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Cancer Research

Multiple Antitumor Mechanisms Downstream of Prophylactic Regulatory T-Cell Depletion

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Abstract

Several reports have shown that prophylactic depletion of regulatory T cells (Treg) using various monoclonal antibodies (mAb) in mice can stimulate potent antitumor immune responses and prevent tumor development. These same depletion methods do not significantly suppress tumor growth in a therapeutic setting. Although different strategies to deplete FoxP3$^+$ Treg have been used, no study has systematically compared these qualitatively for the effector mechanisms they each liberate. Herein, using prophylactic depletion of FoxP3$^+$ Tregs with either anti-CD4, anti-CD25, or anti-FR4 mAbs, we have compared the cellular and effector requirements for elimination of the renal carcinoma RENCA and prevention of methylcholanthrene-induced fibrosarcoma. Collectively from these two models, it was clear that CD8$^+$ T cells and natural killer cells played an important role downstream of Treg depletion. However, whereas all three mAbs quantitatively depleted FoxP3$^+$ T cells to a similar extent, subtle differences in the downstream mechanisms of tumor control existed for all three approaches. In general, neutralization of any lymphocyte subset or effector mechanism was insufficient to alter tumor suppression initiated by Treg depletion, and in some settings, the neutralization of multiple effector mechanisms failed to prevent tumor rejection. These studies reveal that Tregs control multiple redundant elements of the immune effector response capable of inhibiting tumor initiation and underscore the importance of effectively targeting these cells in any cancer immunotherapy. *Cancer Res; 70(7): 2665–74. ©2010 AACR.*

Introduction

A number of studies have now documented tumor rejection following the prophylactic depletion of regulatory T cells (Treg; refs. 1–4). In some models, Treg depletion alone is sufficient for complete tumor eradication, whereas in others, Treg depletion acts as an adjuvant, enhancing vaccination and cytokine treatment. How these antitumor immune responses released by Treg depletion are coordinated is less clear. Tumor rejection triggered by anti-CD25 has been reported to involve CD4$^+$ and CD8$^+$ T cells, depending on the tumor model (1, 2), whereas in another study, peptide vaccination induced tumor-specific CD4$^+$ T cells, making IFN-γ sufficient for CT26 tumor rejection (5). IFN-γ was also important for rejection of Meth A sarcomas induced by treatment with the agonistic α-GITR antibody DTA-1, which is thought to induce tumor immunity by abrogating Treg activity (6). The suppressive activity of Treg on CD8$^+$ T cells has been examined in a model that used a HA-expressing derivative of CT26 called CT44 (7). Here Treg did not influence CD8$^+$ T-cell expansion or cytokine secretion but rather inhibited their cytotoxic activity *in vitro*. Depletion of the entire CD4$^+$ T-cell subset can also induce tumor rejection in some circumstances, for example, both Ag104L^d (4) and Ad5E1/ras (8) tumors were rejected by mice depleted of CD4$^+$ T cells and in both cases tumor rejection was dependent on CD8$^+$ T cells. More recently, evidence points to a critical role for Tregs in dampening natural killer cell effector functions *in vitro* and *in vivo* (9). The mechanisms seemed to involve membrane-bound transforming growth factor-β on Treg downregulating NKG2D expression on NK cells (10, 11) or Treg preventing dendritic cell exposure to interleukin-15Rα (IL-15Rα) and dendritic cell–mediated NK cell proliferation *in vivo* (12). Treg cells were recently reported to constitutively express high amounts of folate receptor 4 (FR4) and administration of anti-FR4 monoclonal antibody (mAb) specifically reduced Treg cells, provoking effective tumor immunity in tumor-bearing mice, but the mechanisms of rejection were not characterized (13).

Because therapeutic CD25$^+$ Treg depletion has failed to consistently cause suppression of established tumors, even when used in combination with other immunotherapies (14), we have chosen to study the mechanisms of tumor suppression

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caused by the early absence of Treg at the time of tumor or carcinogen inoculation. We have focused on the effector cells, cytokines, and cytotoxic molecules required for successful tumor cell elimination following Treg depletion. The experimental renal carcinoma RENCA and spontaneous fibrosarcomas induced by the chemical carcinogen methylcholanthrene (MCA) are both well characterized for host adaptive ($CD8^+$ CTL) and innate (NK/NKT) immune mechanisms that control their respective outgrowth in mice (15–19). In addition, MCA-induced fibrosarcomas were strikingly infiltrated with $FoxP3^+$ Tregs (20), and anti-CD25 mAb significantly reduced MCA-induced fibrosarcoma incidence in an IFN-γ–dependent fashion (21, 22).

Here we show that RENCA and MCA-induced fibrosarcomas are both suppressed following a variety of strategies (anti-CD4, anti-FR4, and anti-CD25) that partially deplete Tregs. Quantitatively these strategies depleted $FoxP3^+$ T cells and triggered tumor rejection to a similar extent, but qualitatively each method revealed distinct tumor rejection mechanisms, with no single lymphocyte subset or effector molecule completely accounting for the tumor immunity in these mouse models of cancer.

Materials and Methods

Mice and tumor cell lines. Wild-type (WT) and gene-targeted BALB/c and C57BL/6 mice were either purchased from the Walter and Eliza Hall Institute Animal Facility or bred and maintained in house. BALB/c and C57BL/6 gene-targeted strains were either derived on a pure background (C57BL/6 perforin, pfp) or backcrossed to the appropriate strains for 10 to 12 generations (Jα18, IFN-γ, TRAIL, DR5, IL-4, IL-13, granzyme B, pfp × IFN-γ). Adult mice (age, 6–12 wk) were used in all experiments, unless otherwise indicated, and experiments were conducted under specific pathogen-free conditions according to the Peter MacCallum Cancer Centre Animal Experimental Ethics Committee guidelines. The BALB/c-derived renal adenocarcinoma cell line RENCA was maintained in RPMI 1640 supplemented with 10% FCS, L-glutamine, and penicillin/streptomycin.

