Clinical Cancer Research

Age Correlates with Response to Anti-PD1, Reflecting Age-Related Differences in Intratumoral Effector and Regulatory T-Cell Populations

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Abstract

Purpose: We have shown that the aged microenvironment increases melanoma metastasis, and decreases response to targeted therapy, and here we queried response to anti-PD1.

Experimental Design: We analyzed the relationship between age, response to anti-PD1, and prior therapy in 538 patients. We used mouse models of melanoma, to analyze the intratumoral immune microenvironment in young versus aged mice and confirmed our findings in human melanoma biopsies.

Results: Patients over the age of 60 responded more efficiently to anti-PD-1, and likelihood of response to anti-PD-1 increased with age, even when we controlled for prior MAPKi therapy. Placing genetically identical tumors in aged mice (52 weeks) significantly increased their response to anti-PD1 as compared with the same tumors in young mice (8 weeks). These data suggest that this increased response in aged patients occurs even in the absence of a more complex mutational landscape. Next, we found that young mice had a significantly higher population of regulatory T cells (Tregs), skewing the $CD8^+$:Treg ratio. FOXP3 staining of human melanoma biopsies revealed similar increases in Tregs in young patients. Depletion of Tregs using anti-CD25 increased the response to anti-PD1 in young mice.

Conclusions: While there are obvious limitations to our study, including our inability to conduct a meta-analysis due to a lack of available data, and our inability to control for mutational burden, there is a remarkable consistency in these data from over 500 patients across 8 different institutes worldwide. These results stress the importance of considering age as a factor for immunotherapy response. *Clin Cancer Res; 24(21); 5347–56. ©2018 AACR.*

See related commentary by Pawelec, p. 5193

Introduction

Anticytotoxic T-lymphocyte associated protein 4 (CTLA4) and anti-programmed-death-receptor-1 (PD1) checkpoint immunotherapies have activity in both BRAF-mutant and wild-type melanoma, and have the unique ability to produce durable disease control. Recent efforts to identify biomarkers for immunotherapy have highlighted the importance of intratumoral immune populations and their function in response to therapy (1–6). Interestingly, subpopulations of immune cells, and overall immune function, decline with age in a well-known process called

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Translational Relevance

In a multinational, multi-institutional cohort of over 500 patients, we found that the chance of disease progression after anti-PD1 treatment decreased by 13% with each decade of life. In animal models, pretreating with anti-CD25 decreased the number of Tregs in the tumors in young mice, and increased their response to anti-PD1. Together, these data reveal the unexpected finding that older patients fare better on anti-PD1, and suggest that treating refractory patients with antibodies that deplete Tregs may increase their chances of response to anti-PD1. Our results stress the importance of age as an important factor in understanding tumor response and resistance to therapy.

immunosenescence (7, 8). This process is the result of multiple factors including thymic atrophy (9, 10), decreases in naïve T cells, and increases in memory T cells with reduced functionality (11, 12), along with decreases in the diversity of antigen recognition by T cells (13). These factors may be at least partly responsible for the increased incidence of most cancers, including melanoma, in older individuals (14). While immunosenescence-driven differences in overall immune function between young and aged individuals have been well characterized, the potential impact of aging on differences in the intratumoral immune populations and response to immunotherapy remain unknown (15). We have recently shown that factors present in the aged microenvironment promote melanoma resistance to targeted inhibition of BRAF, highlighting the importance of considering patient age when predicting both disease progression and response to therapy (16).

In this study, we investigated whether age-mediated differences in intratumoral immune populations played a role in patient response to immunotherapy. The data indicate that younger melanoma patients were more resistant to anti-PD1 inhibition. We show that murine melanomas in young mice, and melanomas in younger patients, have significantly higher levels of forkhead box protein P3 (FOXP3) positive regulatory T cells (Tregs) and decreased $CD8^+$ effector T-cell populations than their older counterparts. In our murine melanoma model, resistance to anti-PD1 could be partially overcome in mice by treatment with anti-CD25. These data were consistent with a study from the Quezada laboratory, showing that CD25 expression is largely restricted to Tregs in mice and in humans, and that depleting Tregs could increase response to anti-PD1 (17). Furthermore, trials of the drug daclizumab in humans have also indicated that CD25 can be effectively depleted, without depleting effector T cells (18).

These data may have significant clinical impact, as they suggest that depleting Tregs preferentially in young patients could enhance the response of these otherwise refractory patients to anti-PD1 therapy.

