealed that these changes were tumor specific (Supplementary Table S1).

## Discussion

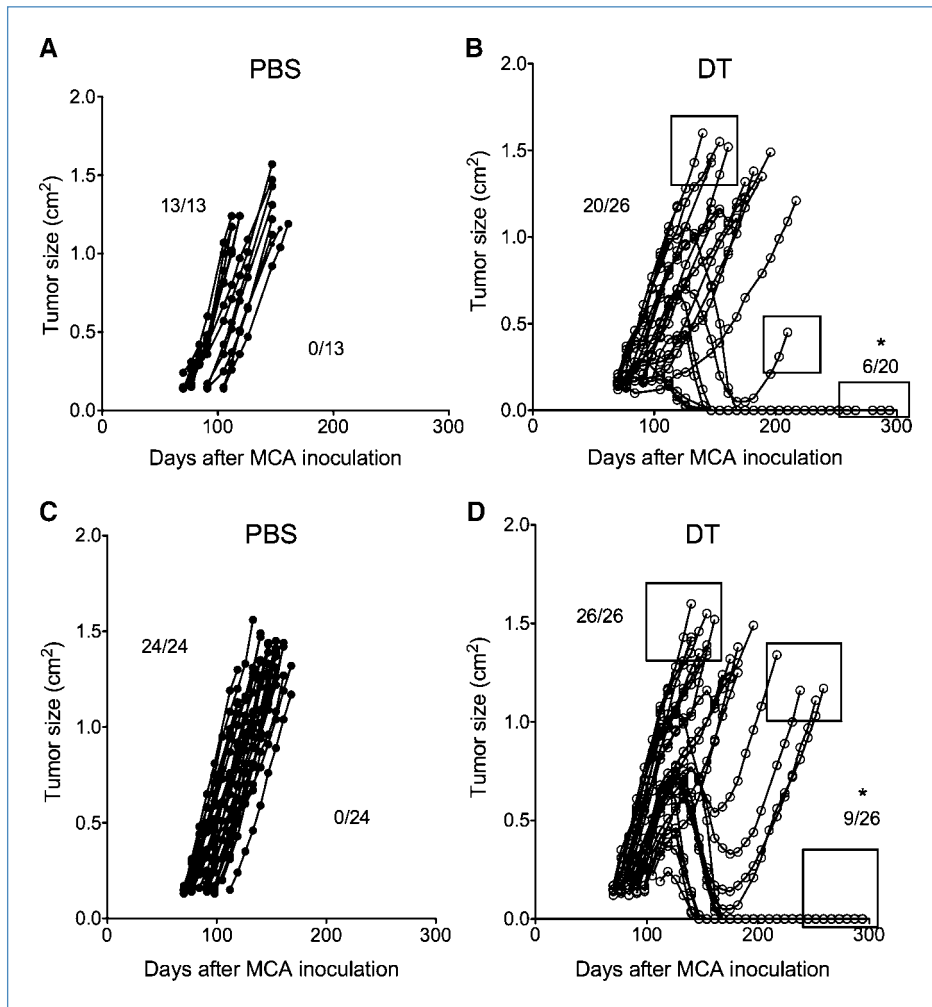
Previous experimental depletion strategies to reduce the numbers of Treg at the time of, or early in, tumor development have resulted in significant tumor suppression; however, Treg depletions are partial and similar approaches in mice and humans have largely failed in affecting established tumors. Mice expressing a DTR-eGFP fusion protein under the control of the *foxp3* gene locus have now allowed conditional and efficient depletion of Foxp3<sup>+</sup> Treg by DT injection. We have used DEREg mice and a mouse model of carcinogenesis to show that conditional and effective Treg depletion can both protect mice from carcinogenesis by innate control, yet permanently eradicate a proportion of *de novo*-established tumors in mice

in a largely CD8<sup>+</sup> T-cell- and IFN- $\gamma$ -dependent manner. Interestingly, tumors displayed a heterogeneous response to Treg depletion, and suppression of established tumors was accompanied by a relative increase in tumor-infiltrating CD8<sup>+</sup> T-cell/B-cell ratio. Complete tumor rejection also occurred in the absence of overt autoimmunity, suggesting that powerful transient Treg depletion strategies may be therapeutic in at least a proportion of spontaneous tumors developing in the host.