Tumor models. Groups of BALB/c or BALB/c gene–targeted mice were injected s.c. with 1×10^5 RENCA cells in RPMI media alone. The depletion schedule and dosage of Tregs with anti-CD4, anti-CD25, or anti-FR4 are outlined in Fig. 1A. Primary tumor growth was measured with digital calipers, and the tumor area was calculated by multiplying the shortest and longest axis of the tumor. Mice were culled when tumors reached 100 mm^2 in the area. Groups of 20 male B6 WT and other gene-targeted mice were inoculated s.c. in the hind flank with 400 μg of 3-MCA (Sigma-Aldrich) in 0.1 mL of corn oil. Development of fibrosarcomas was monitored periodically over the course of 56 to 250 d. Tumors, with a diameter of >3 mm and showing progressive growth, were recorded as positive. The depletion schedule and dosage of Tregs with either rat anti-mouse CD4 (GK1.5), rat anti-mouse CD8 (53-6.7), rat anti-mouse CD25 (PC61), rat anti-mouse FR4 (Th6), or the control rat antibody MAC4 (cIg; all purified in house) are outlined in Fig. 5A and Supplementary Fig. S6A. Anti-ASGM1 was purchased from Wako Pure Chemicals.

Statistical analysis. Statistical significance of tumor-free survival was assessed using a log-rank test ($P < 0.05$).

Results

Treg depletion causes RENCA tumor rejection. To investigate if Tregs influence the growth of RENCA tumors, three depletion strategies were tested: anti-CD4, anti-CD25, or anti-FR4 mAb treatments. An experimental overview outlining treatment schedules and tumor challenges is shown in Fig. 1A. Multiple anti-CD4 injections were used to optimally deplete the larger number of $CD4^+$ T cells, and this protocol was used throughout the RENCA studies. All three strategies equivalently depleted $FoxP3^+$ cells in the lymph node (Supplementary Fig. S1), spleen, and blood (data not shown). Optimal depletion was observed at day 3 after the last mAb administration, and depletion was effective for up to 7 days in each case with respective $FoxP3^+$ and $FoxP3^-$ cells returning equivalently from days 7 to 14 (data not shown). Anti-CD4 was most effective in depleting $FoxP3^+CD4^+$ cells but also depleted most other $CD4^+$ T cells including $FoxP3^-CD4^+$ T cells (Supplementary Fig. S1C). Anti-CD25 and anti-FR4 were approximately equivalent in depleting $FoxP3^+$ Treg, but anti-FR4 also depleted many $FoxP3^-FR4^+$ T cells (~23–5%), comprising largely $CD4^+$ T cells (Supplementary Figs. S1 and S2). Smaller populations of $FoxP3^-$ $CD8^+$ T cells and $FR4^+TCR\beta^-$ cells were also depleted (Supplementary Fig. S2); however, notably FR4 was only expressed on a very small population of NK cells (<10%; ref. 13). Anti-CD4, anti-CD25, and anti-FR4 mAb treatments were all able to stimulate rejection of RENCA tumors compared with untreated mice (Fig. 1B and C) or cIg-treated mice (data not shown). Consistently, tumors grew larger before regressing within 3 weeks in mice treated with anti-CD4 or anti-CD25 compared with those receiving anti-FR4 (Fig. 1B). Although the rate of tumor regression was variable between individual mice, no relapses were observed and no overt signs of autoimmunity were noted (over a 1-year observation period, data not shown; Fig. 1B). Over a large number of mice ($n = 77–114$) observed, anti-CD4 was more powerful in causing rejection of RENCA than anti-CD25 or anti-FR4 (Fig. 1C). A single day −1 and days −1, 0, and +1 protocol of anti-CD4 also enabled complete rejection of all RENCA tumors (data not shown). In some tumor models, anti-CD4 treatment can induce tumor rejection by depleting regulatory CD1d-restricted T cells (23); however, growth of RENCA tumors in $CD1d^{-/-}$ and $J\alpha18^{-/-}$ mice was equivalent to that in WT mice (Fig. 1D). Treg depletion failed to stimulate antitumor immunity when used therapeutically (starting on day 10 post tumor challenge) against RENCA (Supplementary Fig. S3).

$CD8^+$ cells are required for RENCA tumor rejection following Treg depletion. To determine how RENCA tumors were rejected following Treg depletion, mice were first depleted of $CD8^+$ T cells coincident with anti-CD4, anti-FR4, or anti-CD25 treatments and then challenged with RENCA. None of the 10 mice treated with anti-CD4 plus anti-CD8,

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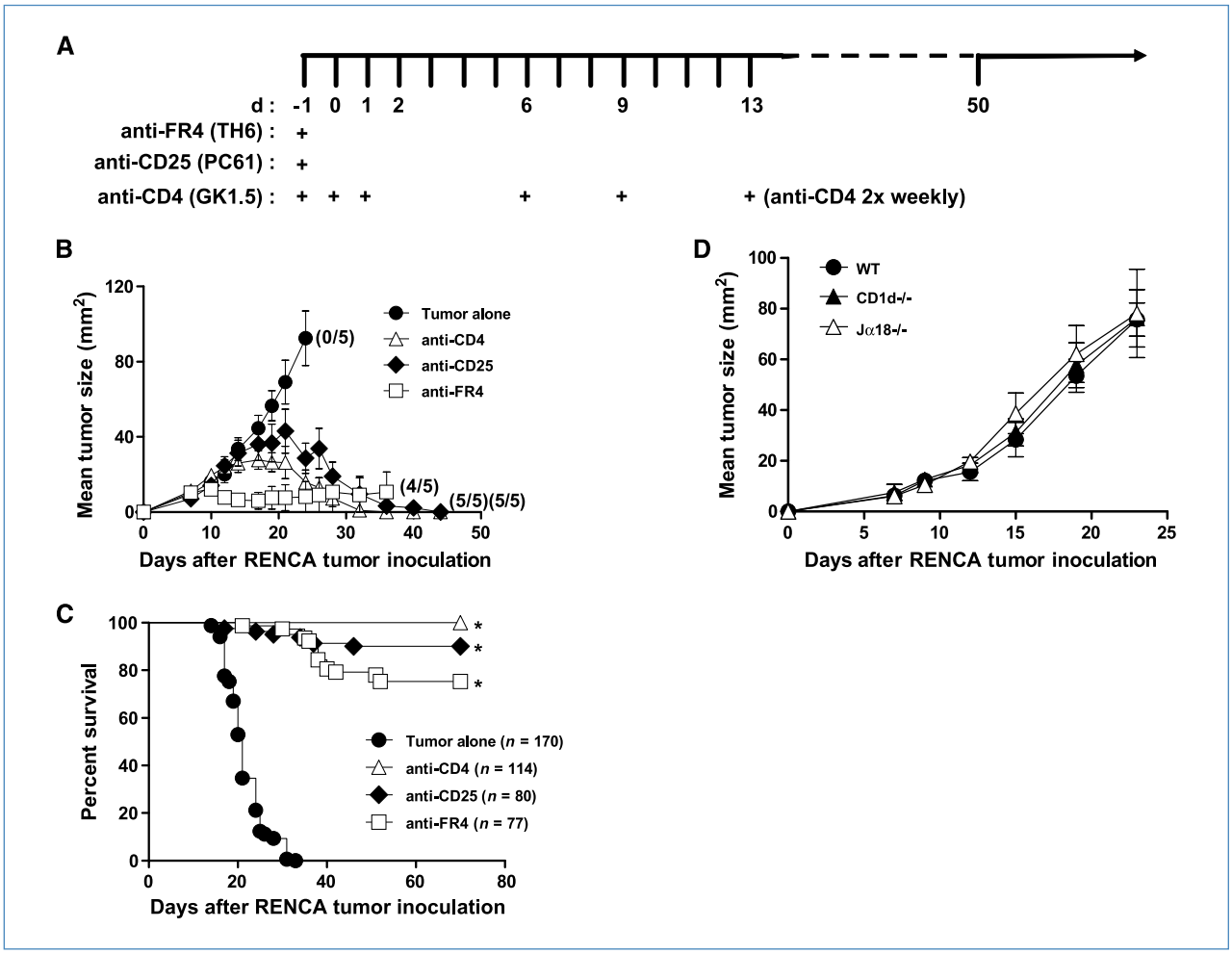