Materials and Methods

Patient data

A total of 538 total (238 <62 and 300 ≥62) patients with primary or metastatic disease were available with age at diagnosis or treatment recorded. Patient samples were stained and quantified by certified pathologists blinded to patient age and gender at NYU and Vanderbilt University. Informed consent was obtained prior to collecting tissue and patient data provided to Wistar Institute was deidentified and collected under Institutional Review Board Exemption 212052858. FOXP3 and CD8 expression were scored by first identifying the densest area of infiltration by cell morphology and then quantifying the number of positively stained cells in a single high-power field (HPF) within the lymphocytic infiltrate at 40× magnification. Patient best response data was obtained from 8 institutions including 6 in the United States: NYU (New York, NY), UCLA (Los Angeles, CA), MD Anderson Cancer Center (Houston, TX), Vanderbilt University Medical Center (Nashville, TN), University of Pennsylvania (Philadelphia, PA) and the H. Lee Moffitt Cancer Center (Tampa, FL). Patient data were also obtained from Westmead Hospital and the Melanoma Institute of Australia (NSW, Australia) as well as Essen University Hospital (Essen, Germany). All patient materials and clinical data were obtained from patients who provided written informed consent under a tissue collection protocol that was approved by an institutional review board, and sharing of deidentified information collected with other research entities is specified in the research consent form. Patient data were recorded on site and deidentified for analysis (19). An additional 11 patients were confirmed nonduplicates, and added to the UCLA cohort from the GEO dataset GSE78220 (20).

Cell lines

Yumm1.7 and Yumm2.1 cells were provided by Dr. Marcus Bosenberg (Yale University, New Haven, CT) and cultured in DMEM containing 10% FCS, 1% penicillin/streptomycin, and 1% L-glutamine. BSC9AJ2 cells were cultured in RPMI media containing 10% FCS, 1% penicillin/streptomycin, and 1% L-glutamine. All cells were grown at 37°C at 5% CO_2. These cells were used in experiments upto 5–10 passages from thawing (between 2015 and 2017). These mouse cells were IMPACT tested (RADIL services) to check for impurities, prior to transplantation in the mice. The supernatants of cells were further routinely collected and tested for mycoplasma (monthly) using a Lonza MycoAlert assay at the University of Pennsylvania Cell Center Services.

In vivo reagents

Rat IgG2AK (#400566) and anti-PD1 (clone RMP1-14) were purchased from BioLegend for *in vivo* use. Two-hundred micrograms of CD25 antibody, clone PC-61.5.3 (#BP0012) and IgG1, clone TNP6A7 (#BP0290) were purchased from BioXCell and injected every 5 days. Three-hundred micrograms IgG2AK and anti-PD1 were diluted in sterile PBS and injected via intraperitoneal injections every 5 days. Cyclophosphamide monohydrate was purchased from Sigma Aldrich (#6055-19-2) and diluted in sterile PBS prior to intraperitoneal injection.

Mice

All animal experiments were performed at the Wistar Institute (AAALAC accredited) and approved by the Institutional Animal Care and Use Committee (112503Y_0). C57BL6 male and female mice were purchased from the Charles River NCI facility. All mice were either 8–10 weeks old or 10 months old at the time of tumor implantation. Yumm1.7 or BSC9AJ2 cells (1×10^5) were injected intradermally onto the backs of mice. Tumor growth assays began 10 days post tumor implantation. Mice were given a total of 3 IgG2AK injections and a total of 4 anti-PD1 injections over the course of the experiments. Mice were weighed prior to injection of

tumors and at the conclusion of the experiment, and tumor volumes were measured every few days by digital caliper readings. Volumes were calculated using the formula (length × width^2) × 0.52.

Antibodies

Antibodies used for flow cytometry are listed in Supplementary Table S1. IHC staining on patient tissue was performed using FOXP3 236A/E7 from eBioscience (#14-4777), CD8 Ab-1 (#MS-457-R7) from Thermo Fisher Scientific, and CD4 4B12 (#PA0427) from Leica Biosystems.

Flow cytometry

Flow cytometry was performed on the LSRII, 18 color flow cytometer by BD Biosciences at the Wistar Institute Flow Facility. For intratumoral immune analysis, experiments were performed in duplicate, and tumors were harvested when volumes reached a range between 500 and 1,000 mm^3 by isolating tumors from stromal capsule and skin, chopping into small fragments, and incubating at 37°C for 40–60 minutes in the Miltenyi Tumor Dissociation Kit, mouse. For intracellular cytokine staining, tumors were processed as before and then incubated for approximately 5 hours at 37°C in 1 mL complete DMEM (10% FCS, 1% L-Glutamine, 1% penicillin/streptomycin) containing BD Bio GolgiBlock (#554724) and the T Cell Activation Cocktail without BFA (#423301) from BioLegend. Spleens were mashed through 70-μm cell strainers directly into MACS buffer (PBS containing 0.5% FCS and 2.5 mmol/L EDTA) and incubated with ACK lysis buffer for 1 minute. All single-cell suspensions were washed and stained with the appropriate concentrations of antibodies in MACS buffer. When appropriate, cells were fixed and stained using the True-Nuclear Transcription Factor Buffer Set form BioLegend (#424401). Cells were rinsed and resuspended in PBS prior to FACS analysis.

