

Anti-TIM3 Antibody Promotes T Cell IFN- γ -Mediated Antitumor Immunity and Suppresses Established Tumors

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Abstract

Strategies to activate and rescue exhausted tumor-specific T cells, including the use of monoclonal antibodies (mAb) that block the negative costimulatory receptors CTLA-4 and PD-1 are proving very effective, but TIM3 has been relatively neglected as a target. Here we report an extensive characterization of the therapeutic activity and mechanism of action of an anti-mouse TIM3 mAb against experimental and carcinogen-induced tumors. For the first time we specifically define the mechanism of antitumor action of anti-TIM3 requiring IFN- γ producing CD8⁺ T cells and CD4⁺ T cells, and a higher ratio of tumor infiltrating CD8⁺:CD4⁺ T cells correlating with therapeutic success. Interestingly, in some models, anti-TIM3 appeared to be effective sometime before the appearance and accumulation of significant TIM3⁺PD-1⁺ T cell populations in tumor bearing mice. Anti-TIM3 displayed modest prophylactic and therapeutic activity against a small fraction of carcinogen-induced sarcomas, but comparative and combination studies of anti-TIM3 with anti-CTLA-4 and anti-PD-1 against experimental and carcinogen-induced tumors suggested that these agents might be well-tolerated and very effective in combination. *Cancer Res*; 71(10); 3540–51. ©2011 AACR.

Introduction

T cell immunoglobulin and mucin domain 3 (TIM3) is a member of a relatively newly described 8 member TIM family (1). Amongst leukocytes TIM3 was first reported as expressed by IFN- γ -secreting T helper 1 (Th1) cells, and subsequently on dendritic cells (DC), monocytes, CD8⁺ T cell and other lymphocyte subsets (1, 2). Stimulation of TIM3 by its ligand results in Th1 cell death, implicating a role for TIM3 in negatively regulating Th1 response (3). Consequently, blockade of TIM3 has been shown to increase IFN- γ -secreting cells in the microenvironment, mediating the pathophysiology of Th1-driven autoimmune diseases (1), whereas TIM3 expressed on macrophages and monocytes has also been implicated in phagocytosis of apoptotic cells and cross-presentation (4). In HIV patients, TIM3 and programmed death-1 (PD-1) mark distinct subsets of exhausted CD8⁺ T cells (5). By contrast, TIM3 has been relatively poorly studied in the context of tumor immunity.

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Given that TIM3 negatively regulates IFN- γ -mediated Th1 responses, the use of anti-TIM3 monoclonal antibody (mAb) may complement therapies relieving T cell anergy/exhaustion/tolerance pathway [anti-PD-1 and anti-Cytotoxic T Lymphocyte Antigen-4 (CTLA-4)] (6). Exhausted T cells are coregulated by multiple inhibitory receptors during an immune response, including the negative costimulatory receptors, CTLA-4, PD-1, and TIM3. These pathways have been most clearly studied in the context of chronic viral infection (7), however our understanding of comparative importance of these pathways in cancer is poor. That said, anti-CTLA-4 and anti-PD-1 appear to be extremely promising therapeutics for the treatment of established melanoma in mice and humans (8). Ipilimumab (anti-human CTLA-4) was reported at the 2010 American Society of Clinical Oncology annual meeting to extend the overall survival in patients with advanced melanoma by 10 months, and an important study showed the considerable therapeutic benefit in combining anti-CTLA-4 and anti-PD-1/PD-L1 in the early treatment of melanoma in the context of a tumor-cytokine vaccine (9).

Dardalhon and colleagues have previously shown that the growth of 4T1 mammary tumors was inhibited in TIM3-deficient mice (10). Furthermore, they also showed that an anti-TIM3 mAb could inhibit the growth of established subcutaneous EL4 lymphoma, suggesting that TIM3 is a potential target for cancer immunotherapy. Very recently 2 studies, 1 in mice (11) and another in humans (12), have shown the importance of TIM3 upregulation on tumor-specific CD8⁺ T cell dysfunction and the potential of restoring antitumor immunity by targeting TIM3. TIM3 expressed on lymphoma endothelium has also been shown to suppress CD4⁺ T cell activation (13). Nonetheless, these earliest studies have not

studied the effect of TIM3 inhibitors in many tumor settings or tumors generated *de novo*, nor have their mechanism of action been explored. Here we provide the first study to show the single agent activity and mechanism of anti-TIM3 in several experimental mouse tumor models and a model of primary carcinogenesis. Our data strongly support the potential complementary effects of this therapy in the context of other immune checkpoint inhibitors.

Materials and Methods

Mice

Inbred wild-type BALB/c (WT), BALB/c SCID, C57BL/6 (B6 WT), and B6 gene-targeted mice were bred and maintained at the Peter MacCallum Cancer Centre (Peter Mac) as described (14–16). Mice 6 to 18 weeks of age were used in all experiments that were carried out according to Peter Mac animal experimental ethics committee guidelines.

Tumor models

Experimental. WT3 sarcoma cells were derived from MCA-inoculated C57BL/6 WT mice as described (17). B16F10 melanoma, TRAMP-C1 prostate carcinoma, MC38 and CT26 colon adenocarcinomas (all from ATCC), WTMCA2, and WT3 sarcomas were maintained, injected, and monitored as previously described (14, 15, 18, 19). Mice were treated with cIg (Mac-4), antagonistic anti-TIM3 (RMT3-23, rat IgG2a), antagonistic anti-PD-1 (CD279; RMP1-14; ref. 20), antagonistic anti-CTLA-4 (CD152; UC10-4F10 kindly provided by Jeffrey Bluestone) or their combination as indicated. RMT3-23 does not deplete TIM3 cells *in vivo* (4, 21). RMT3-23 blocked TIM3-Fc binding to Gal-9 but did not induce apoptosis in Th1 cells *in vitro* even when immobilized (Hideo Yagita, unpublished data). Some mice additionally received anti-CD4 (GK1.5) or anti-CD8 β (53.5.8) as indicated to deplete T cell subsets as previously described (22). MAbs reactive with CD4 (GK1.5), CD8 β (53.5.8), and IFN- γ (H22) were used as indicated. B6 CD11c-DOG (DTR) mice were used as described (15).

MCA carcinogenesis model. Groups of male B6 WT mice were inoculated s.c. in the hind flank with 400 μ g of 3-methylcholanthrene (MCA; Sigma-Aldrich) in 0.1 mL of corn oil as described (17). In some experiments, mice were treated prophylactically with cIg, anti-TIM3, anti-PD-1, or anti-PD-1/anti-TIM3 (100 μ g i.p. each) on days -1, 0 and weekly for 8 weeks. Alternatively, mice were treated with cIg (Mac-4), anti-TIM3, anti-PD-1 or anti-PD-1/anti-TIM3 (100 μ g i.p., twice/wk) for 6 weeks from the second palpable tumor measurement (0.2–0.4 cm², days 84–147 relative to MCA inoculation). Mice were then monitored for fibrosarcoma development over 250 days and recorded as either percentage of tumor free mice or recorded as the growth curves (tumor size in cm²) of individual mice with tumor in each group. Measurements were made with a caliper square as the product of 2 perpendicular diameters (cm²).