Figure 1. Mice depleted of $CD4^+$, $FR4^+$, or $CD25^+$ T cells reject RENCA tumors. The standard mAb and tumor challenge regimen used in all experiments (unless otherwise noted) is depicted in A. Groups of mice received 500 μg (days −1, 0, and +1), thereafter followed by 250 μg of anti-CD4 (i.p.) as indicated, 500 μg of anti-CD25 (i.p.), or 10 μg of anti-FR4 (i.v.) on days indicated. All mice received 10^5 RENCA tumor cells s.c. on day 0. B, representative graph of the mean tumor growth of the various groups (calculated by the product of two perpendicular axes, mm^2) ± SEM and plotted versus time. Number of cured mice in parentheses. C, the survival of all mAb-treated mice pooled from all mice (n = 77–170; pooled from 14–34 experiments) and plotted as percentage survival over time. D, depicts the growth of RENCA tumors in BALB/c WT, $CD1d^{-/-}$, and $J\alpha18^{-/-}$ mice. WT, $CD1d^{-/-}$, and $J\alpha18^{-/-}$ mice were challenged with 10^5 RENCA cells, and tumor growth was subsequently monitored by caliper square measurements. Data are presented as mean tumor area (mm^2) ± SEM (n = 5 mice per group). Statistical differences in survival between tumor-bearing mice treated with the indicated antibodies and nontreated tumor-bearing mice were determined by log-rank test; *, $P < 0.05$.

1 of the 10 anti-FR4 plus anti-CD8, and 0 of the18 mice treated with anti-CD25 plus anti-CD8 were able to reject RENCA tumors, showing that $CD8^+$ T cells were essential for RENCA tumor rejection (all $P < 0.0001$ compared with Treg depletion alone; Fig. 2A). By contrast, sustained NK cell depletion alone had little or no effect on the ability of anti-CD4–treated or anti-FR4–treated mice to reject tumors. Interestingly, NK cell–depleted tumor-bearing mice treated with anti-CD25 were less able to reject tumors compared with tumor-bearing mice treated with only anti-CD25 ($P < 0.0001$); however, none of the nontreated tumor-bearing mice survived ($P < 0.0001$; Fig. 2B). This suggested that NK cells alone were not essential for tumor rejection following anti-CD4 or anti-FR4 treatment but were partially required following anti-CD25 treatment. Depletion of both NK cells and $CD8^+$ T cells abrogated all protection from RENCA afforded by anti-CD4, anti-FR4, or anti-CD25 treatments (Fig. 2C). A role for $CD8^+$ T cells was further supported by showing that anti-CD4–mediated, anti-FR4–mediated, and anti-CD25–mediated protection from RENCA tumor was associated with an increase in the proportion of tumor-infiltrating $CD8^+$ T cells compared with control immunoglobulin-treated tumors (Supplementary Fig. S4A and B). We monitored the *ex vivo* restimulated cytolytic activity of tumor draining $CD8^+$ T cells after anti-CD4, anti-FR4, and anti-CD25 treatments, and it was clear that all three treatments significantly enhanced the tumor-specific cytotoxic activity of $CD8^+$ T cells compared with equivalent control mice bearing RENCA tumors (Supplementary Fig. S4C).

Effector cytokines required for rejection of RENCA tumors following Treg depletion. Next, the role of the major

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Th1 and Th2 polarizing cytokines was investigated. All anti-CD4–treated IFN-$\gamma^{-/-}$ (8 of 8) and WT (114 of 114) mice were able to reject RENCA tumors, whereas control mice of both genotypes (24 of 24 and 170 of 170, respectively) all succumbed to tumor growth (Fig. 3A). Similarly, the majority of anti-FR4–treated IFN-$\gamma^{-/-}$ (11 of 12) and WT (58 of 77) mice were able to reject RENCA tumors (Fig. 3A). In contrast, whereas 90% (72 of 80) WT mice treated with anti-CD25 were able to reject RENCA tumors, no IFN-$\gamma^{-/-}$ mice treated with anti-CD25 (0 of 15 mice) survived tumor challenge ($P < 0.0001$ compared with WT mice treated with anti-CD25; Fig. 3A). Thus, whereas IFN-γ was required for tumor rejection following anti-CD25 treatment, it was dispensable for tumor clearance following anti-CD4 or anti-FR4 treatment.

Anti-CD4 was able to induce rejection of RENCA tumors in 100% of IL-4–deficient and IL-13–deficient mice (Fig. 3B and C, respectively), whereas anti-FR4 caused reduced rejection of RENCA tumors in IL-$4^{-/-}$ (45.5%, 10 of 22; $P = 0.00053$) and IL-$13^{-/-}$ (52.9%, 9 of 17; $P = 0.02923$) mice. Whereas anti-CD25 treatment was able to induce RENCA tumor rejection in 90% of WT mice (72 of 80), only 18.2% (2 of 11) of IL-$4^{-/-}$ mice and 18.2% (2 of 11) of IL-$13^{-/-}$ mice treated in parallel rejected their tumors ($P < 0.0001$ and $P < 0.0001$, respectively; Fig. 3B and C). Thus, Th2 cytokines are key for rejection of RENCA tumors following anti-CD25, partially important after anti-FR4 and not relevant after anti-CD4 treatment. Collectively, these data showed that tumor rejection following anti-CD25 treatment required a combination of both Th1 and Th2 cytokines (IFN-γ, IL-4, and IL-13), whereas tumor rejection following anti-CD4 occurred in the absence of the major Th1 and Th2 polarizing cytokines. In contrast, tumor rejection following anti-FR4 treatment did not require IFN-γ but was partially dependent on Th2 cytokines (IL-4, IL-13).