Statistical analysis

Statistical analysis of intratumoral immune populations by FACS analysis and CD4$^+$ IHC staining was performed using two-sided Student *t* tests assuming unequal variances and a two-way ANOVA when comparing more than two factors. Statistical analysis of FOXP3$^+$ and CD8$^+$ IHC was performed using two-sample Wilcoxon rank-sum (Mann–Whitney) tests. Statistical significance for CD8$^+$ patient percentages was achieved using the Fisher exact test. A linear mixed-effect model with interaction term between treatment and follow-up days, and random effect at mouse level was used to examine whether the tumor growth velocities would be different between treatment groups for tumor growth assays. For best response patient data, significance was determined using a Fisher exact test, and probability was estimated from the logistic regression analysis.

Results

Age correlates with patient response to PD1 inhibition

A total of 538 metastatic melanoma patients treated with anti-PD1 antibody (pembrolizumab, FDA approval 2014) at 7 centers in the United States, Germany, and Australia were evaluated (Supplementary Fig. S1A). The most significant cutoffs, which we note for each analysis, range between the ages of 52 and 66 years old, depending on the analysis and cell type studied. We observed a statistically significant difference in response to pembrolizumab by age (Fig. 1A), where the odds of progressing on pembrolizumab treatment decreased 13% for every decade of patient age at treatment initiation (Fig. 1B). Fifty percent of patients <62 years saw no beneficial response from pembrolizumab treatment. Conversely, only 37% of patients ≥62 years failed to respond to therapy (Fig. 1A). These results were not dependent on gender (Fig. 1C; Supplementary Fig. S1B). In addition, the receipt of prior MAPK inhibitor (MAPKi) therapy, which was more frequently used in younger patients (median age; MAPKi treated: 59 years vs. MAPKi naïve: 64 years), consistent with established age-related differences in BRAF mutation frequency (21), did not impact the association between patient age and pembrolizumab response. Using the most statistically significant cut-off points for each group, younger patients were more likely to progress on treatment in both the MAPKi-naïve (Fig. 1D) and MAPKi-treated cohorts (Fig. 1E), similar to the total patient analysis. Interestingly, we also observed major reductions in complete responses in younger patients with prior MAPK inhibition (Fig. 1E). Overall, there was a significant decrease in likelihood of progressing on pembrolizumab with each decade of life in either MAPKi-naïve or -treated patients (Fig. 1F).

Age correlates with response to PD1 inhibition in genetically identical tumors in mice

To test whether the age-related differences we observed in response to anti-PD1 monotherapy in patients could be recapitulated in animal models, we treated BSC9AJ2 (derived from the BRAF^{V600E}/Trp53^{R172H} mouse; ref. 22) tumors in young (2-month-old) and aged (10-month-old) female mice. Anti-PD1 treatment in young mice did not lead to any significant reduction in tumor growth over mice treated with IgG2AK control (Fig. 2A), consistent with multiple studies in young mice (23, 24). Conversely, aged mice treated with the same dose of anti-PD1 had a modest but significant reduction in tumor growth compared with IgG2AK (Fig. 2B). These data were reproducible and not gender dependent, as results were similar in young and aged male mice (Fig. 2C and D). Importantly, these effects were not dependent on mutational burden, as genetically identical tumors were transplanted into both young and aged mice. It has also been reported that immune checkpoint inhibitor therapies can result in lethal inflammation and death in elderly mouse models when combined with other therapies (25), but no additional distress or significant weight loss was observed (Supplementary Fig. S2A and S2B). Thus, the data shown here suggest that young mice are more resistant to PD1 inhibition *in vivo* compared with aged mice, in keeping with the patient data.

The immune microenvironment is more suppressed in the tumors of young mice, and affects response to anti-PD1

To understand what aspect of the immune microenvironment was driving the observed differences in young and aged mouse response to anti-PD1, we injected the following transplantable melanoma models: male Yumm1.7 (BRAF^{V600E}PTEN$^{-/-}$CDKN2A$^{-/-}$; ref. 26) and female BSC9AJ2 (derived from the BRAF^{V600E}/Trp53^{R172H} mouse; ref. 22), as well as Yumm2.1 (BRAF^{V600E}PTEN$^{-/-}$CDKN2A$^{-/-}$; ref. 26) murine melanomas into 2-month-old and 10-month-old C57BL6 mice. No significant differences in the total percentage of immune infiltrate (CD45$^+$) between young and aged tumors were observed in mice bearing Yumm1.7 or BSC9AJ2 tumors, suggesting observed differences in subpopulations were not the result of overall differences in immune cell infiltration (Supplementary Fig. S3A and