Anti-CD25 and anti-FR4 mAbs depleting Treg before, or at the time of, tumor inoculation can lead to an efficient anti-tumor effector response that suppresses and, in some cases, prevents tumor formation and growth (6, 9, 15–17). However, these depletion strategies do not lead to effective tumor suppression when administered in mice with established tumors. Building on previous observations that MCA-induced fibrosarcomas were strikingly infiltrated with Foxp3<sup>+</sup> Treg (29), and significantly reduced in incidence in an IFN- $\gamma$ -dependent fashion following anti-CD25 mAb pretreatment (30), we have now shown that MCA-induced fibrosarcomas are suppressed by a variety of strategies (anti-CD4, anti-FR4, and anti-CD25) that partially deplete (about 50–70%) Treg at the time of MCA inoculation (32). Quantitatively, these strategies depleted Foxp3<sup>+</sup> T cells and prevented tumor initiation to a similar extent, with a combination of CD8<sup>+</sup> and NK cells



**Figure 4.** Host NK cells and CD8<sup>+</sup> T cells protect mice from fibrosarcoma induction in the absence of Treg. Groups of 15 male DEREg mice were injected s.c. on the flank with either 400  $\mu$ g MCA on day 0. DEREg mice were treated on day 0 with either PBS or DT (1,000 ng) as indicated. Some groups of mice were treated with anti-CD8 and/or anti-asialoGM1 (asGM1) antibody on days -1 and 0 and then weekly to day 56 to deplete CD8<sup>+</sup> T and/or NK cells. Mice were then monitored for tumor development over 250 d and recorded as % tumor-free mice for each group. Statistical differences in % mice tumor-free compared with DT + control Ig (clg) group were determined by log-rank Mantel-Cox test as PBS ( $P < 0.0001$ ), DT + anti-CD8 ( $P = 0.2472$ ), DT + anti-asialoGM1 ( $P = 0.0002$ ), DT + anti-CD8/anti-asialoGM1 ( $P < 0.0001$ ), and DT + anti-asialoGM1 versus DT + anti-CD8/anti-asialoGM1 ( $P = 0.022$ ).