Flow cytometry

Tumor infiltrating leukocytes. WT mice bearing established cIg- or anti-TIM3-treated tumors (>4 mm in diameter) were excised on days as indicated in the experiments. Tumors

excised from mice were digested, and cells were then used for flow cytometry analysis as previously described (23). For surface staining, tumor infiltrating leukocytes (TIL) were stained with FITC-anti-CD3, FITC-anti-CD19, FITC-anti-PD-1, PE-anti-TIM3 (clone 8B.2C12 and RMT3-23), PECy7-anti-CD4, APC-anti-CD11c, APC-anti-TCR- β , APC-anti-CTLA-4, A750-anti-CD45.2, eF450-anti-CD8, eF605-anti-CD11b (eBioscience and BD Pharmingen) in the presence of 2.4G2 (anti-CD16/32, to block Fc-receptors) on ice. 7-AAD (BD Pharmingen) was added immediately before flow cytometry analysis. TIM3 expression profile on CD8⁺ or CD4⁺ cells was from CD45.2⁺7AAD⁻ gated cells, whereas TIM3 expression profile on CD11c⁺ cells was from CD45.2⁺7AAD⁻CD3⁻CD19⁻ gated cells. For intracellular staining, TIL were fixed using BD Cytotfix/Cytoperm and stained with APC-CTLA-4 (eBioscience), suspended in BD Perm/Wash Buffer. Independent analysis of CD4⁺ and CD8⁺ TIL revealed more than 95% to be TCR β ⁺. The proportion of CD45.2⁺ leukocytes amongst all live cells was between 7% to 43% (CT26) and 16% to 62% (MC38) for all samples examined with no significant differences between cIg- and anti-TIM3-treated groups. Cells were acquired on the BD FACSCANTO II (BD Biosciences) and analysis was carried out using FlowJo (Tree Star).

Statistical analysis

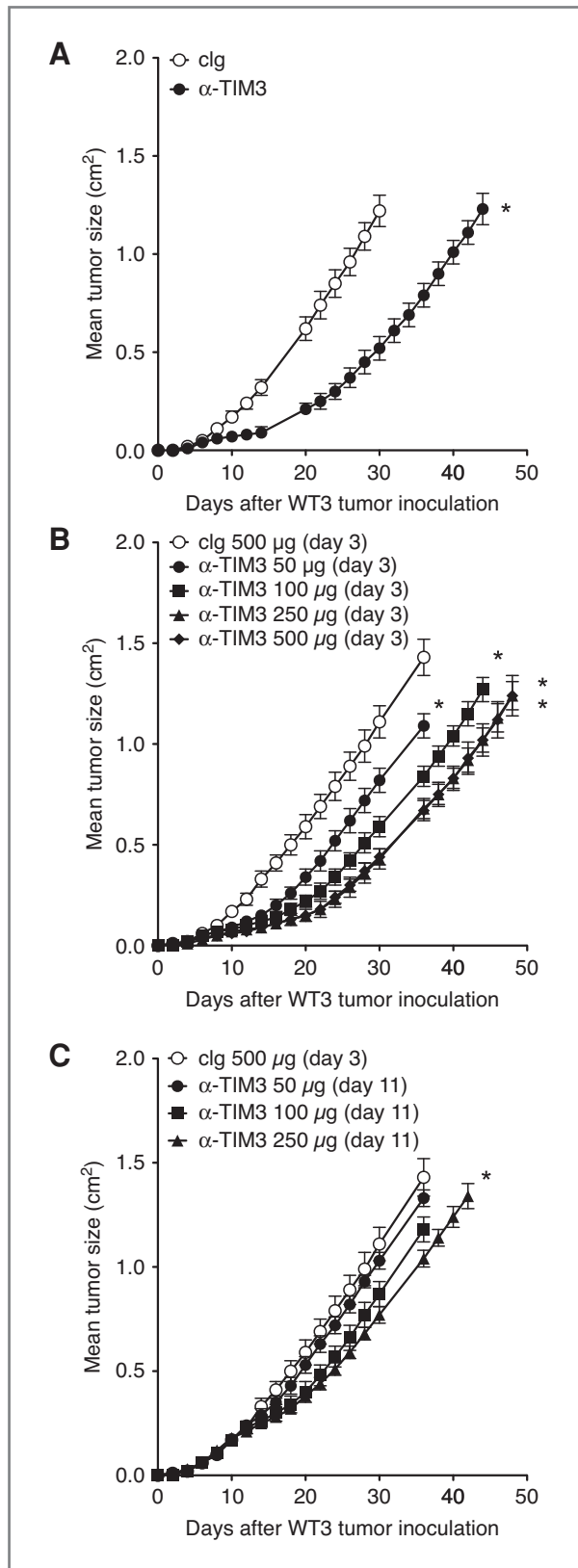
Statistical analyses were carried out using Graph Pad Prism software. Significant differences in tumor growth and metastases were determined by a Mann-Whitney test. Statistical differences in percentage of mice tumor free were determined by a Log Rank Mantel-Cox test. Values of $P < 0.05$ were considered significant.

Results

Anti-TIM3 antitumor activity is T cell and IFN- γ dependent

Initially we wished to determine whether anti-TIM3 was capable of suppressing subcutaneous tumor growth. We screened a series of tumor cell lines (CT26, MC38, WT3, TRAMP-C1, and B16F10) for expression of PDL1 and TIM3 and their corresponding receptors, PD-1 and galectin-9. None of the tumor cell lines expressed PD-1 or TIM3, but all cell lines expressed intracellular galectin-9, and all but TRAMP-C1 expressed cell surface PD-L1 (Supplementary Fig. S1). Transplant of WT3 resulted in robust tumor growth and early therapy (from day 3) with a high dose of anti-TIM3 (250 μ g per injection \times 4) significantly suppressed WT3 growth (Fig. 1A). A dose-response experiment, in the same tumor, showed that 250 μ g anti-TIM3 was the minimal optimal dose with lower doses having reduced therapeutic effects (Fig. 1B). Delayed dosing of anti-TIM3 (from day 11) was considerably less effective at all doses (Fig. 1C).

Next we assessed the mechanism of action of anti-TIM3 by comparing response in WT mice depleted of various lymphocytes or deficient in effector molecules and/or cytokines. From these studies it was clear that anti-TIM3 was largely ineffective in the absence of CD4⁺ or CD8⁺ T cells (Fig. 2A), but effective in the absence of mature B cells (Fig. 2B). Given the role of T



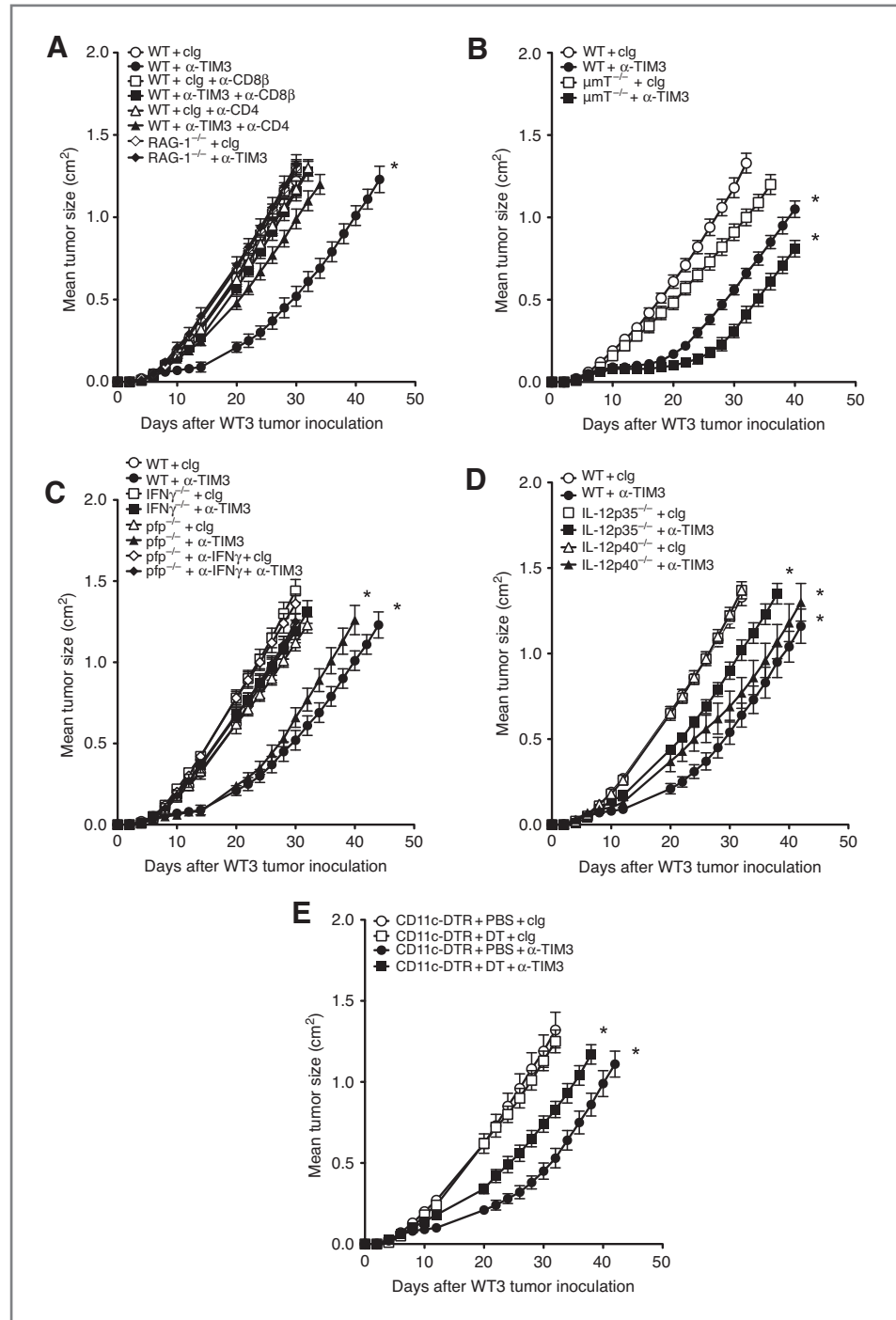
cells, we then examined the importance of the cytotoxic lymphocyte effector molecule, perforin and T cell antitumor cytokine, IFN- γ . Unequivocally, these data showed that anti-TIM3 was ineffective in the absence of functional IFN- γ , but largely suppressive in perforin-deficient mice (Fig. 2C). Further analysis revealed that host IL-12p35 was partially important in the antitumor activity of anti-TIM3 (Fig. 2D), whereas IL-17A was irrelevant (data not shown). In concert with a partial role of IL-12p35, anti-TIM3 was not optimal in mice that were conditionally depleted of CD11c⁺ DC (Fig. 2E).