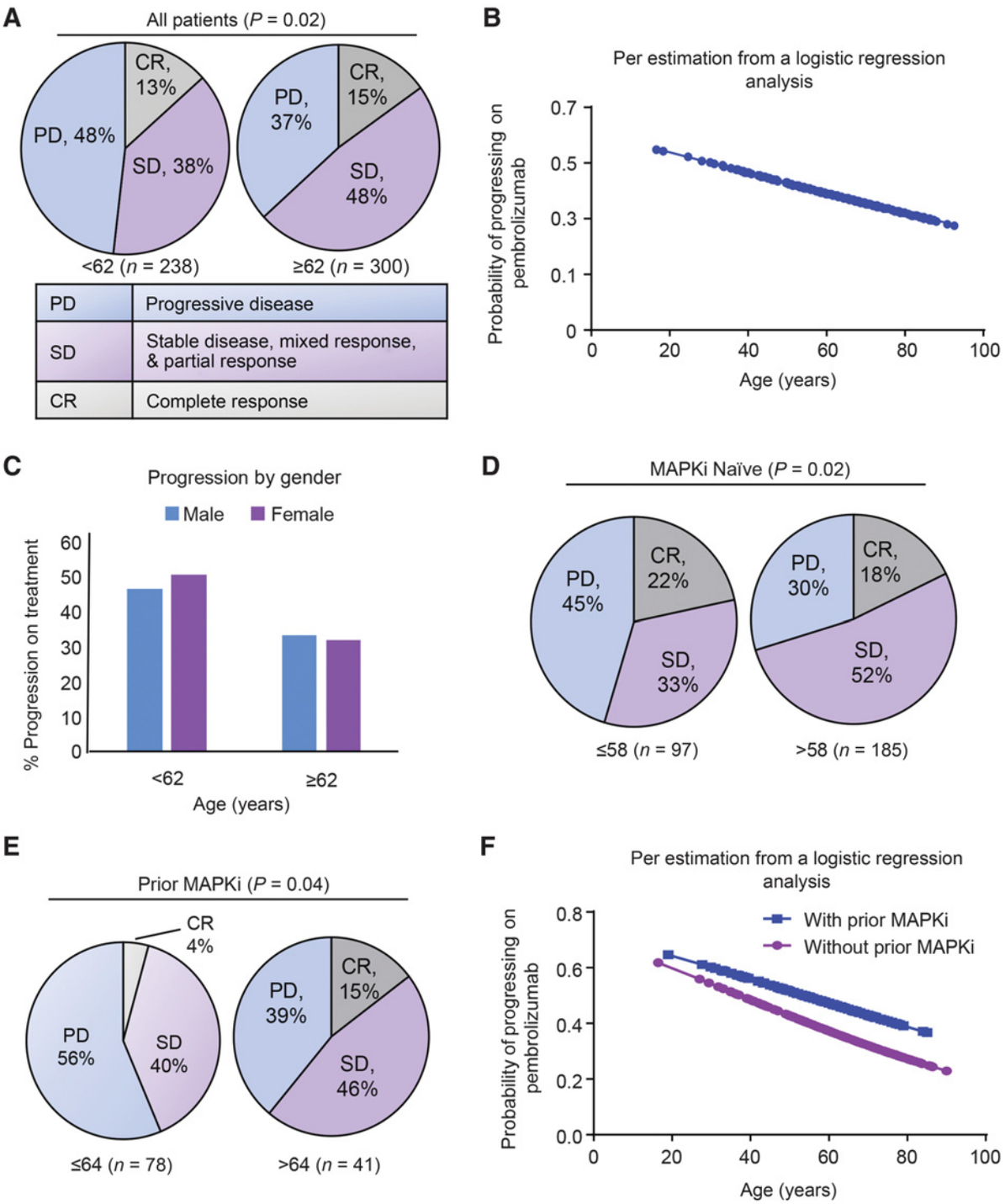

Figure 1.

Age-related efficacy of pembrolizumab in melanoma patients. **A,** Pie charts showing the percentages of patients with the indicated responses between younger and older melanoma patients combined using 62 years old as the most statistically significant cutoff. Significance was determined using a Fisher exact test. **B,** Graph indicating the probability of a patient progressing on pembrolizumab treatment given their age and determined using a logistic regression analysis. **C,** Percentage of patients from **A** who progressed on treatment, separated by gender, and using 62 as the most statistically significant cutoff. Best responses of patients without prior MAPKi therapy (**D**) and with prior MAPKi therapy using most statistically significant cutoffs (**E**). Statistical cutoffs determined using Fisher exact test. **F,** Graph indicating the probability of patients progressing on pembrolizumab treatment, from **D** and **E**, given their age and determined using a logistic regression analysis.