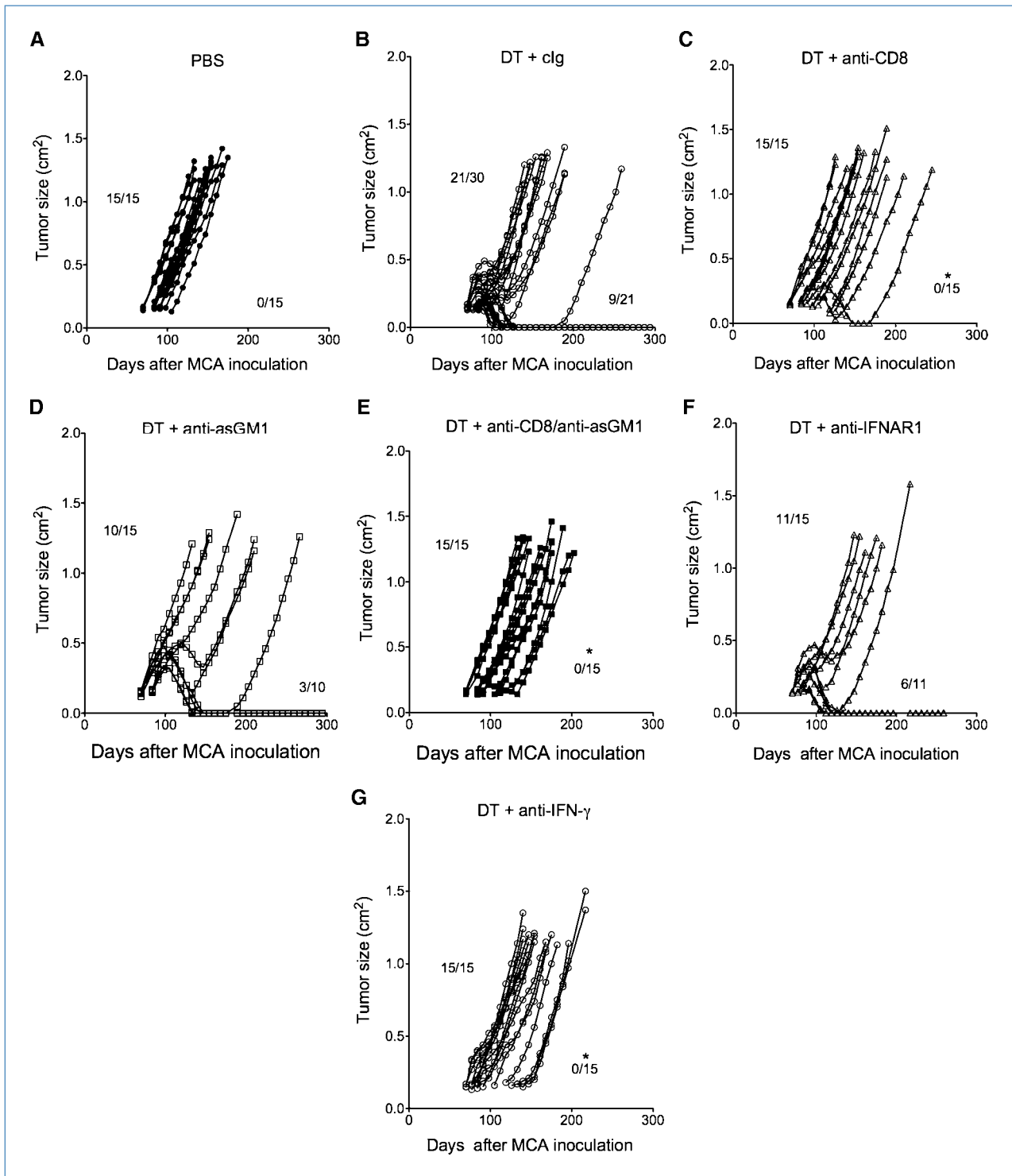
**Figure 5.** Complete Treg depletion cures a proportion of mice with established MCA-induced fibrosarcomas. Groups of 13 to 26 male DEREg mice were injected s.c. on the flank with 400  $\mu$ g MCA on day 0. A and B, when most sarcomas had established, DEREg mice were treated on days 77, 84, 98, and 105 with either PBS or DT (1,000 ng) as indicated. C and D, DEREg mice were treated even later on days 112, 119, 133, and 140 with either PBS or DT (1,000 ng) as indicated. A and D, mice were then monitored for tumor growth, and results were recorded as the tumor growth curves (size in cm<sup>2</sup>) of individual mice in each group. The proportion of mice with developing tumors in each group is shown on the left of each panel, whereas the proportion of cures is shown as a ratio on the right of each panel. Statistical differences in % mice cured in the absence of Treg were determined by Fisher's exact test. \*,  $P < 0.05$ .

and effector mechanisms (perforin and IFN- $\gamma$ ) accounting for tumor rejection (32). Similar mechanisms of tumor prevention have been established herein using the prophylactic, but complete, depletion of Treg in DEREg mice. In particular, a major role for innate NK cell-mediated prevention of MCA-induced sarcomas and experimental B16F10 lung metastases was observed.