We further assessed the mechanism of action of anti-TIM3 in 2 other experimental tumor models. Anti-TIM3 had significant antitumor activity against MC38 colon adenocarcinoma in WT mice; however, this activity was almost completely lost in RAG-1^{-/-} and IFN- γ ^{-/-} mice or WT mice depleted of either CD4⁺ or CD8 β ⁺ T cells (Fig. 3A). The effect of anti-TIM3 was largely intact in mice lacking CD11c⁺ DC, with only a minor reduction in antitumor activity (Fig. 3B). These data were entirely consistent with observations in the WT3 sarcoma model (Fig. 2). To determine more specifically the functional importance of T cells in anti-TIM3 activity, we then isolated purified populations of naive WT or IFN- γ -deficient spleen CD4⁺ and CD8⁺ T cell subsets and adoptively transferred them alone or in combination into RAG-1^{-/-} recipients with MC38 tumors. In RAG-1^{-/-} mice that were treated with clg commencing 1 day after adoptive transfer, MC38 tumors grew regardless of what subsets of T cells were transferred (Fig. 3C). Consistent with a role of T cells in anti-TIM3 antitumor activity, anti-TIM3 was ineffective in RAG-1^{-/-} mice receiving no adoptive transfer (Fig. 3D). Interestingly however, RAG-1^{-/-} mice that received either a combination of WT CD8⁺ T and WT CD4⁺ T cells or WT CD8⁺ T and IFN- γ ^{-/-} CD4⁺ T cells responded to anti-TIM3 and MC38 tumor growth was significantly suppressed (Fig. 3D). By contrast, the adoptive transfer of WT or IFN- γ ^{-/-} CD4⁺ or CD8⁺ T cells alone was insufficient to observe anti-TIM3 antitumor activity (Fig. 3D). Furthermore, the inability of a combination of IFN- γ ^{-/-} CD8⁺ T cells and WT CD4⁺ T cells, or IFN- γ ^{-/-} CD8⁺ T cells and IFN- γ ^{-/-} CD4⁺ T cells, to confer significant anti-TIM3 antitumor activity illustrated the key role of CD8⁺ T cell IFN- γ (Fig. 3D). Evidently, CD4⁺ T cells must be contributing some other key molecule to the antitumor activity of anti-TIM3 which is yet to be determined.

Figure 1. Anti-TIM3 suppresses WT3 in a dose-dependent manner. Groups of B6 mice ($n = 5$) were inoculated subcutaneously with WT3 (5×10^5). A, on days 3, 7, 11, and 15 after tumor inoculation, mice were intraperitoneally treated with either control Ig (clg) or anti-TIM3 (250 μ g). Representative of 2 independent experiments. B, on days 3, 7, 11, and 15 after tumor inoculation, groups of mice were intraperitoneally treated with either clg (500 μ g) or increasing doses of anti-TIM3 (50–500 μ g) as indicated. C, on days 11, 15, 19, and 23 after tumor inoculation, groups of mice were intraperitoneally treated with increasing doses of anti-TIM3 (50–250 μ g) as indicated (compared with clg as in B). Tumor sizes are represented as the mean \pm SEM. Statistical differences in tumor sizes between mice treated with clg or anti-TIM3 therapy were determined by a Mann-Whitney test (*, $P < 0.05$).

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Figure 2. Anti-TIM3 therapy of WT3 is dependent on T cells and IFN- γ . Groups of B6 WT or B6 gene-targeted mice ($n = 4-6$) were inoculated subcutaneously with WT3 (5×10^5). A, therapeutic doses (days 3, 7, 11, and 15) of clg or anti-TIM3 (250 μg i.p.) were compared for antitumor efficacy in WT mice treated with clg, anti-CD4 or anti-CD8 β (100 μg i.p. on days 2, 3, 10, 17, and 24) or RAG-1 $^{-/-}$ mice. B, therapeutic doses (as in A) of clg or anti-TIM3 (250 μg i.p.) were compared for antitumor efficacy in WT and $\mu\text{MT}^{-/-}$ mice. C, therapeutic doses (as in A) of clg or anti-TIM3 (250 μg i.p.) were compared for antitumor efficacy in WT mice, IFN γ $^{-/-}$ mice, $\text{pfp}^{-/-}$ mice, or $\text{pfp}^{-/-}$ mice treated with anti-mIFN- γ (H22, 250 μg i.p. on days 2, 3, 10, 17, and 24). D, therapeutic doses (as in A) of clg or anti-TIM3 (250 μg i.p.) were compared for antitumor efficacy in WT mice, IL-12p35 $^{-/-}$, and IL-12p40 $^{-/-}$ mice. E, therapeutic doses (as in A) of clg or anti-TIM3 (250 μg i.p.) were compared for antitumor efficacy in CD11c-DTR mice treated with PBS or DT (8 ng/g body weight, every 2 days from day 2 to day 14) to deplete CD11c $^{+}$ DC. Tumor sizes are represented as the mean \pm SEM. Statistical differences in tumor sizes between mice treated with clg or anti-TIM3 therapy were determined by a Mann-Whitney test (*, $P < 0.05$).



