

Mitigating Age-Related Immune Dysfunction Heightens the Efficacy of Tumor Immunotherapy in Aged Mice

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Abstract

Although cancer tends to affect the elderly, most preclinical studies are carried out in young subjects. In this study, we developed a melanoma-specific cancer immunotherapy that shows efficacy in aged but not young hosts by mitigating age-specific tumor-associated immune dysfunction. Both young and aged CD4⁺CD25^{hi} regulatory T cells (Treg) exhibited equivalent *in vitro* T-cell suppression and tumor-associated augmentation in numbers. However, denileukin diftitox (DT)-mediated Treg depletion improved tumor-specific immunity and was clinically effective only in young mice. DT-mediated Treg depletion significantly increased myeloid-derived suppressor cell (MDSC) numbers in aged but not young mice, and MDSC depletion improved tumor-specific immunity and reduced tumor growth in aged mice. Combining Treg depletion with anti-Gr-1 antibody was immunologically and clinically more efficacious than anti-Gr-1 antibody alone in aged B16-bearing mice, similar to Treg depletion alone in young mice. In contrast, DT increased MDSCs in young and aged mice following MC-38 tumor challenge, although effects were greater in aged mice. Anti-Gr-1 boosted DT effects in young but not aged mice. Aged antitumor immune effector cells are therefore competent to combat tumor when underlying tumor-associated immune dysfunction is appropriately mitigated, but this dysfunction varies with tumor, thus also varying responses to immunotherapy. By tailoring immunotherapy to account for age-related tumor-associated immune dysfunctions, cancer immunotherapy for aged patients with specific tumors can be remarkably improved. *Cancer Res*; 72(8); 2089–99. ©2012 AACR.

Introduction

Among known factors associated with cancer development, advancing age remains the leading risk (1). Immune therapy for cancer is a scientifically sound approach, but clinical effects have generally been modest. Much of our understanding of tumor immunity comes from studies in young hosts. Few studies have examined the effects of age on response to immune therapy and on tumor-associated immune dysfunction.

Although antitumor immunity declines with age, as do other immune effector functions (2, 3), T-cell functions in aged hosts can sometimes be improved (4). Thus, to the extent that

underlying tumor-associated immune dysfunction can be reversed in aged hosts with cancer, clinically relevant antitumor immune responses could potentially be achieved.

Regulatory T cells (Treg) are key mediators of tumor immune dysfunction, and reducing Treg function is a rational cancer immunotherapy strategy (5–8). Treg contributions to age-related decline in immune responses is contradictory, with some studies showing increases in Treg prevalence and/or function with age in humans and mice (9–12), whereas others show no changes or reduced Treg contributions (13, 14). Myeloid-derived suppressor cells (MDSC) are regulatory cells that also increase in tumors (15–18) and suppress antitumor immunity (19). They are a heterogeneous population usually identified as CD11b⁺Gr-1⁺ cells in mice (17).

Although Treg depletion is an effective approach to improving antitumor immunity and responses to immunotherapy (6, 8), conflicting studies report the effect of Treg depletion as cancer immunotherapy in aged hosts (20, 21). MDSC depletion is another potentially effective approach to reverse cancer-associated immune dysfunction. Whereas MDSCs contribute to immunopathology in aged hosts including in cancer (22, 23), the subject remains little studied, as does potential interactions between MDSCs and Tregs.

We examined immune dysregulatory mechanisms in young versus aged hosts with cancer and assessed age-specific responses to Treg depletion, MDSC modulation, or both. We

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showed that aged and young hosts with cancer responded differently to those immune therapies in a tumor-dependent fashion, but that, more importantly, clinically significant anti-tumor immune responses can be achieved even in aged tumor-bearing mice using age-specific immune therapies. These data suggest means to improve the efficacy of tumor immunotherapy in the population most at risk for cancers and show that age and tumor interactions with the aged host are significant considerations in preclinical and clinical testing of immunotherapeutic strategies.

Materials and Methods

Mice

Four- to 8-week old (National Cancer Institute repository) and 17- to 19-month old (NIA repository) C57BL/6 (BL6) mice were purchased. Young Foxp3-IRES-red fluorescent protein (FIR) mice (24) provided by Richard Flavell and Foxp3^{DTR} mice (25) from Alexander Rudensky were aged in our facility. Young mice are 2 to 6 months old. Aged mice are 22 to 26 months old. All animal studies were approved by the The University of Texas Health Science Center at San Antonio (UTHSCSA) Institutional Animal Care and Use Committee.

Tumor

B16F10 melanoma and MC-38 colon carcinoma were purchased from the American Type Culture Collection. We engineered OVA-expressing B16F10 cells (26) and MC-38 cells (27), herein referred to as B16 and MC-38, respectively, for simplicity. Tumor challenge was injection of 250,000 B16 or 1×10^6 MC-38 tumor cells subcutaneously as described (26). Tumor growth was measured with Vernier calipers and volume calculated as $(\text{length} \times \text{width}^2)/2$.

Treatments

Starting 2 days after tumor challenge, BL6 mice were injected i.p. every other day with 5 μg denileukin diftitox (DT; Eisai) or PBS control to deplete Tregs, and/or with 200 μg of anti-Gr-1 monoclonal antibody (mAb; clone RB6.8C5-18) or isotype control to deplete Gr-1⁺ cells. Foxp3^{DTR} mice were injected with 15 μg diphtheria toxin per kilogram or PBS once, and sacrificed 7 to 9 days later as indicated. B16-bearing mice were treated with 25 or 50 mg 5-fluorouracil per kilogram or PBS once, 1 week after tumor challenge, and sacrificed 7 days later.

Flow cytometry

We isolated and stained cells and carried out cell sorts as previously described (28), using LSR II and FACSAria flow cytometers and FACSDiva software (BD Bioscience). Anti-CD11b (M1/70), anti-interferon (IFN)- γ (XMG1.2), anti-CD25 (PC61), anti-CD69 (H1.2F3), anti-CD4 (GK1.5), anti-CD3 (500A2), anti-Gr-1 (RB6-8C5), Ly-6C (AL-21), and matched isotype control antibodies (Ab) were from BD Pharmingen. Anti-CD62L (MEL14), anti-CD44 (1M7), anti-interleukin (IL)-17A (17B7), and anti-Foxp3 (FJK-16a) were purchased from eBioscience; anti-CD8 α (5H10) from Caltag Laboratories; Ly6-G (1A8) and anti-CD44 (1M7) mAbs from Biolegend. CD8⁺

OVA-specific T cells were detected with OVA-specific pentamers (ProImmune).

Suppression assays

Treg suppression assays were done as previously described (26). Briefly, Tregs (CD4⁺CD25^{hi} from BL6 mice or CD4⁺Foxp3⁺CD25^{hi} or CD4⁺Foxp3⁺CD25^{lo} from FIR mice) were sorted from spleens after CD4⁺ T-cell enrichment (StemCell). For MDSC suppression assays, CD11b⁺Gr-1^{hi} cells were sorted from spleens. Carboxyfluorescein succinimidyl ester-labeled CD4⁺ responder T cells from naïve mice were incubated at 3×10^4 cell per well in 96-well plates with Tregs or MDSCs, and CD3/CD28 T Cell Expander beads (Invitrogen). After 4 days incubation at 37°C, responder cell proliferation was measured by carboxyfluorescein succinimidyl ester dilution by fluorescence-activated cell sorting (FACS).