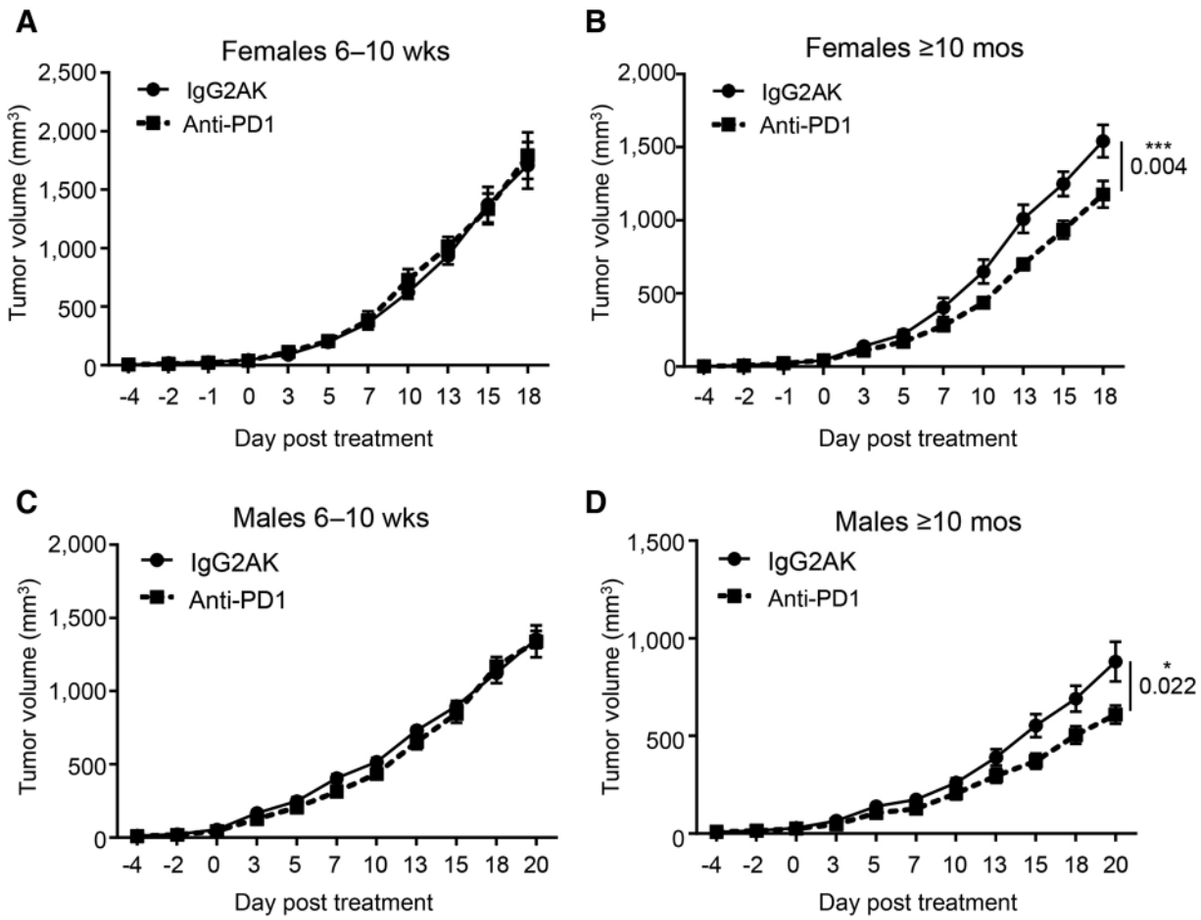

Figure 2.

Differential response to anti-PD1 in young and aged mice. Mice bearing BSC9AJ2 tumors treated with 3 doses of 300 μg rat IgG2AK ($n = 5$) or 4 doses of 300 μg anti-PD1 ($n = 5$) every 5 days, starting on day 0. Response rates in young (8- to 10-week-old; **A**) and aged (10-month-old; **B**) female mice, and in young (8- to 10-week-old; **C**) and aged (10-month-old; **D**) male mice. All experiments used a linear mixed-effect model to determine significance. Error bars, SEM.