Because elimination of Treg with mAbs before tumor challenge can result in protection in a proportion of mice and yet their same depletion in mice with established tumors had no therapeutic effect, it was thought that Treg might often serve as the dominant immune escape mechanism early in tumor progression but not in established tumors. However, because there are no mAbs that can specifically deplete all Foxp3<sup>+</sup> Treg, the lack of regression in established tumors following depletion raised the question of whether not enough Treg were being eliminated to relieve suppression on antitumor immune cells or whether effector cells responsible for mediating antitumor response were concomitantly depleted. Importantly, we showed that even the anti-FR4 mAb (depletes approximately 60–70% of Foxp3<sup>+</sup> Treg) was largely ineffective in regressing established MCA-induced sarcomas. This inability

of Treg depletion strategies to affect established tumors developed *de novo* in the mouse is consistent with the failure of Treg depletion strategies in humans thus far to result in significant clinical benefit (34). Nevertheless, the consensus view is that Treg remain one of the major obstacles to successful cancer immunotherapy, and it is most encouraging that complete transient depletion of Treg in the DEREg mice did generate a significant number of complete responses of tumors established *de novo*. This rejection of established tumors required CD8<sup>+</sup> T cells and IFN- $\gamma$  and was largely independent of type I IFNs. Further conditional depletion/neutralization of other cells and pathways will be required to explore the context in which CD8<sup>+</sup> T cells suppress tumors in the absence of Treg.

Strikingly, a significant heterogeneity in tumor response was observed between various individual mice in the cohorts depleted of Treg. Our data from six mice rejecting larger established MCA-induced sarcomas indicated that these responding mice display an increase in the ratio of tumor-infiltrating CD8<sup>+</sup> T cells to B cells. By collecting more MCA-induced tumors, we now wish to more broadly and definitively characterize this response—distinguishing the quality and quantity of the effector CD8<sup>+</sup> T-cell response in the nonresponding and



**Figure 6.** Host CD8<sup>+</sup> T cells and IFN- $\gamma$  reject established fibrosarcomas in the complete absence of Treg. Groups of 15 to 30 male DEREG mice were injected s.c. on the flank with 400  $\mu$ g MCA on day 0. When most sarcomas had established, DEREG mice were treated on days 77, 84, 98, and 105 with either PBS (A) or DT (1,000 ng; B) as indicated. Some groups of mice were treated with anti-CD8 and/or anti-asialoGM1 antibody weekly from days 77 to 126 to deplete CD8<sup>+</sup> T and/or NK cells. Other groups of mice were treated weekly from days 77 to 126 with control Ig anti-IFNAR1 or anti-IFN- $\gamma$  to neutralize these cytokines. Mice were then monitored for tumor growth over 300 d, and results were recorded as the tumor growth curves (size in cm<sup>2</sup>) of individual mice in each group. The proportion of mice with developing tumors in each group is shown on the left of each panel, whereas the proportion of cures is shown as a ratio on the right of each panel. Statistical differences in % mice cured in control Ig- and DT-treated mice in the absence of Treg were determined by Fisher's exact test. \*,  $P < 0.05$ .



**Table 1.** Lymphocyte infiltrates in MCA tumors of Treg-depleted mice

Group	CD8 <sup>+</sup> (%)	CD4 <sup>+</sup> (%)	Treg, Foxp3 (%)	B, CD19 (%)	NK (%)	CD8 <sup>+</sup> :B
PBS ( <i>n</i> = 10)	21.7 ± 3.3	15.2 ± 2.4	5.6 ± 0.8	7.6 ± 1.4	11.1 ± 1.6	3.8 ± 0.7
DT-NR ( <i>n</i> = 13)	38.4 ± 4.0	17.0 ± 1.7	0.9 ± 0.2	6.6 ± 2.2	6.7 ± 1.1	15.6 ± 4.5
DT-R ( <i>n</i> = 6)	45.2 ± 7.8	16.5 ± 2.9	0.4 ± 0.1	3.8 ± 1.9	5.1 ± 0.6	32.9 ± 12.5

NOTE: A group of 35 DEREK mice bearing established MCA tumors (>50 mm<sup>2</sup>) was treated with either PBS or 1,000 ng DT. Tumors in the DT-treated group were classified as nonresponders or responders (if they showed two consecutive decreases in tumor size). Tumors from PBS-treated (*n* = 10) or DT-treated nonresponder (*n* = 13) or responder (*n* = 6) mice were harvested at the second measurement, and single-cell suspensions were generated for FACS analysis. Percentage within the live (7-AAD<sup>-</sup>) CD45<sup>+</sup>CD11b<sup>-</sup> population ± SE.