Bone marrow cultures

At sacrifice, femurs were flushed, washed in PBS, and bone marrow (BM) cells were cultured in 10% fetal calf serum/RPMI at 5×10^5 cells per well in 4 ng/mL GM-CSF (PeProTech Inc.) at 37°C in 5% CO₂. Cells were recovered for counting and FACS analysis on day 4.

Cytokine assays

Serum cytokines were assessed by a custom multiplex BioPlex Pro assay kit (Bio-Rad) according to the manufacturer's protocol. Cytokines levels were measured with a Luminex 200 analyzer (Luminex Corp.).

Statistics

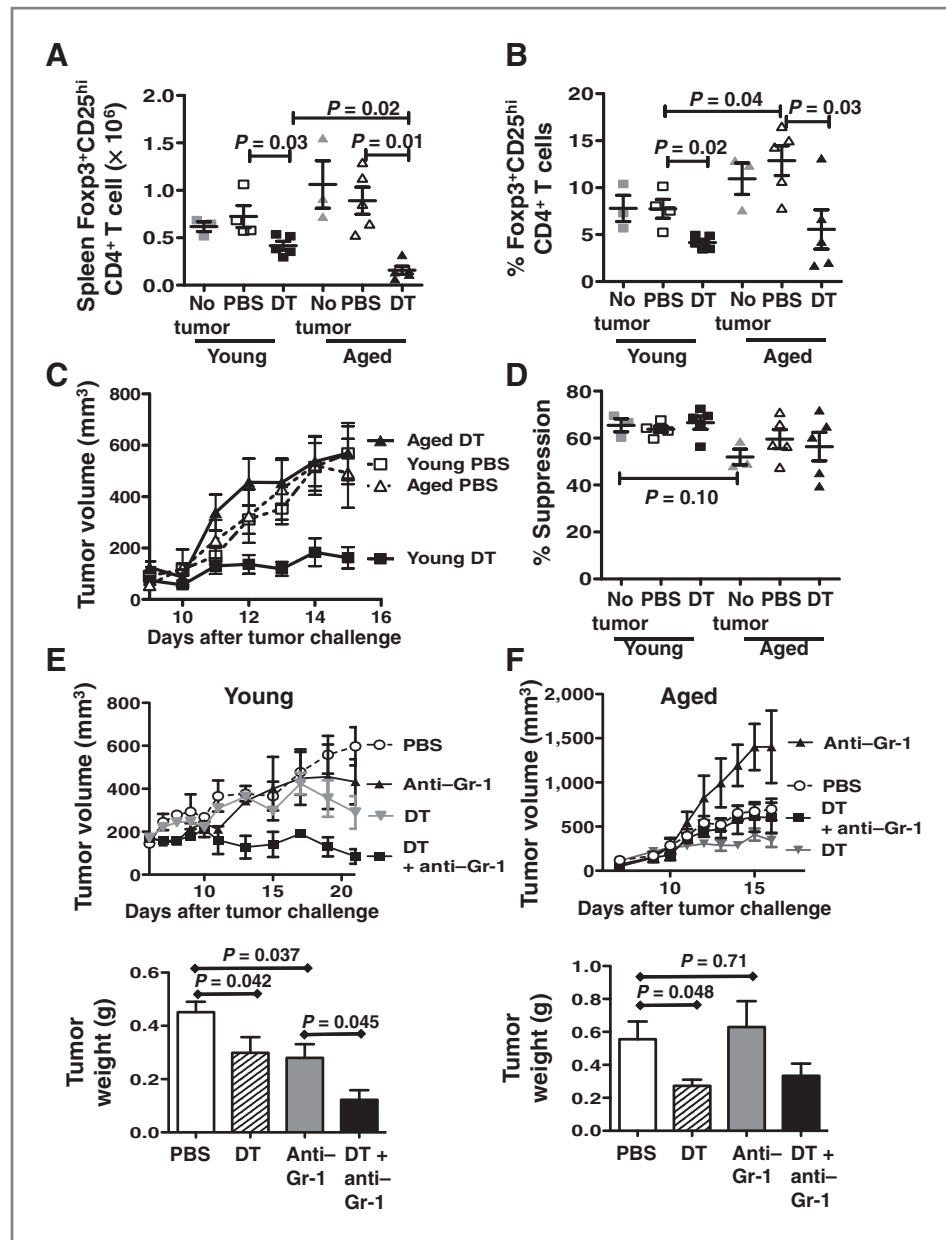
Statistical analyses were conducted with Prism software (GraphPad Inc.). For tumor growth measurements, we used a one-way ANOVA comparing treatment arms. For all other single measurement assays, we used a 2-tailed Mann-Whitney test. $P < 0.05$ was considered significant.

Results

Treg depletion is clinically efficacious in young but not in aged B16-bearing mice

As CD4⁺CD25⁺Foxp3⁺ Tregs contribute to immune dysfunction in cancer (6, 8), we first showed that naïve young and aged mice had comparable numbers of CD4⁺Foxp3⁺CD25^{hi} spleen phenotypic Tregs ($P = 0.1$, Fig. 1A). B16-challenged aged mice had a higher prevalence of spleen CD4⁺Foxp3⁺CD25^{hi} T cells compared with young mice (Fig. 1B) but absolute numbers were similar ($P = 0.5$, Fig. 1A). Following challenge, B16 tumor grew similarly in young and aged mice in the absence of specific treatment (Fig. 1C). DT treatment, which depletes young mouse Treg efficiently (29), significantly reduced B16 growth in young mice as expected ($P = 0.0001$, young DT vs. PBS), but strikingly had no effect in aged mice ($P = 0.4$, Fig. 1A). DT reduced numbers (Fig. 1A) and prevalence (Fig. 1B) of CD4⁺Foxp3⁺CD25^{hi} T cells in spleens of young mice as expected and was even more effective in depleting Tregs in aged mice (Fig. 1A and B). Spleen CD4⁺CD25^{hi} Tregs at baseline and after tumor

Figure 1. Treg depletion reduces B16 tumor growth in young but not aged mice. A–D, young and aged C57BL/6 mice challenged with B16 melanoma and treated with PBS or 5 μ g DT. Numbers (A) and prevalence (B) of spleen Foxp3⁺CD25^{hi} phenotypic Tregs in young versus aged naïve (no tumor, $n = 3$) or tumor-bearing mice treated with PBS or DT ($n = 4$ or 5). C, tumor growth. D, spleen CD4⁺CD25^{hi} Treg suppressive activity in young and aged mice at 1:1 effector:Treg ratio. E and F, tumor growth (top) and final tumor weight (bottom) from young (E) or aged (F) C57BL/6 mice challenged with MC-38 carcinoma and treated with either PBS, DT, anti-Gr-1 antibody or DT+anti-Gr-1. Values are mean \pm SEM.



challenge from young and aged mice were comparably suppressive *in vitro*, with no significant change after DT treatment (Fig. 1D).