S3B). We could not assess Yumm2.1 tumors as they consistently regressed, due to their very immunogenic nature as seen in other studies (23), and as demonstrated here by their high level of $CD45^+$ infiltration (Supplementary Fig. S3C). We did not observe any age-related differences in the $CD4^+FOXP3^-$ T helper (Th) cells, but $CD4^+FOXP3^+$ T cells were significantly decreased in aged mice in both Yumm1.7 and BSC9AJ2 models (Fig. 3A and B). As FOXP3 is preferentially expressed on regulatory T cells (Tregs; refs. 27, 28), these data suggested that Tregs may be upregulated within the tumors of younger mice. $CD8b^+$ T cells were consistently and significantly decreased in tumors from young mice (Fig. 3C). This resulted in an increased $CD8^+$:Treg ratio in aged mice, which has previously been shown to increase response to immunotherapy (Fig. 3D and E). Consistent with a more suppressed immune environment, total numbers of $CD8b^+TNF\alpha^+$ and $CD8b^+IFN\gamma^+$ T cells, as measured by *ex vivo* incubation with PMA and ionomycin, were significantly reduced in the tumor-bearing young mice (Fig. 3F and G). The observed differences in TNFα-expressing cells and CD8:FOXP3 ratios were also limited to the tumor, as similar differences were not observed in the spleen (Fig. 3H and I; Supplementary Fig. S3D and S3E). Overall, our data suggested that the major change in the immune microenvironment between young and aged is a decrease in Tregs in tumors in aged mice. It is important to note that these ratios are not reflected in other organs such as the spleen, where in fact, Tregs are elevated, consistent with data from other aging studies.

Melanoma tumors from younger individuals have lower $CD8^+$:FOXP3 ratios

In an independent cohort of primary and metastatic melanoma samples from NYU and Vanderbilt, FOXP3 ($n = 268$) and CD4/CD8 ($n = 84$) IHC positivity was evaluated by blinded pathologists (F. Darvishian and R. Al-Rohil) in the area of densest immune cell infiltrate (Fig. 4A; Supplementary Fig. S4A). We observed that intratumoral $FOXP3^+$ cells decreased following 50 years of age (Supplementary Fig. S4B; Fig. 4B). In conjunction, we observed the percentage of patients whose immune infiltrate contained less than 20% $CD8^+$ cells to be significantly higher in younger individuals (Fig. 4C). Lower numbers of $CD8^+$ T cells have been observed within tumors of patients who progress on PD1 therapy (2, 29). Consistent with this observation, the