Modulation of CD8/CD4 cell ratio in MC38 tumor bearing mice following anti-TIM3 treatment

To further identify the targets of anti-TIM3, we first assessed the expression of TIM3 and PD-1 on TIL from mice bearing MC38 tumors (days 14, 18, 22, and 26). TIM3 was expressed on CD4 $^{+}$ and CD8 $^{+}$ T cells (Fig. 4A) and CD3 $^{-}$ CD19 $^{-}$ CD11c $^{-}$, and CD3 $^{-}$ CD19 $^{-}$ CD11c $^{+}$ TIL at different levels (data not shown). The majority of CD8 $^{+}$ T cells at the

commencement of treatment were TIM3 $^{+}$ PD-1 $^{+}$. Interestingly, regression of MC38 tumor after 3 doses of anti-TIM3 was associated with the maintenance of the CD8:CD4 ratio in the TIL following treatment (day 26; Fig. 4B). However, the effect of anti-TIM3 in modulating the TIM3 expression on immune cells could not be assessed in B6 mice because the only clone available to label immune cells specifically in B6 mice was also being used therapeutically (RMT3-23). To

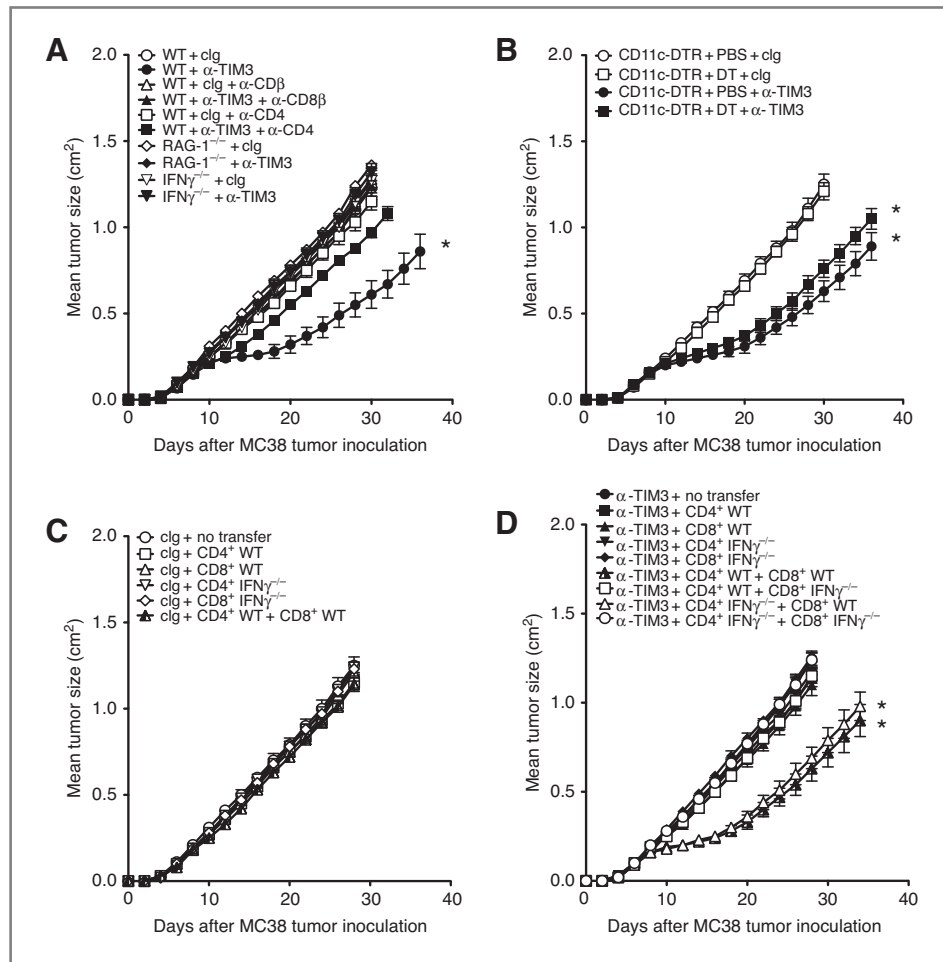


Figure 3. Anti-TIM3 therapy suppresses established MC38 tumor growth and requires T cells and IFN- γ . Groups of B6 WT or B6 gene-targeted mice ($n = 5$) were inoculated subcutaneously with MC38 (1×10^6). A, therapeutic doses (days 8, 12, 16, and 20) of clg or anti-TIM3 (250 μg i.p.) were compared for antitumor efficacy in WT mice treated with clg, anti-CD4 or anti-CD8 β (100 μg i.p. days 7, 8, 15, and 22) or RAG-1 $^{-/-}$ or IFN- γ $^{-/-}$ mice. B, therapeutic doses (as in A) of clg or anti-TIM3 (250 μg i.p.) were compared for antitumor efficacy in CD11c-DTR mice treated with PBS or DT (8 ng/g body weight, every 2 days from day 6 to day 22) to deplete CD11c $^{+}$ DC. C, therapeutic dose (days 7, 11, 15, and 19) of clg (250 μg i.p.) was compared for antitumor efficacy in RAG-1 $^{-/-}$ mice following no adoptive transfer or receiving purified spleen CD4 $^{+}$ and CD8 $^{+}$ T cell subsets alone (from WT or IFN- γ $^{-/-}$ mice) or in combination on day 6 after tumor inoculation as indicated (1×10^6 cells). D, therapeutic dose (days 7, 11, 15, and 19) of anti-TIM3 (250 μg i.p.) with adoptive transfer as in C. Tumor sizes are represented as the mean \pm SEM. Statistical differences in tumor sizes, between mice (A and B) treated with clg or anti-TIM3 therapy and (C and D) receiving no adoptive transfer versus T cell transfer, were determined by a Mann-Whitney test (*, $P < 0.05$).

overcome this limitation we additionally assessed anti-TIM3 activity in tumors growing in BALB/c mice.

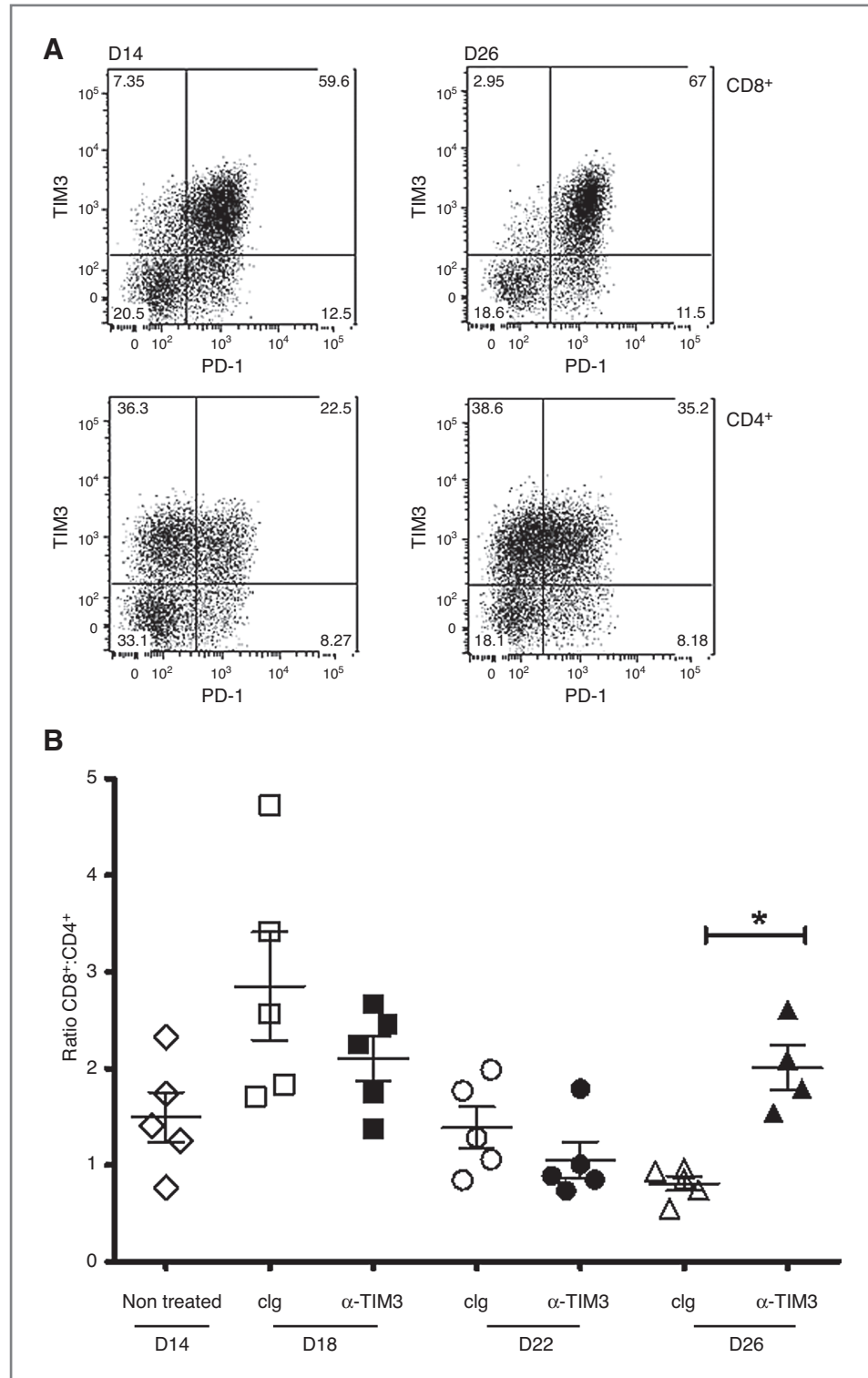
Anti-TIM3 mAb therapy suppresses tumor growth in BALB/c mice by a similar mechanism