Treg depletion improves tumor antigen-specific immunity in young but not aged hosts with B16

Untreated, aged B16-bearing mice have a higher prevalence of IL-17⁺ (Fig. 2C, Supplementary Fig. S1) and activated T cells (Supplementary Fig. S2) compared with young mice, consistent with greater inflammation in age (27, 30). DT increased tumor-antigen-specific CD8⁺ T cells (Fig. 2A) in spleens of young but not aged B16-bearing mice. DT increased the prevalence and numbers of IFN- γ - and IL-17-producing CD4⁺ and CD8⁺ spleen T cells in young B16-bearing mice while reducing these

cells in aged mice (Fig. 2B and C; Supplementary Fig. S1), suggesting differential age-related regulatory control of these cytokines producing T cells in B16-bearing mice. Finally, DT increased the prevalence of CD44⁺CD62L⁺ effector memory CD8⁺ T cells in young but not aged mice and their activation as measured by CD69 expression (Supplementary Fig. S2A and S2B). DT increased the prevalence and activation of naïve CD62L⁺CD4⁺ T cells in young mice but reduced CD69 expression in aged mice (Supplementary Fig. S2B and S2D). Together, these data are consistent with better immune improvement in DT-treated young versus aged hosts with B16 melanoma.

Lack of efficacy of DT in aged mice could result from: (i) a defect in aged Treg in hampering antitumor immunity, (ii) a defect in aged effector cells to produce significant

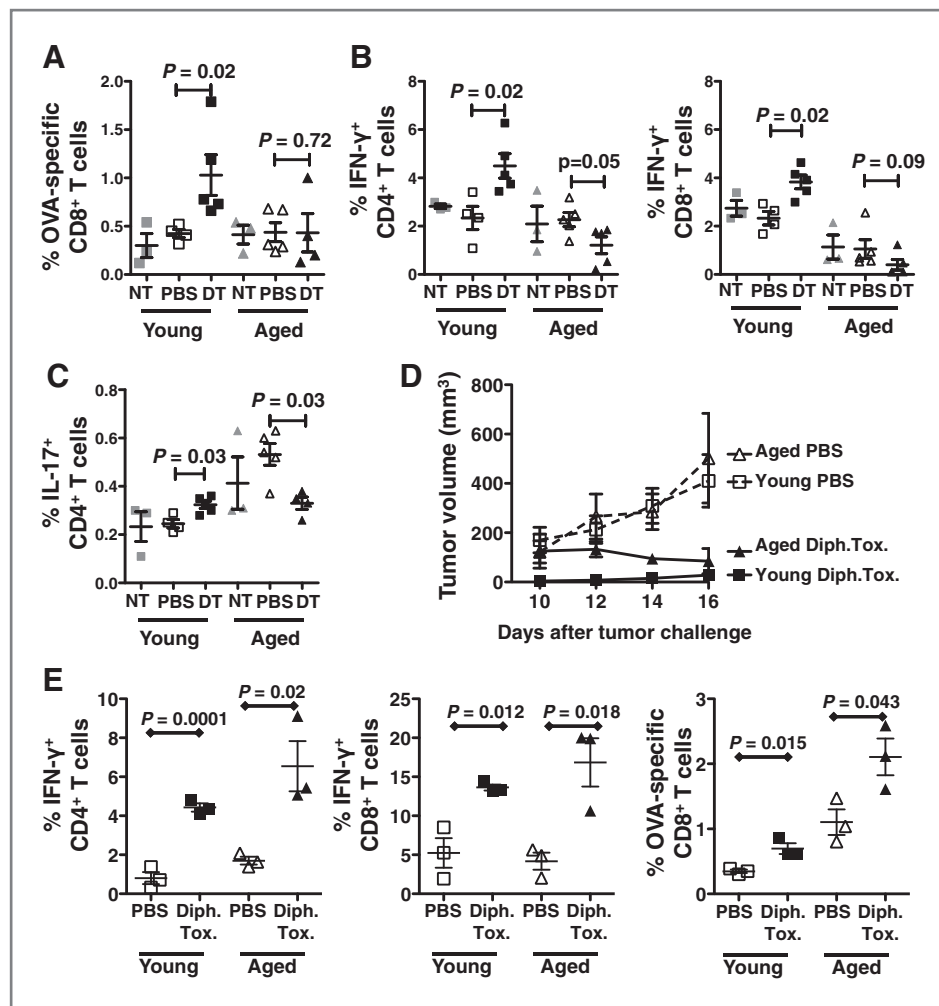


Figure 2. DT-mediated Treg depletion improves antitumor immunity in young but not aged tumor-bearing mice. A–C, young and aged C57BL/6 mice challenged with OVA-expressing B16 melanoma and treated as in Fig. 1. A, spleen OVA-specific CD8⁺ T cells prevalence. B, spleen IFN- γ ⁺ CD4⁺ and CD8⁺ T cells prevalence. (NT, no tumor; naïve controls). C, spleen IL-17⁺ CD4⁺ T cells prevalence. D and E, young and aged Foxp3^{DTR} mice challenged with B16 and treated with 15 μ g diphtheria toxin (Diph. Tox.)/kg or PBS. D, tumor growth in young and aged Foxp3^{DTR} mice. E, prevalence of IFN- γ ⁺ CD4⁺ and CD8⁺ T cells and OVA-specific CD8⁺ T cells. Values are mean \pm SEM.

antitumor immunity following Treg depletion, or (iii) additional age-dependent regulatory mechanisms preventing effective antitumor immunity after Treg depletion.

Functional Foxp3⁺CD25^{lo} Tregs increase with age

To test for potential additional Treg populations, we used FIR mice in which Tregs are identified as red fluorescent protein⁺Foxp3⁺ cells (24). Foxp3⁺CD4⁺ T-cell prevalence increased with age (Fig. 3A and B) and, in particular, Foxp3⁺CD4⁺ T cells expressing low levels of CD25 (CD4⁺Foxp3⁺CD25^{lo}; Fig. 3C), previously described as being equally suppressive as conventional CD4⁺Foxp3⁺CD25^{hi} Tregs in young naïve mice (31). CD4⁺Foxp3⁺CD25^{lo} Tregs from aged naïve mice were functional but less suppressive than conventional CD4⁺Foxp3⁺CD25^{hi} Tregs *in vitro* (Fig. 3D).