The role of cytotoxic effector molecules in tumor rejection. Perforin was found to be dispensable for RENCA tumor rejection in anti-CD25–treated mice (Fig. 4A). A minor reduction in tumor rejection was noted in either anti-CD4–treated or anti-FR4–treated pfp$^{-/-}$ mice compared with WT mice (Fig. 4A; $P < 0.0002$ and $P = 0.0104$, respectively); however, anti-FR4–treated pfp$^{-/-}$ mice were still able to mediate tumor

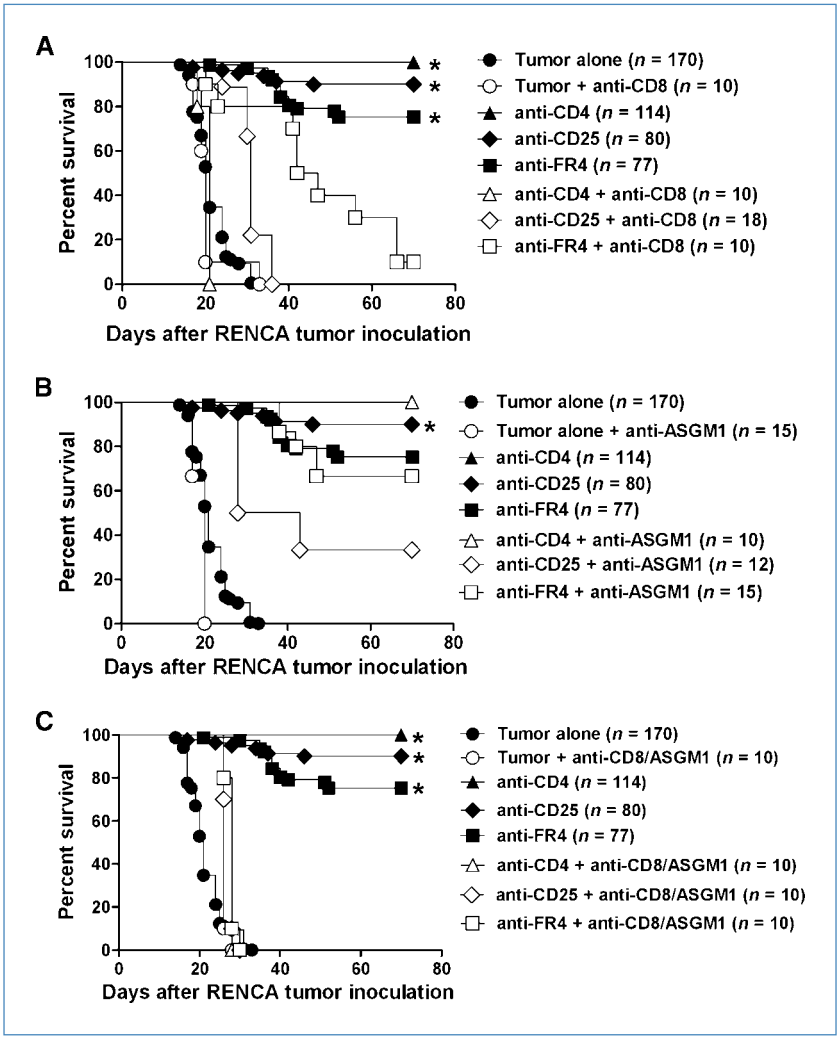

Figure 2. CD8$^+$ T cells are required for tumor rejection. Groups of BALB/c mice were injected s.c. with 10^5 RENCA cells and treated with mAbs, as outlined in Fig. 1A. A, the indicated groups of mice were additionally depleted of CD8$^+$ T cells by i.p. injection of 100 μg of anti-CD8 on days –1, 0, and 1 and twice weekly thereafter. B, the indicated groups of mice were alternatively depleted of NK cells by i.v. injection of 100 μg of anti-ASGM1 on days –1 and 0 and every 7 d thereafter until tumors regressed or mice were culled. C, the indicated groups of mice were depleted of both CD8$^+$ T and NK cells as in A and B. The survival of all antibody-treated mice was calculated from multiple experiments (pooled from 2 to 34 experiments) and is plotted as percentage survival over time. Statistical differences in survival between tumor-bearing mice treated with the indicated antibodies and tumor-bearing mice depleted of CD8$^+$ T cells and/or NK cells and treated with the indicated antibodies were determined by log-rank test; *, $P < 0.05$.

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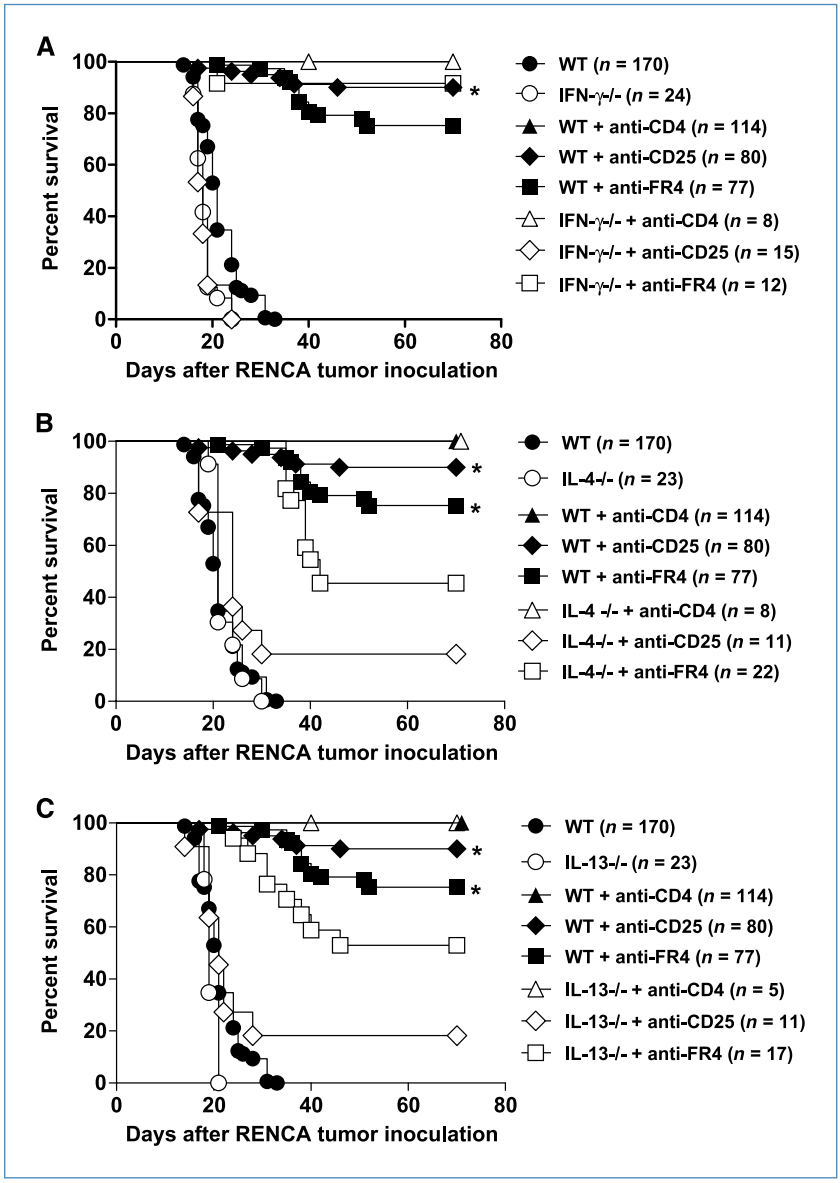