The antitumor activity of anti-TIM3 and the critical role of both CD4 $^{+}$ and CD8 $^{+}$ T cell subsets and IFN- γ in anti-TIM3 antitumor activity was also validated in the subcutaneous BALB/c CT26 colon adenocarcinoma (Supplementary Fig. S2A and B) and WTMCA2 fibrosarcoma (Supplementary Fig. S2C). In contrast to a recent report suggesting that TIM3 $^{+}$ PD-1 $^{+}$ cells comprise the major CD8 $^{+}$ TIL (11) and our data in the MC38 tumor model, we observed that majority of CD8 $^{+}$ and CD4 $^{+}$ cells in CT26 TIL were TIM3 $^{-}$ PD-1 $^{-}$ during early tumor development (days 7 and 11), with the existence of low

number of TIM3 $^{+}$ PD-1 $^{-}$ cells, TIM3 $^{-}$ PD-1 $^{+}$ cells, and TIM3 $^{+}$ PD-1 $^{+}$ cells (Supplementary Fig. S3A and B). The appearance of TIM3 $^{+}$ PD-1 $^{+}$ cells dominated the majority of CD8 $^{+}$ TIL only at a later tumor stage (day 15). Analysis of CT26 TIL on day 11 after tumor inoculation showed that anti-TIM3 treatment down modulated TIM3 expression on CD4 $^{+}$ and CD8 $^{+}$ T cells, but had little impact on TIM3 expression on CD3 $^{-}$ CD19 $^{-}$ CD11c $^{-}$, and CD3 $^{-}$ CD19 $^{-}$ CD11c $^{+}$ cells (Supplementary Fig. S4). A more complete analysis revealed that administration of 3 doses of anti-TIM3 reduced the frequency of TIM3 $^{+}$ PD-1 $^{+}$ cells and increased the frequency of TIM3 $^{-}$ PD-1 $^{+}$ cells amongst CD8 $^{+}$ TIL (Fig. 5). Amongst, CD4 $^{+}$ TIL, the administration of anti-TIM3 reduced the frequency of TIM3 $^{+}$ (both PD-1 $^{-}$ cells and PD-1 $^{+}$ cells), and increased the frequency of TIM3 $^{-}$ (both PD-1 $^{-}$ cells

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Figure 4. Modulation of CD8/CD4 cells ratio in MC38 tumor-bearing mice following anti-TIM3 treatment. A and B, groups of B6 mice ($n = 4$ or 5) were inoculated subcutaneously with MC38 (1×10^6) on day 0. Following tumor inoculation, tumor-bearing mice were i.p. treated with either clg or anti-TIM3 (250 μ g) on days 14, 18, 22, and 26. At the indicated time points, tumors were excised and FACS analyses on TILs were carried out. A, the FACS plots of TIM3 and PD-1 expression on CD8⁺, CD4⁺, or CD3⁻CD19⁻CD11c⁺ gated TILs obtained from day 26 tumor-bearing mice are shown. Data shown are representative of 5 independent analyses. B, the CD8⁺:CD4⁺ ratio of TILs obtained from the tumor bearing mice that were untreated or treated with either clg or anti-TIM3 at the indicated time points are shown. Each symbol represents a single mouse. Statistical differences in the CD8⁺:CD4⁺ ratio between clg- and anti-TIM3-treated mice were determined by a Mann-Whitney test (* $P < 0.05$).



and PD-1⁻ cells; Fig. 5). Collectively, the TIL analyses suggested that the anti-TIM3 was modulating TIM3 on CD4⁺ and CD8⁺ TIL in CT26 tumors and that the earliest injections of anti-TIM3 (days 3, 7, and 11) were predominantly targeting TIM3⁺PD-1⁻ T cells.

Comparative and combined effect of anti-TIM3 with anti-PD-1 and anti-CTLA-4

Anti-CTLA-4 and anti-PD-1 appear to be extremely promising therapeutics for the treatment of established melanoma in mice and humans. Thus, next we made a comparison