DT depletes both CD25^{hi} and CD25^{lo} Foxp3⁺ Tregs in tumor-bearing aged mice

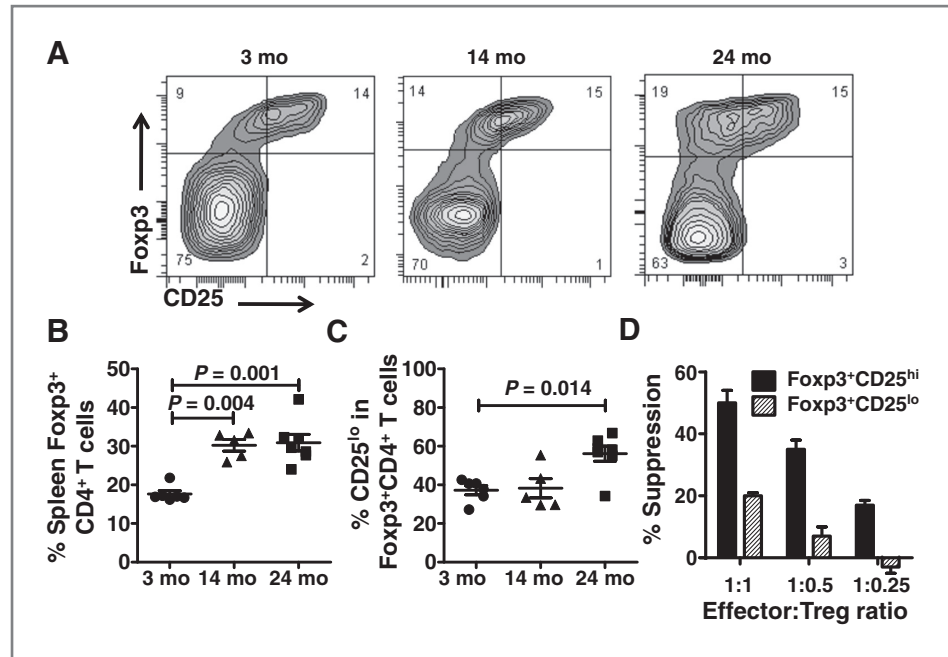
Because DT targets IL-2 receptor-expressing cells and not Tregs specifically (6), it was possible that DT failed to deplete this Foxp3⁺CD25^{lo} Treg subpopulation prominent in aged tumor-bearing mice. Aged FIR mice were challenged with B16 and treated with DT. Tumor growth in aged FIR

mice was comparable with that in wild-type (WT) mice, and DT treatment was again clinically ineffective (Fig. 4A). Two days after the last DT injection, both CD25^{hi} and CD25^{lo}Foxp3⁺CD4⁺ spleen T cells were reduced, although conventional Foxp3⁺CD25^{hi} Tregs were preferentially depleted (Fig. 4B). The proportion of CD25^{hi} Tregs among total Foxp3⁺ cells was significantly reduced by DT treatment ($P = 0.01$, Fig. 4C).

CD25^{hi} and CD25^{lo} Foxp3⁺CD4⁺ T cells are equally suppressive in aged B16-bearing mice

We tested the suppressive function of CD25^{hi} and CD25^{lo} Foxp3⁺CD4⁺ populations and found that both Treg subsets were equally suppressive in aged B16-bearing mice (Fig. 4D) in contrast to their differential suppression capacity in aged naïve mice (Fig. 3D), suggesting that tumors can alter the function of a specific Treg subset, in this case making it more suppressive. Nonetheless, DT treatment did not affect the suppressive function of remaining Tregs in aged mice (Fig. 4D). As in WT mice, DT treatment did not significantly increase tumor specific or IFN- γ ⁺CD8⁺ T cells in spleens of DT-treated B16-bearing FIR mice (Fig. 4E). Thus, lack of clinical efficacy of DT treatment in aged B16-bearing mice

Figure 3. CD25^{lo}Foxp3⁺ Tregs are increase in aged naïve mice. A, Foxp3 versus CD25 expression in CD4⁺ T cells from spleen of one representative 3-, 14-, and 24-month old naïve FIR mice. B, percentage of Foxp3⁺CD4⁺ T cells in 3-, 14-, and 24-month old naïve FIR mice (n = 5 to 6 mice per group). C, percentage of CD25^{lo} cells among Foxp3⁺CD4⁺ T cells. D, Foxp3⁺CD25^{hi} versus Foxp3⁺CD25^{lo} Treg suppressive activity from 24-month old mice.



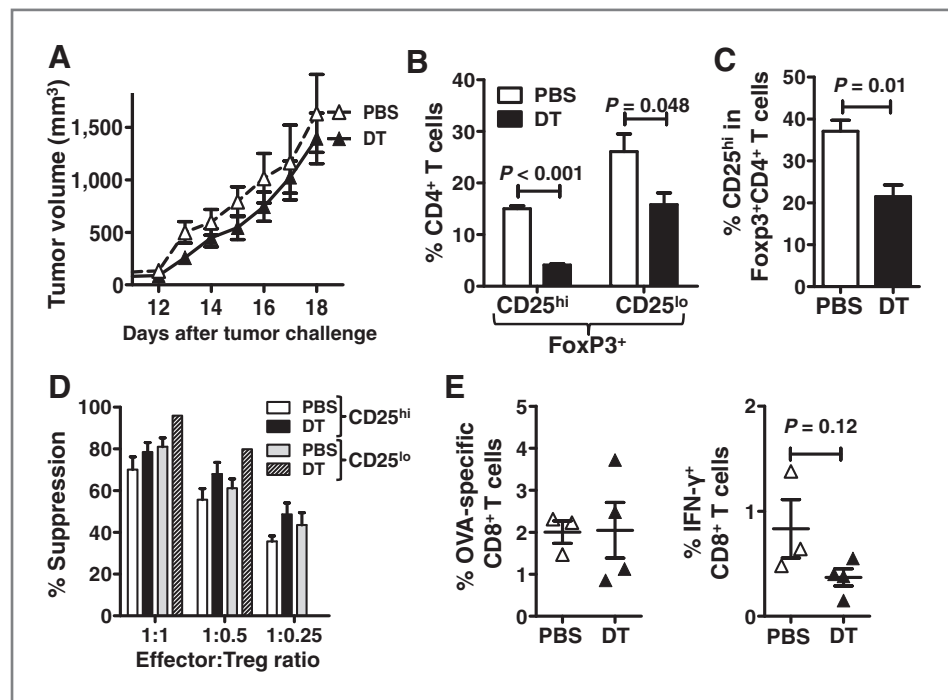
is not due to lack of depletion of a specific Treg subpopulation.

Treg depletion increases MDSC numbers in B16-bearing aged mice

As differential Treg depletion seemed unlikely to explain differential treatment effects, we tested DT treatment effects on MDSCs as they are reportedly increased in aged mice and contribute to immune dysfunction in cancer (32), including in

aged hosts (22). Consistent with prior reports (22, 33), aged mice had a higher prevalence (Fig. 5A) and numbers (Fig. 5B) of spleen CD11b⁺Gr-1^{hi} MDSCs at baseline and after tumor challenge. DT treatment slightly reduced the numbers and prevalence of CD11b⁺Gr-1^{hi} MDSC in young tumor-bearing mice (Fig. 5A and B). By striking contrast, DT significantly increased the prevalence and numbers of CD11b⁺Gr-1^{hi} MDSCs in aged B16-bearing mice (Fig. 5A and B). MDSCs were also significantly more suppressive in aged versus young mice,

Figure 4. DT depletes CD25^{hi} and CD25^{lo} Foxp3⁺ Tregs in tumor-bearing aged mice. Twenty-two-month old FIR mice were challenged with B16 tumor cells and treated with either PBS or DT. A, B16 tumor growth in PBS (n = 3) versus DT (n = 4) treated mice. B, percentage of Foxp3⁺CD25^{hi} and Foxp3⁺CD25^{lo} Tregs in spleen CD4⁺ T cells at day 18. C, prevalence of CD25^{hi} T cells among Foxp3⁺CD4⁺ T cells. D, Foxp3⁺CD25^{hi} and Foxp3⁺CD25^{lo} Treg suppressive activity from mice treated with PBS or DT. E, OVA-specific and IFN-γ⁺ CD8⁺ spleen T cells prevalence.