Abbreviations: NR, nonresponders; R, responders.

responding groups. The increase in CD8<sup>+</sup> T cells, even in the nonresponding group depleted of Treg, begs further questions as to why these mice do not respond. Exploring what contribution tumor-infiltrating B cells might make to suppressing the CD8<sup>+</sup> T-cell response in untreated or treated nonresponding tumors is one avenue, but the potential energy or exhaustion of tumor-infiltrating CD8<sup>+</sup> T-cell populations and their proliferative capacity and multifunctionality are worthy of further investigation. Indeed, there is far more tumor microenvironment biology and genetics to explore that will improve our prediction of those tumors that will be sensitive or refractory to Treg depletion. The heterogeneity in such responses also provides an excellent platform on which to biopsy tumors and explore links between tumor-intrinsic genetic mutations and tumor-extrinsic responses that dictate the growth and fate of established tumors that undergo immune attack. It was beyond the scope of this study to examine the relative effect of Treg among other regulatory leukocytes, such as myeloid-derived suppressor cells (MDSC), tumor-associated macrophages (TAM), type I/II NKT cells, mast cells, and subsets of dendritic cells, which could potentiate tumor progression depending on their activation status. The relative hierarchy and importance of Treg, MDSC, and TAMs in immune suppression and their temporal cross-regulation during the course of tumor progression still remain to be elucidated.

We have described tumor prevention and rejection in several experimental tumors (including subcutaneous MC38 and EG7 and B16F10 lung metastases) and at least one carcinogen-induced tumor model using the DEREK mice. Treg infiltrates have been observed in a variety of mouse and human cancers, with high Treg frequency correlating with poor prognosis in several epithelial carcinomas such as ovarian, breast, and hepatocellular carcinoma (35). It will be interesting to determine whether total depletion of Treg in mouse models of these cancers can reduce or eradicate established disease. Paradoxically, Treg correlate with a good prognosis in some hematopoietic cancers, such as diffuse large B-cell lymphoma, and thus, the DEREK mice will be a useful tool to explore the role of Treg in these diseases.

No autoimmunity with Treg depletion was observed in mice conditionally depleted of Treg, and rejecting established subcutaneous MCA-induced sarcomas or the other experi-

mental tumors used here. This may be explained by the potential compensatory effect of other nontransgenic regulatory cells or the fact that several tumors used, including MCA-induced sarcomas, express tumor antigen of relatively high affinity and thus are effectively targeted without the host needing to mount a strong self-antigen response. Comparative experiments in Foxp3-DTR knock-in mice, where autoimmunity is more easily generated on Treg depletion (36), will be of interest.

Recently, clinical studies, where the inhibitory checkpoints on T cells, such as CTLA-4 and PD1, have been blocked using mAbs, have shown the efficacy of these approaches. Many strategies that attenuate Treg function are also being explored, including (a) blocking Treg suppression, (b) blocking Treg trafficking, and (c) blocking or subverting Treg differentiation (37, 38). Our data support further effort to consider these and other approaches, where human Treg may be deeply and selectively depleted even for transient periods. Our future work will explore why some tumors are refractory to host immunity after Treg depletion and whether those might be sensitive to other immunotherapeutic responses.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

We thank Michelle Stirling for maintenance of the mice at the Peter MacCallum Cancer Centre and Robert Schreiber (Washington University School of Medicine, St. Louis, MO) for providing the H22 and MAR1 mAbs.

## Grant Support

National Health and Medical Research Council of Australia (NH&MRC) Program Grant (454569) and the Victorian Cancer Agency. M.W.L. Teng was supported by a NH&MRC Peter Doherty Fellowship. M.J. Smyth received support from a NH&MRC Australia Fellowship. S.F. Ngiow was supported by a Malaysian government ASTS PhD scholarship.

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Received 05/17/2010; accepted 07/06/2010; published OnlineFirst 10/05/2010.

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