Figure 3. IL-4, IL-13, and IFN-γ are required for tumor rejection following anti-CD25 but not anti-CD4 or anti-FR4 treatment. Groups of BALB/c WT (A–C), IFN-$\gamma^{-/-}$ (A), IL-$4^{-/-}$ (B), or IL-$13^{-/-}$ (C) mice were injected s.c. with 10^5 RENCA cells on day 0. In all panels, WT and gene-targeted mice were treated as described in Fig. 1A. The survival of WT or gene-targeted mice after mAb treatment was calculated from multiple experiments (pooled from 1 to 34 experiments) and is plotted as percentage survival over time. Statistical differences in survival between WT tumor-bearing mice treated with the indicated antibodies and gene-targeted tumor-bearing mice treated with the indicated antibodies were determined by log-rank test; *, $P < 0.05$.

rejection compared with control-treated mice ($P < 0.0001$). Tumors grew at a similar rate in WT and pfp$^{-/-}$ mice, and all control-treated mice succumbed to tumor growth with similar kinetics. Very similar results were obtained in TRAIL$^{-/-}$ mice, wherein anti-CD4 and anti-CD25 treatments were able to induce RENCA tumor rejection almost equivalently to WT mice, providing further evidence that this effector pathway was also not essential (Fig. 4B).

We next used three strategies to investigate the contribution of death receptor signaling to tumor regression: blocking antibodies, RENCA cells overexpressing the antiapoptotic molecule FLIP (24), and where possible gene-targeted mice. In WT mice, treatment with a combination of anti-FasL, anti-TRAIL, and anti-tumor necrosis factor-α (TNF-α; called anti-FTT) had no significant effect on tumor rejection following either anti-CD4 or anti-CD25 treatment, implying that FasL, TRAIL, and TNF-α were not essential for tumor clearance (Supplementary Fig. S5A). Anti-CD25–treated mice were able to reject RENCA-FLIP and RENCA-Flag tumors with similar efficiency, whereas anti-CD4 was able to induce the rejection of all RENCA-Flag tumors and most RENCA-FLIP tumors (not statistically different, $P > 0.05$; Supplementary Fig. S5B). These results suggested that tumor rejection following anti-CD25 or anti-CD4 treatment occurred independently of death receptor signaling.

As expected, anti-CD25–treated pfp$^{-/-}$IFN-$\gamma^{-/-}$ mice failed to effectively eliminate RENCA tumors, with only 2 of 17 mice surviving tumor challenge ($P < 0.0001$ compared with anti-CD25–treated WT mice tested in parallel), in keeping with a requirement for IFN-γ in tumor rejection following anti-CD25 treatment (Supplementary Fig. S6A). In contrast, all (10 of 10) anti-CD4–treated pfp$^{-/-}$IFN-$\gamma^{-/-}$ mice were able

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to reject RENCA tumors. The effect of the simultaneous blockade of the pfp, IFN-γ, FasL, TRAIL, and TNF-α pathways on tumor clearance was also investigated, and anti-CD4 mAb–treated mice were still able to reject RENCA tumors (Supplementary Fig. S6B) and RENCA-FLIP and RENCA-Flag tumors (Supplementary Fig. S6C). Thus, following CD4 depletion, tumor rejection still occurred in the collective absence of all five effector molecules.

Treg depletion suppresses the development of MCA-induced sarcoma. To investigate if Tregs also influence the development of spontaneous tumors, two variations of the same three depletion strategies (anti-CD4, anti-CD25, or anti-FR4 mAb treatment) were tested (experimental overview outlining treatment schedules and MCA challenges; Fig. 5A and Supplementary Fig. S7A). As shown in BALB/c mice, similarly effective depletions of populations of FoxP3$^+$ T cells were observed for each treatment schedule in C57BL/6 spleen, lymph nodes, and blood (data not shown). Anti-CD4, anti-CD25, and anti-FR4 mAb treatments were all able to prevent the development of MCA-induced sarcomas compared with control mAb (Fig. 5B–D and Supplementary Fig. S7B–D). Consistently, anti-FR4 reduced sarcoma formation regardless of whether it was given before MCA inoculation or twice weekly for 4 weeks from the point of MCA inoculation (Fig. 5D and Supplementary Fig. S7D). Anti-CD4 and anti-CD25 were also effective (Fig. 5B and C and Supplementary Fig. S7B and C), but anti-CD25 seemed less effective when given in a prolonged fashion (Supplementary Fig. S7C). No overt signs of autoimmunity were noted (over a 1-year observation period) in any of these mice that remained tumor free (data not shown).