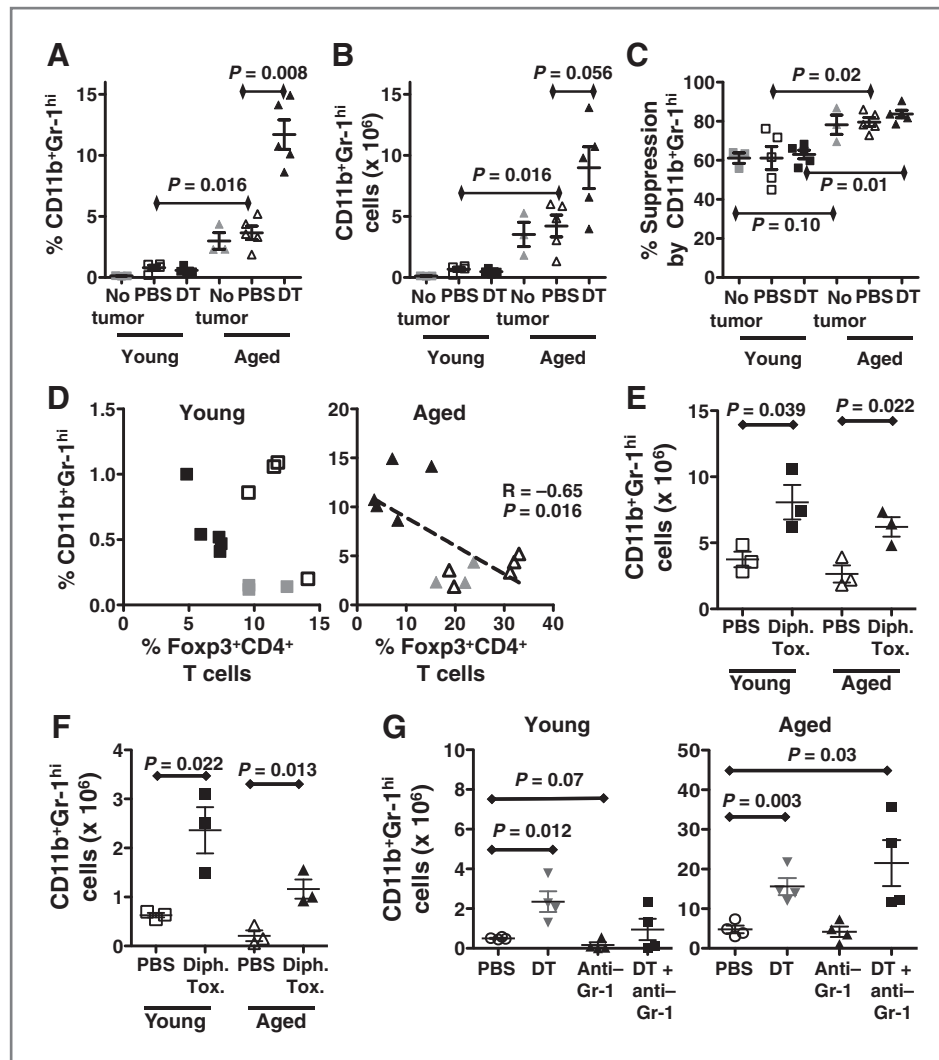


Figure 5. DT-mediated Treg depletion increased CD11b⁺Gr-1^{hi} MDSC in B16-bearing and naïve aged mice. A–D, young and aged mice challenged with B16 cells and treated as in Fig. 1. Prevalence (A) and numbers (B) of spleen CD11b⁺Gr-1^{hi} phenotypic MDSC in young versus aged naïve (no tumor) or tumor-bearing mice treated with PBS or DT. C, spleen CD11b⁺Gr-1^{hi} MDSC suppression at 1:1 effector:MDSC ratio. D, negative correlation between spleen CD11b⁺Gr-1^{hi} MDSCs and Foxp3⁺CD4⁺ splenic Tregs in aged but not in young tumor-bearing mice. E, spleen CD11b⁺Gr-1^{hi} cell numbers in young and aged B16-bearing Foxp3^{DTR} mice treated with PBS or diphtheria toxin (Diph. Tox.). F, spleen CD11b⁺Gr-1^{hi} cell numbers in young and aged naïve Foxp3^{DTR} mice treated with PBS or diphtheria toxin (Diph. Tox.). G, spleen CD11b⁺Gr-1^{hi} cell numbers in young or aged C57BL/6 mice challenged with MC-38 carcinoma and treated with PBS, DT, anti-Gr-1, or DT+anti-Gr-1.

both at baseline and after B16 challenge, which was not affected by DT treatment (Fig. 5C).

MDSC prevalence was inversely correlated with Treg prevalence only in aged B16-bearing mice (Fig. 5D) suggesting that Tregs could regulate MDSC numbers in aged B16-bearing mice and that Treg depletion in aged mice leads to an undesired increase in MDSCs, thus negating the beneficial antitumor effects of DT-mediated Treg depletion observed in young mice. Increased spleen MDSCs following DT treatment and their highly suppressive character was confirmed in aged B16-bearing FIR mice (Supplementary Fig. S3A–S3C). Because MDSC subsets could have differential function, we then showed that DT had similar effects on CD11b⁺Gr-1^{med} MDSC (Supplementary Fig. S3D).

MDSC depletion is clinically efficacious in aged but not in young B16-bearing mice