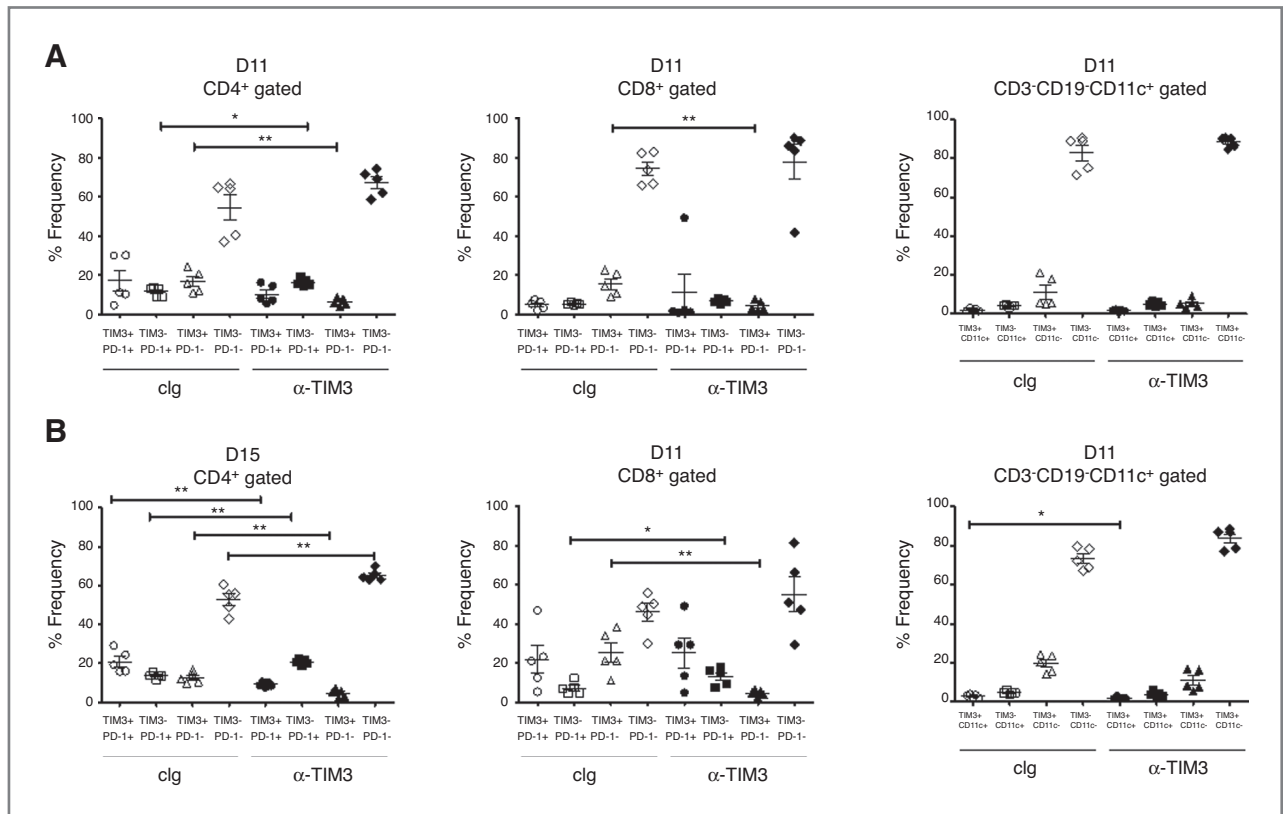


Figure 5. Modulation of TIM3 expression on CD4⁺, CD8⁺, and CD11c⁺ TILs following anti-TIM3 treatment. Groups of BALB/c mice (n = 5) were inoculated subcutaneously with CT26 (5 × 10⁵) on day 0. Following tumor inoculation, groups of tumor bearing mice were i.p. treated with either cIg or anti-TIM3 (250 μg) either on days 3 and 7 (A) or days 3, 7, and 11 (B). On day 11 (A) and day 15 (B), tumors were excised and FACS analyses on TILs were carried out. The frequency of TIM3 and/or PD-1 expression on CD8⁺, CD4⁺, or CD3⁺CD19⁻CD11c⁺ gated TIL at the indicated time points are shown. Each symbol represents a single mouse. Statistical differences between the frequency of various populations in cIg-treated versus anti-TIM3-treated mice were determined by a Mann-Whitney test (*, P < 0.05; **, P < 0.01).

between these mAbs and anti-TIM3 in a series of experimental tumor models. Early treatment of subcutaneous WT3 fibrosarcoma produced a nearly identical therapeutic effect of anti-TIM3 compared with anti-CTLA-4 and anti-PD-1 (Fig. 6A). This was also the case for the treatment of subcutaneous B16F10, although the effect of each of the 3 mAbs was quite inferior (Fig. 6B), consistent with the reduced leukocyte infiltrate found in this tumor (data not shown). Consistent with the minimal effect of anti-CTLA-4 against established tumors, delayed treatment of TRAMP-C1 prostate tumors (commencing on day 10) with anti-CTLA-4 was only weakly effective, but the same regimen of either anti-PD-1 or anti-TIM3 significantly slowed TRAMP-C1 tumor growth (Fig. 6C). In a similar manner, delayed anti-CTLA-4 treatment of subcutaneous MC38 adenocarcinomas (commencing day 14–0.45 cm²) was largely ineffective, whereas anti-PD-1 or anti-TIM3 had quite profound effects on tumor growth (Fig. 6D). Given the weaker therapeutic effect of each mAb monotherapy against B16F10 tumors, we finally explored whether these agents could act in synergy. Each dual therapy was more effective than any monotherapy (compare Fig. 6B with E), whereas the 3 mAbs, anti-CTLA-4, anti-PD-1, and anti-TIM3, produced a further suppression of B16F10 tumor growth,

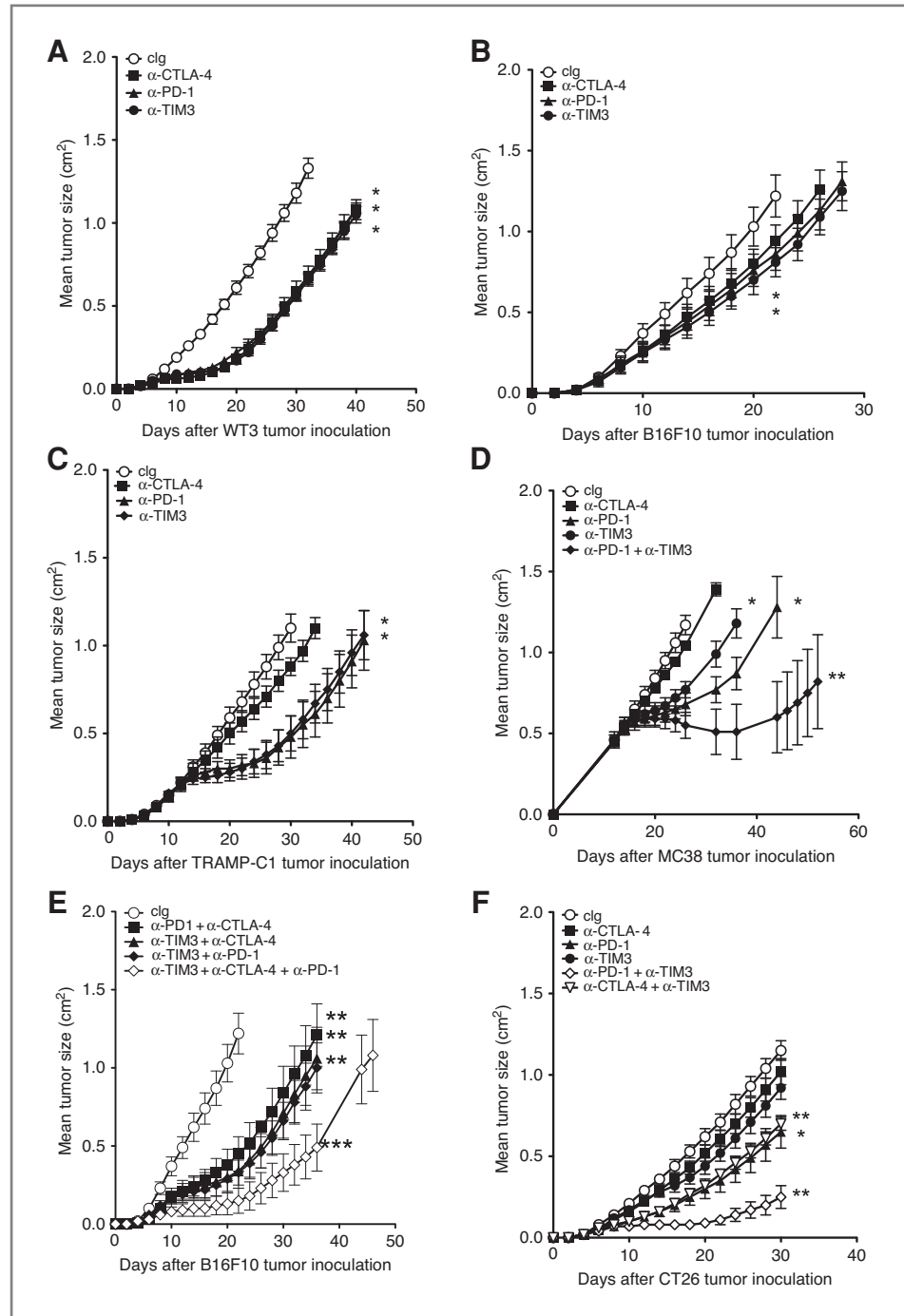
significantly prolonging the survival of all mice in this group (Fig. 6E). It should be noted that no treated mice displayed any signs of autoimmunity, including vitiligo (data not shown). Synergy between anti-TIM3 and anti-PD-1 was also noted against CT26 colon carcinomas, while anti-CTLA-4 and anti-TIM3 were less effective (Fig. 6F).