CD8$^+$ and NK cells collectively account for MCA-induced sarcoma suppression following anti-FR4 treatment. Anti-FR4 prevented MCA-induced sarcoma in mice depleted of either CD8$^+$ T cells (Fig. 6A) or NK cells (Fig. 6B), showing that neither CD8$^+$ T cells nor NK cells were essential for tumor rejection stimulated by Treg depletion. By contrast, combined CD8$^+$ T-cell and NK cell depletion resulted in a complete loss of the protection afforded by anti-FR4–mediated Treg depletion (Fig. 6C). Type I NKT cells are thought to be upstream of NK cells in natural host immune protection from MCA-induced sarcoma (18), but interestingly anti-FR4–mediated Treg depletion in type I NKT cell–deficient Jα18$^{-/-}$ mice was still effective in affording mice protection from MCA-induced sarcoma (Fig. 6D). Interestingly, combined CD8$^+$ T-cell and NK cell depletion also resulted in a complete loss of the protection afforded by anti-CD25–mediated (Supplementary Fig. S8A–D) and anti-CD4–mediated Treg depletion (data not shown). Thus following Treg depletion, either CD8$^+$ T cells or NK cells can act to prevent MCA-induced sarcomas.

Effector mechanisms required for suppression of MCA-induced sarcoma following anti-FR4 treatment. Anti-FR4 treatment prevented MCA-induced sarcoma in pfp$^{-/-}$ (Supplementary Fig. S9A), granzyme B$^{-/-}$ (Supplementary Fig. S9B), DR5$^{-/-}$ (Supplementary Fig. S9C), TNF-α$^{-/-}$ (Supplementary Fig. S9D), and IL-4$^{-/-}$ (Supplementary Fig. S9E) mice, showing

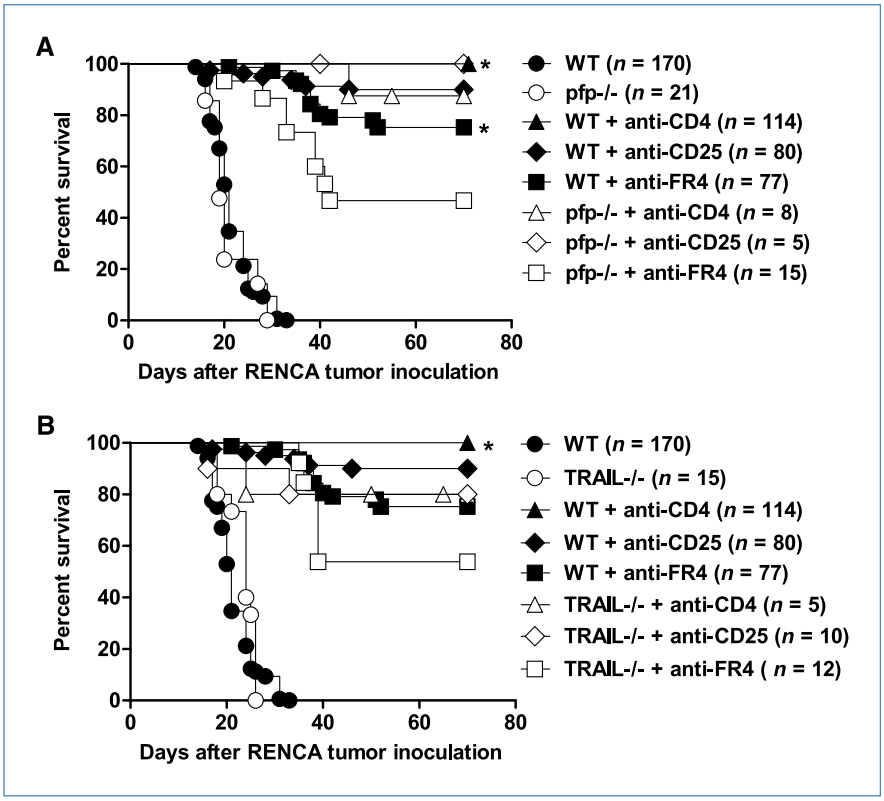

Figure 4. Differential requirements for perforin and TRAIL in tumor rejection following anti-CD4, anti-FR4, or anti-CD25 treatment. Groups of BALB/c WT (A and B), pfp$^{-/-}$ (A), or TRAIL$^{-/-}$ (B) mice were injected s.c. with 10^5 RENCA cells on day 0. In all panels WT and gene-targeted mice were treated as described in Fig. 1A. The survival of WT or gene-targeted mice after mAb treatment was calculated from multiple experiments (pooled from 1 to 34 experiments) and is plotted as percentage survival over time. Statistical differences in survival between WT tumor-bearing mice treated with the indicated antibodies and gene-targeted tumor-bearing mice treated with the indicated antibodies were determined by log-rank test; *, $P < 0.05$.

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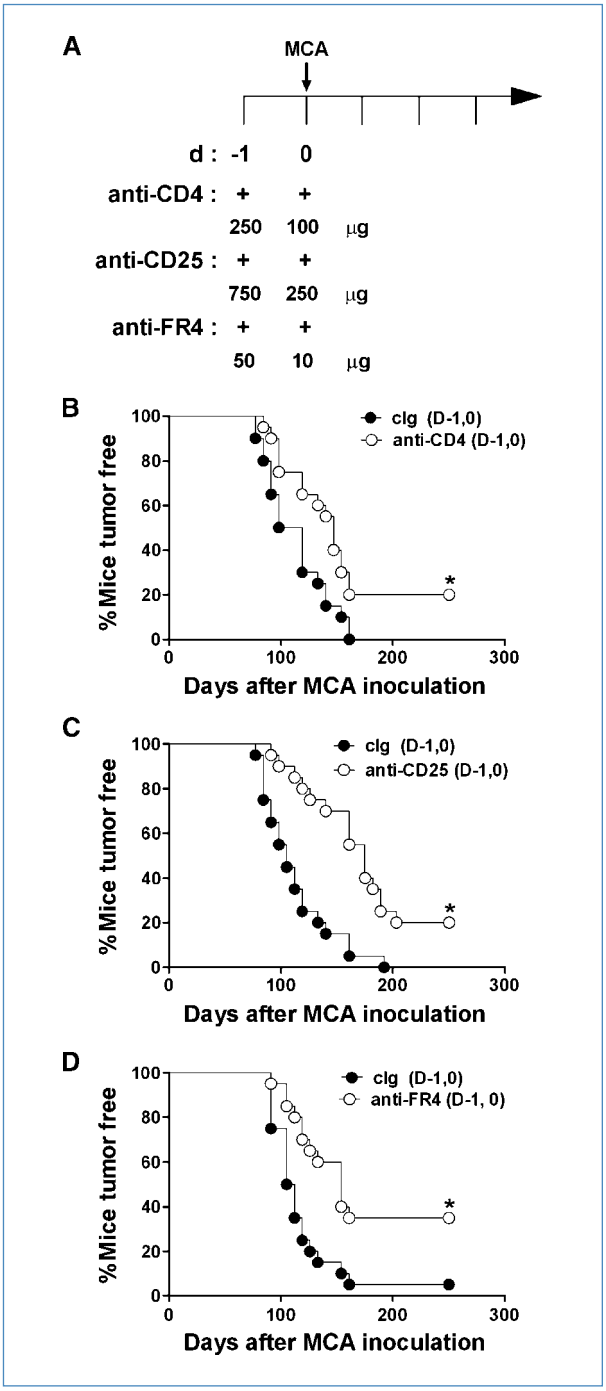