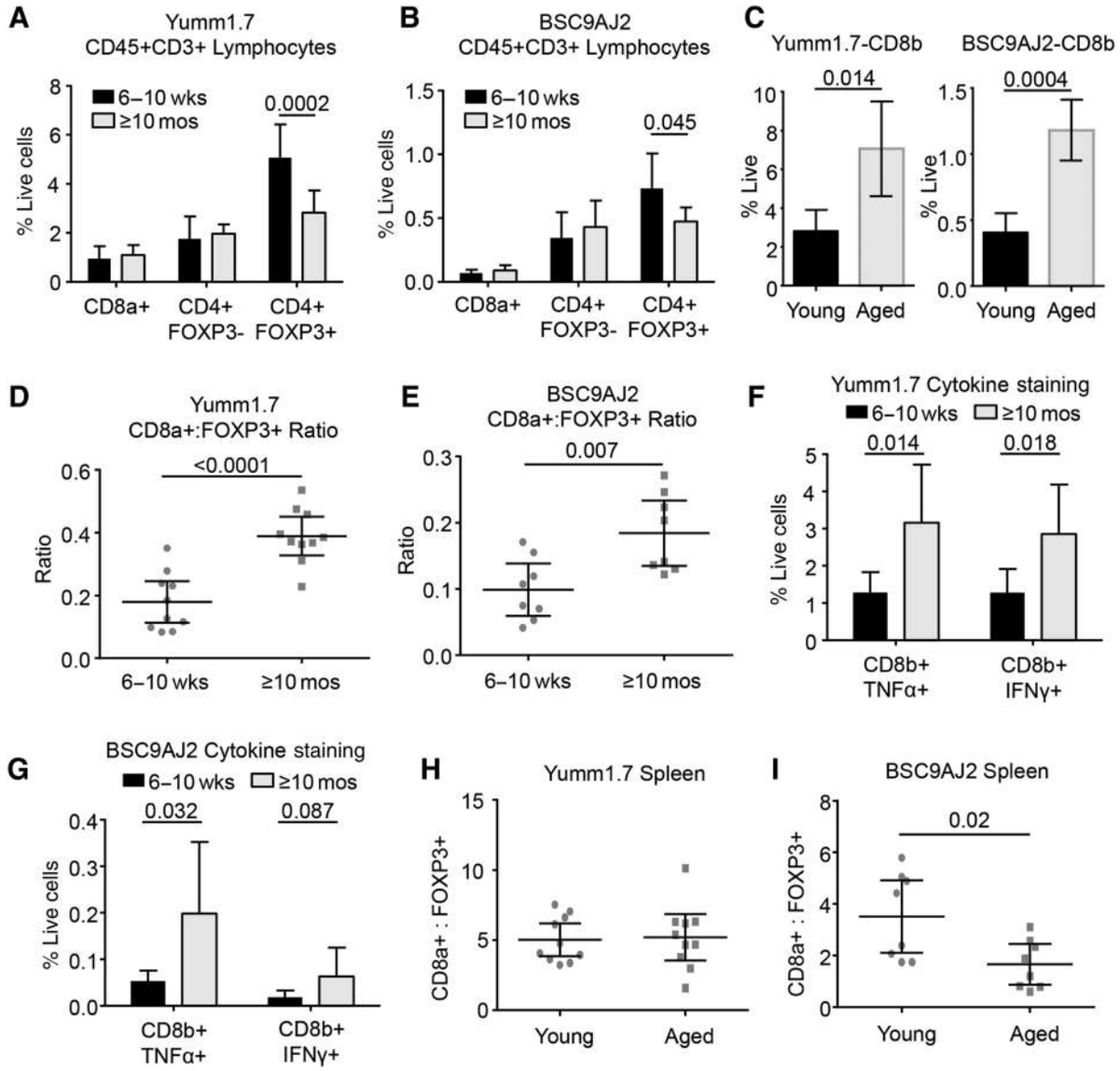

Figure 3.

Effect of age on intratumoral immune populations in mice. FACS analysis of lymphocyte populations within tumors from young (8- to 10-week-old) and aged (10-month-old) C57/Bl6 male mice bearing Yumm1.7 murine melanomas (**A**) and females bearing BSC9AJ2 murine melanomas (**B**). Experiments were performed in duplicate, and melanomas were injected intradermally and harvested when tumor volumes were between 500 and 1,000 mm^3. Significance determined by two-way ANOVA. Error bars, = 95% CI. $CD8b^+$ levels as a percentage of live cells in young and aged mice in both models (**C**). Significance determined by two-tailed Student *t* test assuming unequal variance. Error bars, 95% CI. Ratios of $CD45^+CD3^+CD8a^+$ cells and $CD45^+CD3^+CD4^+FoxP3^+$ cells within individual tumors from young and aged male mice bearing Yumm1.7 tumors (**D**) and female mice bearing BSC9AJ2 tumors (**E**). Significance determined by two-tailed *t* test assuming unequal variance. Error bars, 95% CI. $CD45^+CD8b^+$ cells from Yumm1.7 (**F**) and BSC9AJ2 (**G**) tumors expressing TNFα and IFNγ following 5-hour incubation with PMA and ionomycin prior to FACS analysis. Significance determined by two-tailed Student *t* test assuming unequal variance. Error bars, 95% CI. Ratios of $CD45^+CD3^+CD8a^+$ cells to $CD45^+CD3^+CD4^+FoxP3^+$ cells within the spleens of mice bearing Yumm1.7 (**H**) or BSC9AJ2 tumors (**I**). Error bars, 95% CI.

percentage of $CD8^+$ cells was significantly lower in patients under the age of 66 years (Fig. 4D; Supplementary Fig. S4C), which translated to lower CD8:FOXP3 ratios in young patients (Fig. 4E; Supplementary Fig. S4D). Taken together, we consistently observe significant age-related differences in lymphocyte populations within patient melanoma tumors. These data, consistent with the response data we show in Fig. 1, indicate that older patients have increased $CD8^+$:Treg ratios.

Depleting Tregs in using anti-CD25 enhances response to anti-PD1

Our data suggest that we might be able to achieve a better response to anti-PD1 by increasing $CD8^+$:Treg ratios in young mice. To accomplish this, we depleted Tregs using an antibody against CD25, which targets all $CD25^+$ cells. While CD25 is temporarily expressed on activated effector T cells, it is predominantly expressed at higher levels on Tregs. Mice were treated