Anti-TIM3 suppresses carcinogen-induced tumor growth

Given the effect of anti-TIM3 against established experimental tumors, we next assessed whether tumors induced *de novo* would respond to anti-TIM3 therapy. MCA-induced fibrosarcoma is a well-characterized model of *de novo* carcinogenesis and has been used to determine the prophylactic and therapeutic activity of a number of monoimmunotherapies and combination immunotherapies (16, 24, 25). Prophylactic anti-TIM3 had a modest effect in delaying MCA-induced fibrosarcomas compared with cIg (P = 0.0029) and previous approaches (Fig. 7A; ref. 16), including anti-PD-1 (P = 0.0001). Together, anti-TIM3 combined with anti-PD-1 prevented the development of a significant proportion of tumors (~30%) compared with either anti-PD-1 (P = 0.046) or anti-TIM3 (P = 0.0018) alone (Fig. 7A). Despite modest tumor prevention

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Figure 6. Comparative effect of anti-TIM3 against experimental tumors. Groups of B6 mice ($n = 5$) were inoculated subcutaneously with (A) WT3 (5×10^5), (B and E) B16F10 (1×10^5), (C) TRAMP-C1 (5×10^5), (D) MC38, and (F) CT26. On days 3, 7, 11, and 15 (A, B, E, and F), days 10, 14, 18, and 22 (C), or days 14, 18, 22, and 26 (D) after tumor inoculation, mice were intraperitoneally treated with either clg, anti-TIM3, anti-CTLA-4, anti-PD-1, or their combination (100 μ g) as indicated. Tumor sizes are represented as the mean \pm SEM. A–D and F, statistical differences in tumor sizes between mice treated with clg and single mAb therapy were determined by a Mann–Whitney test (*, $P < 0.05$). D–F, statistical differences in tumor sizes between mice treated with single mAb therapy or a dual combination were determined by a Mann–Whitney test (**, $P < 0.05$). E, statistical differences in tumor sizes between mice treated with dual mAb therapy or triple combination were determined by a Mann–Whitney test (***, $P < 0.05$).



capability, anti-TIM3 was effective in suppressing the growth of a small percentage (4/30, $\sim 13\%$) of established MCA-induced sarcomas when treatment commenced on the second week of palpable tumor (0.20–0.45 cm^2 ; Fig. 7B and C). Anti-PD-1 was not any more effective as a monotherapy (Fig. 7D), but the combination of anti-PD-1 and anti-TIM3 eradicated 1 of 15 established tumors and significantly suppressed the tumor growth of 3 of 15 other tumors for between 35 and 100 days (Fig. 7E). Established MCA-induced

fibrosarcomas generally displayed a low percentage of TIM3⁺PD-1⁺, TIM3⁺PD-1⁻, and TIM3⁻PD-1⁺, CD4⁺ and CD8⁺ cells in the TIL (Supplementary Fig. S5). This may partially explain the modest therapeutic effect of single agent anti-TIM3 or anti-PD-1 against similar established tumors. Ours are the first data to show the effectiveness of anti-PD-1 and anti-TIM3 against tumors established *de novo* in mice, supporting a previous study exclusively employing experimental tumor transplants (11), and

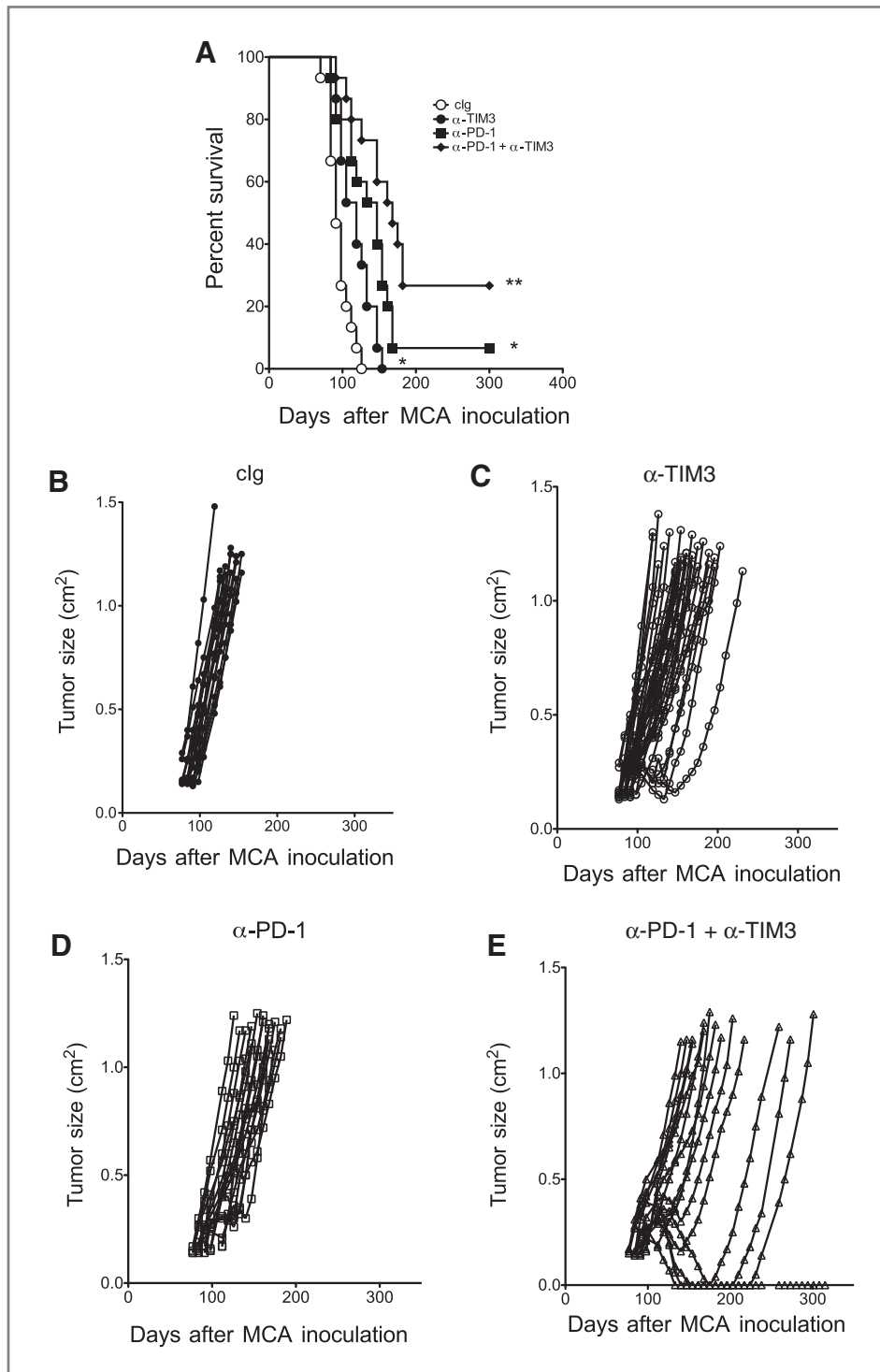


Figure 7. Anti-TIM3 suppresses the growth of established MCA-induced sarcomas. Groups of 15 to 30 male B6 WT mice were injected subcutaneously on the flank with 400 μg MCA on day 0. A, mice were treated prophylactically with clg, anti-TIM3, anti-PD-1, or anti-PD-1/anti-TIM3 (100 μg i.p.) on days -1, 0 and weekly for 8 weeks. B-E, when sarcomas had established (the second week of palpable tumor—0.20–0.45 cm²) B6 mice were treated i.p. with 100 μg clg, anti-TIM3, anti-PD-1, anti-PD-1/anti-TIM3 twice a week for 6 weeks. Mice were then monitored for tumor development over 350 days and recorded as either percentage of tumor free mice (A) or recorded as the growth curves (tumor size in cm²) of individual mice with tumor in each group (B-E). Statistical differences in percentage of mice tumor free were determined by a Log Rank Mantel-Cox test (*, monotherapy compared with clg; **, anti-PD-1/anti-TIM3 compared with either alone).

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strongly suggesting the combination of anti-PD-1 and anti-TIM3 may be synergistic.

Discussion

TIM3 is a promising target for cancer immunotherapy, but the mechanism of action of TIM3 inhibitors, such as anti-

TIM3 mAb, and their activity in mouse models of spontaneous cancer have never been determined. For the first time we specifically define the mechanism of antitumor action of anti-TIM3 requiring CD4⁺ T cells and IFN-γ-producing CD8⁺ T cells, with a comparatively minimal model-dependent requirement for host CD11c⁺ DC. Anti-TIM3 displayed modest prophylactic and therapeutic activity against a small fraction

of MCA-induced sarcomas, but comparative and combination studies of anti-TIM3 with anti-CTLA-4 and anti-PD-1 against experimental and carcinogen-induced tumors suggested that these agents might be well tolerated and very effective in combination.