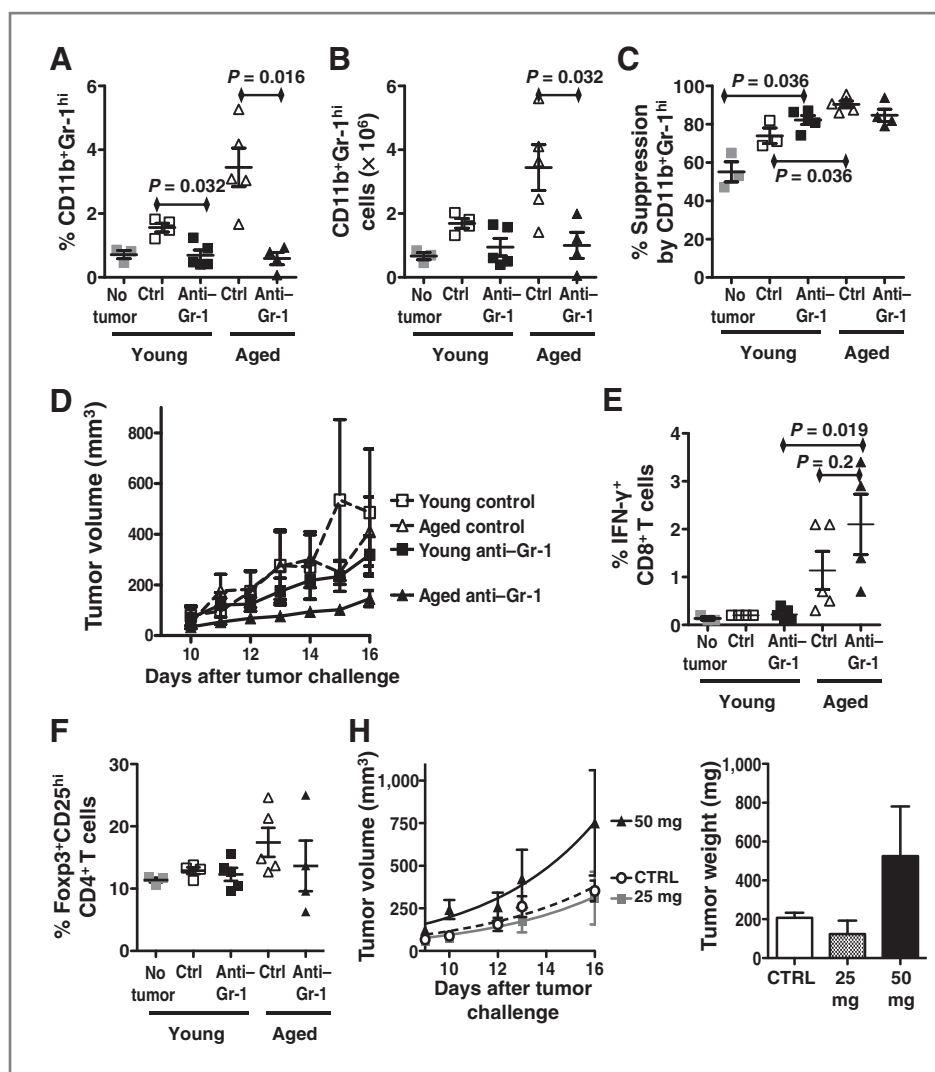
We tested effects of MDSC depletion given their deleterious effects. Anti-Gr-1 antibody depleted CD11b⁺Gr-1^{hi} MDSC in spleens of young and aged B16-bearing mice (Fig. 6A and B). Similar to DT treatment, anti-Gr-1 antibody did

not significantly affect the suppressive function of remaining MDSCs in young or aged mice (Fig. 6C). However, by contrast to DT-mediated Treg depletion, anti-Gr-1-mediated depletion of MDSC resulted in significantly slower tumor growth in aged but not young B16-bearing mice ($P = 0.007$ for aged, Fig. 6D). MDSC depletion yielded significantly more IFN- γ ⁺CD8⁺ T cells in aged versus young mice (Fig. 6E) suggesting improved antitumor immunity as a mechanism for treatment efficacy, without significant effect on the prevalence of Foxp3⁺CD25^{hi} Tregs (Fig. 6F) or Treg function (Supplementary Fig. S4A) in both young and aged mice. Surprisingly, anti-Gr-1 antibody increased CD11b⁺Gr-1^{med} MDSC in young but not aged hosts (Supplementary Fig. S4B).

Combining Treg depletion with MDSC depletion increases clinical response in aged B16-bearing mice

As MDSC seem to contribute more to cancer-related immune dysfunction in aged versus young mice with B16, and because DT-mediated Treg depletion increased these dysfunctional MDSC in aged B16-bearing mice, we hypothesized that

Figure 6. MDSC depletion improves antitumor immunity in aged mice. A–F, young and aged mice challenged with B16 and treated with isotype control or anti-Gr-1 antibody. Prevalence (A) and numbers (B) of CD11b⁺Gr-1^{hi} spleen MDSC in tumor-bearing mice. C, spleen MDSC suppressive function at 1:1 effector:MDSC ratio. D, tumor growth. E, IFN- γ ⁺CD8⁺T cells prevalence. F, Foxp3⁺CD25^{hi}CD4⁺Tregs prevalence. H, tumor growth and final tumor weight in young C57BL/6 mice challenged with B16 and treated with a single dose of 25 mg or 50 mg/kg 5-fluorouracil or with PBS (Ctrl).



combining both Treg and MDSC depletion would significantly boost antitumor immunity and clinical efficacy in aged mice. Thus, we challenged aged BL6 mice with B16 and treated with anti-Gr-1 antibody alone or combined with DT. Combination therapy significantly reduced tumor growth and weight in aged mice better than anti-Gr-1 treatment alone (Fig. 7A). Reduced tumor growth was associated with increased IFN- γ ⁺CD4⁺ and IFN- γ ⁺CD8⁺ T-cell prevalence (Fig. 7B), and increased tumor-specific CD8⁺ T-cell prevalence in spleen and tumor draining lymph nodes (DLN; Fig. 7C). Anti-Gr-1 treatment again reduced MDSCs in spleen and tumor (Fig. 7D). Combination therapy reduced CD25^{hi}CD4⁺ phenotypic Tregs similar to DT treatment alone (Fig. 7E). In confirmation, combination therapy significantly reduced tumor growth in aged FIR mice over anti-Gr-1 antibody alone (Supplementary Fig. S4C and S4D).

Tregs restrain MDSC numbers in young and aged naive and B16-bearing hosts

DT clearly depletes Tregs, but is not Treg specific (6). To test specific Treg contributions to MDSC effects, we chal-

lenged syngeneic Foxp3^{DTR} mice with diphtheria toxin, which depletes only Tregs (34). Following a single injection of diphtheria toxin, young and aged B16-bearing mice experienced similar MDSC increases 9 days later (Fig. 5E) confirming Treg-mediated MDSC control in B16 tumor, but not just in aged hosts. To test whether effects depended on tumor, we depleted Tregs from young and aged naive Foxp3^{DTR} mice and found similar MDSC increases (Fig. 5F). Thus, DT has an age- and tumor-specific effect on MDSC control, but this effect might not be exclusively from Treg depletion.

High-order Treg depletion improves immune and clinical responses to B16 in aged hosts

Treg depletion is essentially complete in Foxp3^{DTR} mice treated with diphtheria toxin (34; and data not shown), contrasting to lesser Treg depletion with DT (Fig. 1A and B). This high-order Treg depletion in aged Foxp3^{DTR} mice improved clinical tumor control (Fig. 2D), IFN- γ production, and anti-tumor immunity (Fig. 2E). Thus, with sufficient Treg reduction