Figure 5. Treg depletion suppresses the development of MCA-induced sarcoma. Groups of male C57BL/6 mice ($n = 20$) were injected s.c. with MCA (400 μg) on day 0. The schedules for depleting Tregs on days −1 and 0 (A) with anti-CD4 (B), anti-CD25 (C), anti-FR4 (D), or clg (B–D) mAbs are depicted. Anti-CD4 and anti-CD25 mAbs were injected i.p., whereas anti-FR4 mAbs were injected i.v. Tumor growth was monitored weekly by caliper square measurements, and the percentage of tumor-free mice of each group of mice is shown plotted against days after MCA inoculation. Statistical differences in the proportion of tumor-free mice treated with anti-CD4, anti-CD25, or anti-FR4 and tumor-free mice treated with clg (groups of 20 mice per treatment) were determined by log-rank test; *, $P < 0.05$.

that neither pfp, granzyme B, TRAIL, TNF-α, nor IL-4 was essential for tumor rejection stimulated by Treg depletion. By contrast, loss of IFN-γ (Supplementary Fig. S9F) somewhat reduced the effectiveness of Treg depletion, and mice deficient for both pfp and IFN-γ showed no protection afforded by anti-FR4–mediated Treg depletion (Supplementary Fig. S9G). Given the importance of IL-4 and IFN-γ in anti-CD25–mediated rejection of RENCA, we also examined MCA sarcoma induction in these mice following anti-CD25 treatment. Clearly, like anti-FR4 treatment, IFN-γ, but not IL-4, was essential for the prevention of MCA-induced sarcomas stimulated by anti-CD25 (Supplementary Fig. S10A and B).

Discussion

In this study we examined the mechanism of host immune suppression of experimental RENCA tumors or chemical carcinogen–induced fibrosarcomas following Treg depletion. Three methods of early depletion of regulatory FoxP3+ T cells were used (anti-CD4, anti-FR4, or anti-CD25 treatment) and collectively, while it was clear that $CD8^+$ T cells and NK cells played an important role downstream of Treg depletion, each method revealed subtly different effector pathways of tumor rejection. The tumors used in this study did not express FR4, CD4, or CD25, and thus, the effect of the mAbs was not directly on the tumor but rather on the host (data not shown). In all cases, $CD8^+$ T cells were required for RENCA tumor rejection; however, following anti-CD25 treatment tumor rejection required an unusual combination of IFN-γ, IL-4, and IL-13 and occurred independently of the tested death-inducing pathways (perforin, TRAIL, FasL, and TNF-α). By contrast, tumor rejection following anti-CD4 or anti-FR4 depletion did not require any of the cytokines examined (IFN-γ, IL-4, and IL-13 were dispensable). Tumor rejection following anti-FR4 depletion seemed to partially involve perforin and TRAIL pathways, but neither pathway was critical. Despite the large number of cytokines and effector pathways tested, the current study failed to determine how RENCA tumors were eliminated following CD4 depletion.

Early depletion using anti-CD25, anti-CD4, and anti-FR4 mAbs was also effective in preventing MCA-induced fibrosarcoma. Further analysis of anti-FR4 treatment revealed that Treg depletion remained largely effective in preventing tumor formation in single effector molecule (pfp, granzyme B, IL-4, DR5, TNF-α)–deficient mice or those mice deficient in $CD8^+$ T cells, type I NKT cells, or NK cells alone. By contrast, all Treg depletion strategies were completely ineffective in mice depleted of both $CD8^+$ T cells and NK cells, anti-FR4 and anti-CD25 were less effective in IFN-γ–deficient mice, and anti-FR4 was not effective in mice doubly deficient for pfp and IFN-γ. Collectively, this work highlights the redundancy and diversity of effector responses that must be considered when removing key checkpoints in immune regulatory control.

The finding that pfp was not essential for RENCA tumor rejection following Treg depletion was interesting in the light of the fact that all methods of depletion enhanced the proportion of $CD8^+$ tumor-infiltrating lymphocytes and increased the cytotoxic capacity of CTL in the tumor-draining lymph

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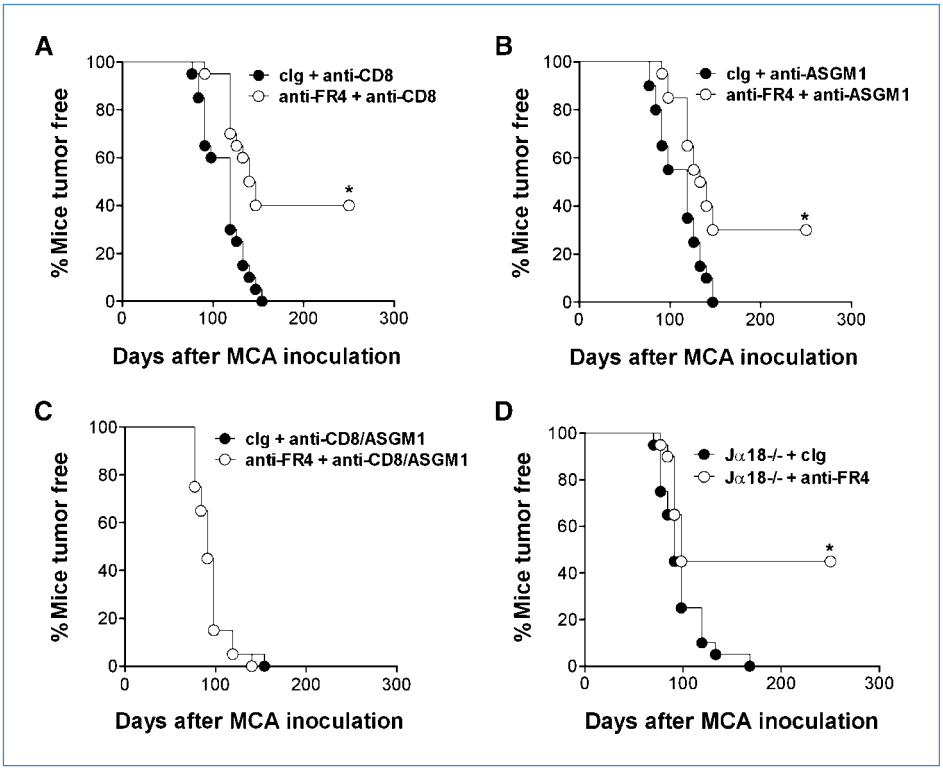