There has been a multitude of studies that have assessed the mechanism of action of anti-CTLA-4 in mice (26–28) and more recently in man (29–31). Anti-PD-1 and anti-PD-L1 have also been studied in some depth in the mouse and humans (32–34). Studies of the mechanism of action of anti-PD-1 and anti-CTLA-4 combinations have also been explored in mice (9). By contrast, no studies have specifically assessed the mechanism of action of anti-TIM3 in tumors, rather characterizing the comparatively defective cytokine effector function of TIM3⁺PD-1⁺ CD8⁺ T cell populations in experimental tumors in mice (11) or NY-ESO-1 specific TIM3⁺PD-1⁺ CD8⁺ T cells in advanced melanoma in humans (12). We have defined in the mouse in 3 different experimental tumor models that anti-TIM3 requires the activity of both CD8⁺ and CD4⁺ T cells and that, in particular, the IFN- γ production by the CD8⁺ T cells is critical to the efficacy of anti-TIM3 therapy.

The aforementioned previous studies (11, 12) have implied the target of anti-TIM3 therapy might be on TIM3⁺PD-1⁺ T cell populations found in established tumors, however interestingly, in our studies anti-TIM3 appeared to be effective when administered sometime before the appearance and accumulation of significant TIM3⁺PD-1⁺ T cell populations in tumor bearing mice. Early (days 7–11) CT26 subcutaneous tumors contained CD8⁺TIM3⁺PD-1⁻ cells, but very few detectable TIM3⁺PD-1⁺ T cells, and yet tumors clearly responded to anti-TIM3 therapy. The lack of TIM3⁺ T cells early in tumor progression could be a timing issue, as the expression of TIM3 may be very transient. However, the target and mechanism of anti-TIM3 might also be model dependent, given that anti-TIM3 was also effective against established MC38 tumors, where TIM3⁺PD-1⁺ T cells were the predominant T cell population amongst TIL at the commencement of treatment. In this model, a maintenance of the CD8:CD4 ratio of MC38 TIL over time correlated with the response to anti-TIM3. However, strictly discounting TIM3⁺PD-1⁻ or TIM3⁺PD-1⁺ T cells as a possible target of anti-TIM3 is not possible at the present time.

Interestingly, IFN- γ production from CD8⁺ cells, but not IFN- γ from CD4⁺ cells, was crucial for antitumor effect of anti-TIM3. Our TIL analysis of CT26 tumors suggested that anti-TIM3 alone reduced the frequency of TIM3⁺ T cells in the tumor. It has been previously shown that amongst T cells, CD8⁺TIM3⁻PD-1⁺, and CD8⁺TIM3⁻PD-1⁻ T cells are major producers of IFN- γ (11), and thus we suggest the increase in frequency of these CD8⁺ cells in the TIL of anti-TIM3-treated mice is a possible mechanism of anti-TIM3 in suppressing tumor growth. Clearly, CD4⁺ T cells were essential for anti-TIM3 efficacy, but the changes in TIM3 expression on CD4⁺ TIL were less evident post-anti-TIM3 therapy, and the key molecules that this subset contributes to the antitumor activity of anti-TIM3 remain to be discovered.

Despite the fact that only a small proportion (5%–20%) of CD4⁺ and CD8⁺ T cells in TIL of early tumors are effective

targets of TIM3, anti-TIM3 is active as a single agent, when administered early and even more so in combination with anti-PD-1. In both CT26 and MC38 tumor models, the reduction of TIM3⁺PD-1⁻ cells was associated with an increased frequency of TIM3⁻PD-1⁺ cells. The combination effect of anti-TIM3 and anti-PD-1 in suppressing tumors may lead to the maintenance of TIM3⁻PD-1⁻ T cells amongst TIL and this possibility now requires exploration. Although our studies have shown that TIM3⁺PD-1⁻ TIL, in addition to TIM3⁺PD-1⁺ TIL, may be targets of anti-TIM3, it remains an interesting question why these T cells do not undergo massive cell death, when tumor cells express galectin-9 and PD-L1 (3). Any potential soluble form of TIM3 (splice variant) and the signaling events that maintain the exhausted phenotype of T cells might be worthy of further research. The anti-TIM3 mAb (RMT3-23) does not deplete TIM3 cells *in vivo* (4, 21) and the same mAb has been shown to upregulate IFN- γ production by ConA-stimulated splenic T cells (35), but it is not clear whether proliferation may be enhanced by this mAb.

Our TIL analysis also indicated that the frequency and TIM3 expression of CD11c⁺ TIM3⁺ TIL were not greatly affected by anti-TIM3. These data were in concert with anti-TIM3 therapeutic activity only showing a minor dependence on CD11c⁺ DC, which was somewhat model dependent (MC38 vs. WT3). However, it was previously reported that TIM3 was involved in phagocytosis and cross-presentation of antigen (4), and both of these functional properties of TIM3 may have importance in antitumor immunity. Phosphatidylserine (PS), which is associated with cell death, has been reported to be another ligand for TIM3, other than galectin-9 (4). Thus, the effect of anti-TIM3 on tumor cell death and cross-presentation of tumor antigens by DC may be worthy of further exploration. Using overexpression studies, Dardalon and colleague proposed that the TIM3/galectin-9 pathways correlated with the expansion of CD11b⁺Ly-6G⁺ myeloid derived suppressor cells (MDSC; 10). Comparatively, we intervened using an anti-TIM3 mAb, which is therapeutically more relevant, but analysis of MDSC and other populations in the mechanism of action of anti-TIM3 mAb will be the subject of future studies using 4T1.2 (where MDSC in the tumor and secondary lymphoid organs are prevalent) and other tumor models.

The effects of anti-TIM3 in combination with anti-PD-1 and/or anti-CTLA-4 against established B16F10, MC38, and CT26 tumors were broad and encouraging and these approaches are worthy of further preclinical development to optimize the regimen schedule and further understand the mechanism of action of each combination. Established B16F10 tumors grow rapidly and are extremely difficult tumors to treat with any single therapy. The antitumor effect achieved with the anti-TIM3/anti-CTLA-4/anti-PD-1 combination is approximating what can be achieved with complete regulatory T cell depletion (data not shown), an intervention that provokes many arms of antitumor immunity. Anti-CTLA-4 is more effective against mouse tumors when administered at the time of tumor inoculation, and appears comparatively more effective in humans, so our experiments possibly

under estimate the potential therapeutic benefit of these combinations.

Encouragingly, our data in experimental tumor models were strongly supported by the combined anti-PD-1 and anti-TIM3 treatment of fibrosarcomas established *de novo* by MCA induction, despite the relatively low frequencies of detectable TIM3⁺ and PD-1⁺ T cell populations amongst the TIL of such tumors. MCA-induced fibrosarcomas are a model of both inflammation-induced cancer and cancer immune surveillance (17). It is rare for investigators to test therapies in models of *de novo* carcinogenesis where tumors are established in the host prior to treatment. We have shown that dramatic therapeutic effects are not observed with monotherapies (including anti-PD1 or anti-TIM3) in this model. This is despite the fact that anti-PD1 is an extremely promising therapeutic in some human cancers. In this light, the combination therapy (anti-TIM3 and anti-PD1) effect is quite significant in the MCA-induction model. A larger study with pretreatment biopsy correlating target expression amongst TIL with therapeutic activity of these immunotherapies in this model is warranted. Therapeutic studies in other spontaneous tumor models, such as the TRAMP transgenic model of prostate cancer, will be valuable to assess the broader utility of these combinations. With the rapid clinical development of anti-PD-1 and anti-PD-L1 in humans, the pro-

spect of combining this approach with anti-TIM3 appears very appealing.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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