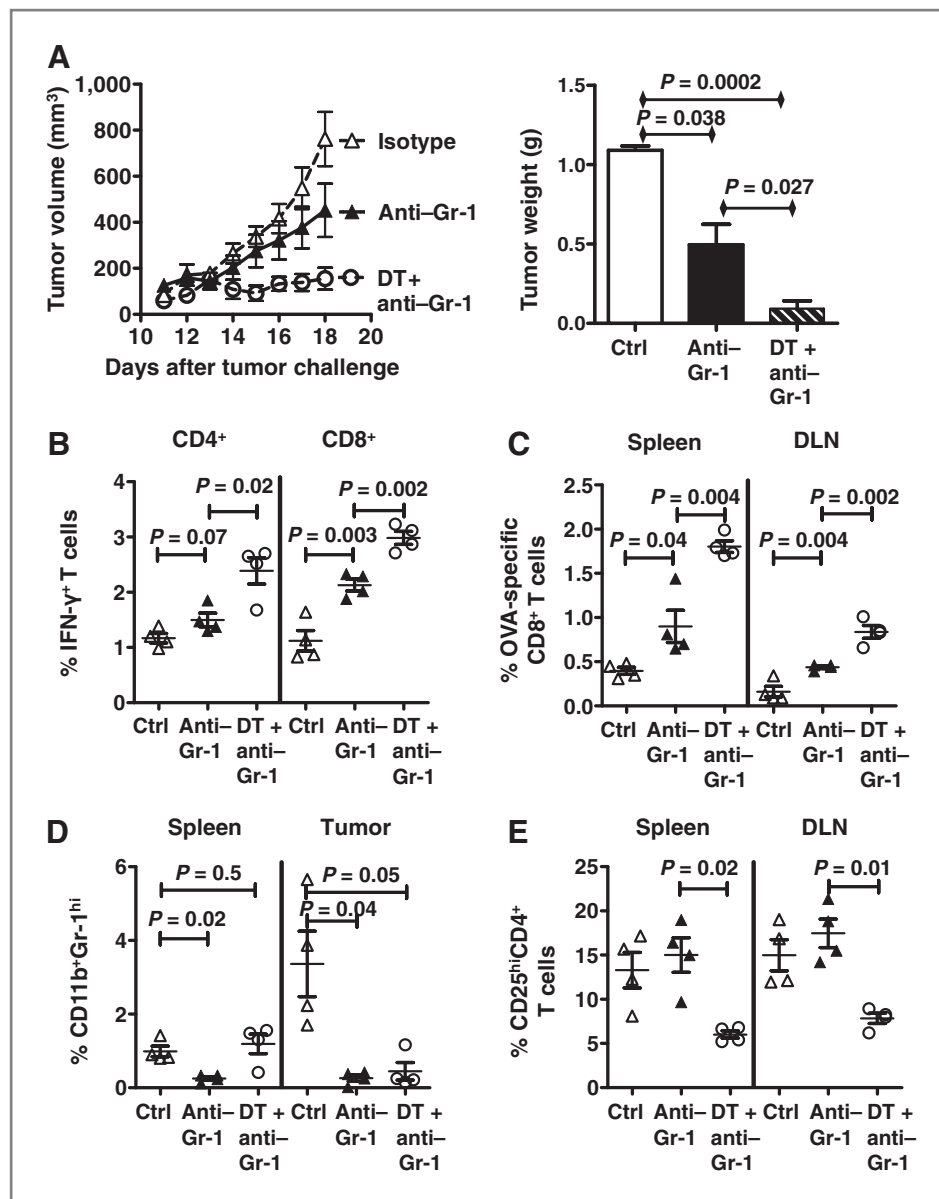


Figure 7. Combination of Treg and MDSC depletion slows tumor growth and improves antitumor immunity in aged mice. Aged mice challenged with B16 melanoma and treated with isotype control, anti-Gr-1 antibody alone, or in combination with DT ($n = 4$ per group). A, tumor growth (left) and final tumor weight (right). B, spleen IFN- γ^+ CD4⁺ and IFN- γ^+ CD8⁺ T cells prevalence. C, percentage of OVA-specific CD8⁺ T cells in spleen and tumor-DLN. D, percentage of CD11b⁺Gr-1^{hi} MDSC in spleen and tumor. E, percentage of CD25⁺CD4⁺ Tregs in spleen and DLN.

alone, aged hosts can mount clinically effective antitumor immunity (Fig. 2D and E).

Treatment effects are tumor dependent

Because DT treatment effects could be tumor- as well as age dependent, we assessed treatment effects in MC-38 colon carcinoma. Unlike results in B16, DT-mediated Treg depletion was beneficial to tumor growth (Fig. 1E and F) and antitumor immunity (Supplementary Fig. S5A–S5F) in young and aged mice. Anti-Gr-1 antibody further boosted clinical effects in young but not aged hosts (Fig. 1E and F) although DT increased CD11b⁺Gr-1^{hi} MDSC in both (Fig. 5G). A significant increase in CD11b⁺Gr-1^{med} was also observed only in young MC-38-bearing mice treated with DT (Supplementary Fig. S3E). Tumor weights (Fig. 1F) were similar in aged B16-bearing mice treated

with PBS and anti-Gr-1-treated mice despite volumetric differences.

DT and Treg depletion affect myelopoiesis *in vitro*

To test whether Treg depletion effects on MDSCs related to altered myelopoiesis, we cultured BM from B16-bearing Foxp3^{DTR} mice and MC-38-bearing WT mice with 4 ng/mL GM-CSF for 4 days. High-order Treg depletion or DT treatment skewed BM differentiation toward the Ly-6C^{hi}Ly-6G[−] subset in B16-bearing Foxp3^{DTR} mice (Supplementary Fig. S6A), and MC-38-bearing C57/BL6 mice (Supplementary Fig. S7A and S7B). These data establish that Tregs and DT treatment can alter myelopoiesis in tumor-bearing mice, but additional work is required to establish the *in vivo* significance. GM-CSF, VEGF, M-CSF, IL-6 (among others) can drive MDSC generation *in vivo*

(32), but we found similar serum levels of these cytokines in B16 (Supplementary Fig. S6B) or MC-38 (Supplementary Fig. S7C) bearing young or aged mice following high-order Treg depletion or DT treatment, and equivalent increases in GM-CSF⁺ T lymphocytes in B16-bearing (Supplementary Fig. S6C and S6D) and MC-38-bearing mice (Supplementary Fig. S7D). Furthermore, DT did not alter GM-CSF, VEGF, M-CSF (not shown), or IL-6 in young or aged B16-bearing mice (Supplementary Fig. S8). These data establish that Tregs control factors related to MDSC generation in tumor-bearing mice.

5-fluorouracil does not treat B16 melanoma effectively

Finally, we tested an agent contemplated for MDSC depletion in humans, 5-fluorouracil. In striking contrast to a published report (35), 5-fluorouracil did not deplete MDSC in B16 melanoma and worsened clinical tumor response (Fig. 6H) with no effect on non-Treg T cells, B cells, or non-MDSC myeloid cell subsets (not shown), and no alteration of MDSC or Treg function *in vitro* (Supplementary Fig. S9).

Discussion

Although increasing age is the biggest risk factor for cancer, surprisingly few studies have addressed the specific consequences of age on antitumor immunity, and fewer still have examined age effects on tumor immunotherapy. Age-dependent immune alterations including the generally decreased performance of effector T cells could reduce antitumor immunity. Naive T cells from older individuals show functional defects such as impaired ability to proliferate, produce relevant cytokines, and reduced differentiation into effector T cells (12–13). Thus, many immunotherapies effective in young hosts are less so in aged hosts. For example, tumor rejection mediated through OX40 signals decreases with age, as does effector T-cell differentiation (36). However, effective cancer immunotherapy for aged individuals is a realistic goal, as some age-associated immune defects are reversible. As examples, tumor immunity in aged mice can be rescued with sufficient cosignaling (37), and reduced T-cell priming boosted by the immune cosignaling CD137 (41BB) pathway (38).