Figure 6. $CD8^+$ and NK cells collectively account for MCA-induced sarcoma suppression following anti-FR4 treatment. Groups of male C57BL/6 or C57BL/6 $J\alpha18^{-/-}$ mice ($n = 20$) were injected s.c. with MCA (400 μg) and treated i.v. with anti-FR4 mAbs [on days −1 (50 μg) and 0 (10 μg), as outlined in Fig. 5A]. The indicated groups of mice were additionally depleted of $CD8^+$ T cells by i.p. injection of 100 μg of anti-CD8 on days −1, 0, and +1 and thereafter twice weekly for 4 wk (A); NK cells by i.p. injection of 100 μg of anti-ASGM1 on days −1, 0, and +1 and thereafter weekly for 4 wk (B); or $CD8^+$ T cells and NK cells together (C). D, type I NKT cell–deficient C57BL/6 $J\alpha18^{-/-}$ mice were treated with control immunoglobulin or anti-FR4 on days −1 and 0 as above. Mice were monitored for tumor development, and proportion of tumor-free mice is plotted against days after MCA inoculation. Statistical differences in the proportions of tumor-free mice between groups depleted of $CD8^+$ T cells or NK cells treated with anti-CD4, anti-CD25, or anti-FR4 and tumor-free mice depleted of $CD8^+$ T cells or NK cells treated with cIg (groups of 20 mice per treatment) were determined by log-rank test; *, $P < 0.05$.

nodes. Furthermore, two previous studies suggested that Tregs control antitumor $CD8^+$ T-cell responses by inhibiting cytotoxicity (7, 25). However, whereas RENCA cells are susceptible to pfp-mediated lysis *in vitro*, RENCA-specific $CD8^+$ T cells can protect mice from growth of RENCA tumors *in vivo* independently of pfp (24). Furthermore, the cytokines IFN-γ, IL-4, and IL-13 were more crucial for tumor rejection in the RENCA model, as mice lacking these cytokines were unable to clear tumors following anti-CD25 treatment. Our finding that tumor rejection in the RENCA model is more dependent on cytokines indicates that Treg may affect the antitumor immune response at a number of levels, depending on the model system under study. In concert, another recent study has suggested that Tregs act to inhibit the accumulation of IFN-γ–producing $CD8^+$ T cells within tumor tissues rather than by inhibiting CTL cytotoxic activity (26). Notably, in our study, whereas anti-FR4 was equally effective in WT and pfp-deficient mice, a role for pfp in the carcinogen-induced fibrosarcoma model was revealed by the reduced effectiveness of anti-FR4 in $pfp^{-/-}$ IFN-$\gamma^{-/-}$ mice compared with IFN-γ–deficient mice.

Unexpectedly, rejection of RENCA tumors following anti-CD25 treatment required both Th1 and Th2 type cytokines. Generally Th1 and Th2 responses are thought to represent opposite poles of an immune response (27), and in some cases, they directly antagonize each other's functions. A role for IFN-γ in rejection of tumors following Treg depletion has been previously documented (5); however, a role of IL-4 and IL-13 has not previously been described. It is clear that under some circumstances Th2-type responses to tumors can be beneficial (28–32), including in the RENCA model (33). Furthermore, evidence that Th1 and Th2 responses can act in concert to eliminate tumors has been reported (34), including in the RENCA model (35). Collectively, these observations support that notion that in some cases Th1 and Th2 cytokines can act in concert to generate effective antitumor responses. The tumor cytokine microenvironment resulting in mice that had been depleted with anti-CD4, anti-CD25, or anti-FR4 may regulate the type of effector response mounted; however, precisely determining Treg factors that suppress effector responses in these tumors will require the use of conditional knockouts of candidate suppressor molecules expressed by Treg and/or adoptive transfer techniques.

Models that use carcinogens, like MCA, may be considered more relevant for deconstructing the relationship between the immune system and developing tumors. It was revealing

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that such early and transient Treg depletion was sufficient to confer protection in the MCA model, and these data would suggest that the $FR4^{hi}FoxP3^{+}$ cell subset of Tregs very effectively depleted by this protocol is particularly important in suppressing the immune response to carcinogen inoculation. Certainly our preliminary analyses indicate that anti-FR4 is not effective in significantly suppressing established tumor growth of *de novo* MCA-induced sarcomas (data not shown). A key question arising from the studies of Betts and colleagues (21) and Nishikawa and colleagues (22) using MCA-induced sarcomas was this: what type of effector cells promoted tumor suppression after depletion of Tregs? Tregs induced by immunization inhibited NK and NKT cells capable of preventing the development of MCA-induced tumors (22). Here we have shown for the first time that $CD8^{+}$ T cells, NK cells, and type I NKT cells alone are not essential, rather a combination of $CD8^{+}$ T cells and NK cells seemed to account for the immune effector function stimulated by Treg depletion by anti-FR4 or anti-CD25. Extending the work of Betts and colleagues (21), we assessed a variety of effector pathways potentially downstream of Treg depletion, including IFN-γ, pfp, IL-4, granzyme B, TNF-α, and TRAIL. No pathway alone was absolutely essential, although in agreement with Betts and colleagues (21) IFN-γ was important. Our data support recent findings in K-ras–driven tumorigenesis, which indicate the central role for $FoxP3^{+}$ Tregs in preventing lung adenocarcinomas (36) and a previous report indicating the role of $CD25^{+}$ Tregs in promoting erbB2-driven mammary carcinoma (37).

Overall, the study presented here shows that a number of redundant effector mechanisms can potentially eradicate tumors when Treg suppression mechanisms are prophylactically abrogated. Clearly the finding that the method of Treg depletion can significantly influence the downstream immune responses raises some significant issues for the application of various Treg depletion strategies in priming immunity in the context of prophylactic vaccines or minimal residual disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Multiple Antitumor Mechanisms Downstream of Prophylactic Regulatory T-Cell Depletion

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