A complementary approach to boosting effector arms of immunity in cancer immunotherapy is to reduce tumor-associated immune dysfunction (5, 6). As we and others have previously demonstrated the utility of depleting Tregs as a means to reverse tumor-associated immune dysfunction to treat cancer in (young) mouse models (6), we tested the concept in aged hosts.

In our B16 melanoma model, Treg depletion with DT was immunologically and clinically effective in young mice as expected. Disappointingly, though, depleting Tregs with DT in aged hosts did not improve antitumor immunity or immune-mediated tumor rejection. For humane reasons, we studied tumor growth, not survival, but tumor growth and weights are good surrogates for survival in B16 melanoma. We showed that Treg numbers and *in vitro* function of young and aged Tregs were comparable, and that DT equally depleted young and aged Tregs. Thus, poor clinical efficacy of Treg depletion in aged hosts could be attributed to a

defect in aged antitumor effector cells, other immunosuppressive mechanisms, or to tumor-specific factors (21). To assess for additional regulatory populations, we found an age-associated increase in CD25^{lo}Foxp3⁺ Tregs as reported, and showed that they were more suppressive in the tumor environment than in naïve mice. It is likely that Tregs contribute differentially to immunopathology in a tumor-dependent fashion as seen in both young and aged hosts (6, 20, 21), but effects of distinct Treg subsets remain to be defined. We further found an age-associated increase in MDSC that were more suppressive *in vitro* compared with young counterparts as reported (22). Surprisingly, DT-mediated Treg depletion increased MDSC numbers in aged but not young hosts with B16 melanoma, suggesting a mechanism for DT treatment failure, and suggesting that MDSC depletion combined with Treg depletion could be useful cancer immunotherapy in aged hosts in this model.

In support, anti-Gr-1 antibody depleted MDSC effectively in young and aged hosts, but improved antitumor immunity and was clinically effective only in aged hosts with B16. Consistent with increased MDSC reducing DT efficacy in aged hosts, addition of anti-Gr-1 antibody improved Treg depletion efficacy in aged, but not young hosts in this model. These data confirm that aged effector cells remain competent and can mediate important effector functions provided that the impeding immune dysfunction is reduced. As anti-Gr-1 antibody is not specific for MDSC, dysfunctional contributions from other Gr-1⁺ cells, such as plasmacytoid dendritic cells, are not entirely excluded and require further investigation.

As DT only incompletely depletes Tregs, and targets IL-2 receptor-expressing cells, not Tregs specifically, we used Foxp3^{DTR} mice in which specific, high-order Treg depletion is possible. High-order Treg depletion in young and aged naïve or B16-bearing hosts equally boosted MDSCs. These data confirm that Treg constrain tumor MDSC, but show that DT-mediated Treg depletion differs from high-order Treg depletion in MDSC effects, which could be due to better Treg depletion in Foxp3^{DTR} mice, lack of depletion of a specific Treg subset by DT, DT effects on another IL-2 receptor-expressing cell, or reduced Treg function from the greater inflammation in Foxp3^{DTR} versus DT-treated mice. Understanding DT-mediated effects on MDSC restraint helps define mechanisms of treatment responses, important to clinical translation, as DT is U.S. Food and Drug Administration (FDA)-approved for some cancers, and depletes Tregs in humans (6). Understanding mechanisms of MDSC restraint by Tregs in cancer furthers basic knowledge of cancer immunopathology. Both approaches are important to developing optimal, translatable cancer immunotherapies.

MDSCs are myeloid cells generated through cooperation of numerous factors (32). We found that DT and high-order Treg depletion had similar effects on *in vitro* myelopoiesis, including skewing to potentially more immunosuppressive Ly-6C⁺ MDSC, and increasing *in vivo* T-cell GM-CSF production but without clear effects on global GM-CSF, VEGF, M-CSF, or IL-6. Thus, Tregs and DT each can affect myelopoiesis, which might affect immunotherapy outcomes. DT effects on MDSCs deserve additional attention to help translate concepts, as

selective high-order Treg depletion in humans is currently unachievable and could pose autoimmune risks in any regard.

High-order Treg depletion alone also improved immune and clinical responses to B16 in aged hosts, further showing the underlying immune competence of aged antitumor effector cells, despite the difficulty in translating this approach clinically. In contrast to DT effects, though, high-order Treg depletion was clinically and immunologically efficacious despite increases in MDSCs. This result could be due to Treg-specific effects in Foxp3^{DTR} mice, to alterations in the inflammatory environment from high-order Treg depletion, or differential MDSC functions after DT versus high-order Treg depletion, among other factors. If clinical effects are specifically from high-order Treg depletion, as opposed to from effects on some IL-2-expressing cell population, that suggests that IL-2 receptor targeting agents for human Treg depletion such as DT or daclizumab might be ultimately inferior to agents that specifically target human Tregs (which do not yet exist). Understanding this point could greatly improve Treg management strategies.

Strikingly, in another tumor, MC-38 colon carcinoma, MDSC depletion was effective in young hosts and further boosted utility of DT-mediated Treg depletion. By contrast, and distinct from B16 results, DT alone was useful in aged hosts but not further augmented by MDSC depletion. Reminiscent of Foxp3^{DTR} results, DT improved immunity in young and aged hosts, despite increasing MDSC in both. Increased antitumor immunity correlated with clinical outcomes supporting the concept that immune dysfunction was improved with the distinct approaches. These data suggest that immune dysfunction is not only age-dependent but also tumor-dependent, affecting both Tregs and MDSC. Our preliminary *ex vivo* studies did not disclose a noticeable difference in myelopoiesis in BM of mice bearing MC-38 or B16, or in cytokines affecting MDSC generation following Treg depletion in each tumor. Additional work is required to understand specifics of MDSC generation and immunopathogenesis in individual tumors as a function of age and as a consequence of distinct treatment modalities.

Because MDSC depletion alone and in combination with Treg depletion was effective in distinct tumors and hosts, we assessed a translational approach. 5-fluorouracil, is FDA approved and thus relatively easily tested as human cancer immunotherapy. Our surprising finding that 5-fluorouracil does not deplete MDSC in B16 and worsens the clinical course suggests that MDSC depletion agents will also have variable

effects depending on the tumor and host. Thus, unlike DT, which depletes Tregs in humans analogous to animal models, MDSC depletion studies in preclinical models might not predict human clinical effects as well.

Together, our data show that the function of aged anti-tumor effector T cells can be improved by reducing age-related immune dysfunction, with positive clinical benefits. Strategies to reduce immune dysfunction must be tailored to account for age-related and tumor-related immune dysfunctions for optimal utility. Thus, improved and efficacious cancer immune therapy for aged hosts, who are at the greatest risk for cancer, is a realistic goal that can be met with a better understanding of the specific effects of age on basal and tumor-related immune dysfunction. Strategies we propose are clinically translatable as FDA-approved agents such as DT, cyclophosphamide, and daclizumab deplete Tregs (6), and FDA-approved agents such as 5-fluorouracil kill MDSCs in some models (35).

Disclosure of Potential Conflicts of Interest

T.J. Curiel is a consultant on the advisory board of Eisai.

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