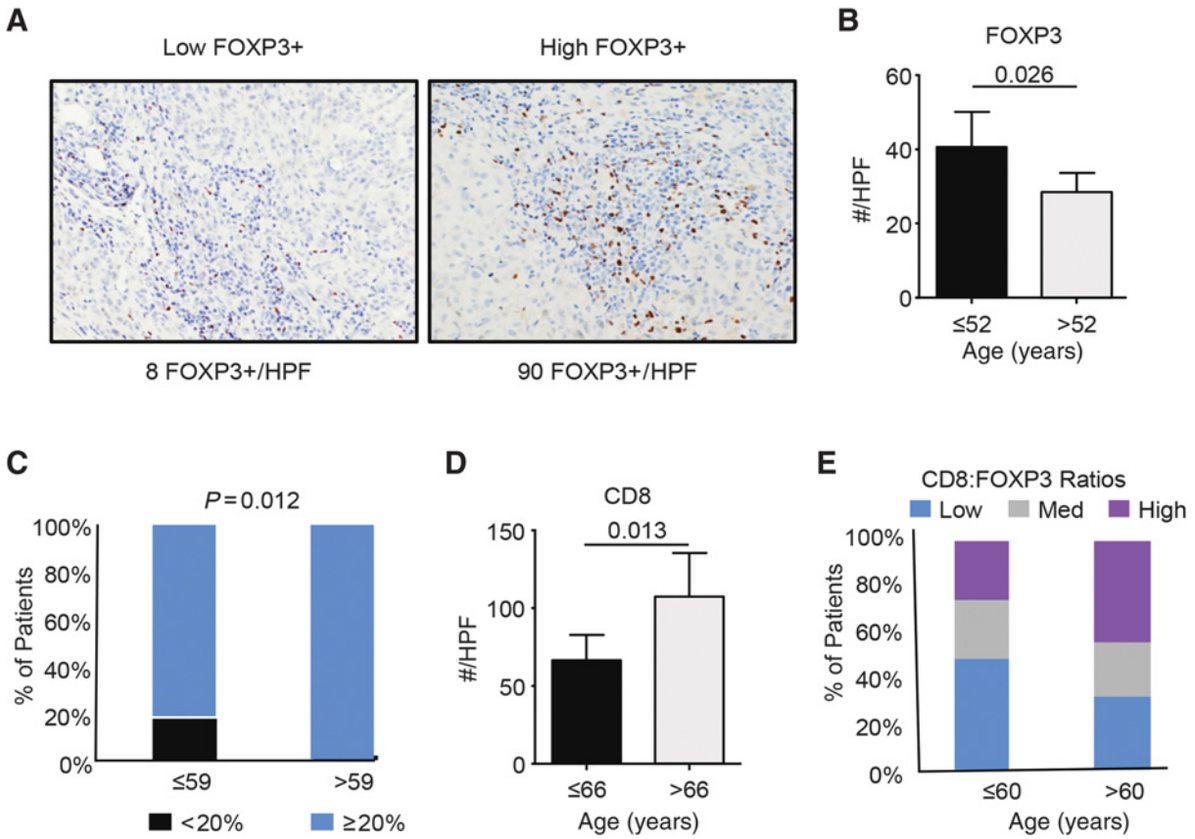

Figure 4.

Lymphocyte populations in patient melanomas. **A,** Representative images of IHC staining of FOXP3$^+$ cells in patient melanoma tumors at 40× magnification. **B,** Quantification of FOXP3$^+$ cells performed by board-certified pathologists identifying the densest area of immune infiltrate within tumors and counting the positively stained cells at 40× magnification. Significance from two-sample Wilcoxon rank-sum (Mann–Whitney) tests. Error bars, 95% CI. **C,** Graph indicating the percentage of patients in each age group, with CD8$^+$ cells representing less than 20% of the total immune cell infiltrate. Significance from Fisher exact test. Error bars, 95% CI. **D,** Quantification of CD8$^+$ cells following outlier removal. Cells counted and significance determined as in **C**. Outliers determined and removed as identified by ROUT (Q = 0.1%) outlier test. Error bars, 95% CI. **E,** Percentages of patients from **D** whose CD8:FOXP3 ratio is low (<median − 1), med (median ± 1), or high (>median + 1) using median age of 60 as the cutoff.

with IgG, anti-CD25, anti-PD1, or an anti-PD1/anti-CD25 combination. Anti-CD25 treatment was started 5 days before tumor inoculation, and repeated every 5 days thereafter. Serum from anti-CD25–treated mice was measured to confirm Treg depletion (Fig. 5A). A linear mixed-effect model with random intercept and random slopes was used to compare the velocities of tumor growth between groups (Fig. 5B). We observed that anti-CD25 alone delayed tumor growth, but that the combination of anti-CD25 and anti-PD1 was the most effective therapy, with response rates comparable with those seen in aged mice (Fig. 5B and C; Supplementary Fig. S5A). Importantly the combination was not toxic, as there was no change in body weight or distress (Supplementary Fig. S5B). These effects could also be replicated using low doses of cyclophosphamide, which has also previously shown to deplete Tregs (Supplementary Fig. S6A–S6E). Together, the results presented indicate that the intratumoral microenvironment is more suppressed in young mice, and suggest that preconditioning the tumor microenvironment by depleting Tregs can sensitize melanoma tumors in young mice to PD1 inhibition.

Discussion

Recently, a surge of immune checkpoint blockade molecules have become available, in particular those targeting the PD-1/PD-L1 axis, not only for the treatment of metastatic melanoma, but for other cancer types as well (30–35). While these produce impressive responses in some patients, they also are hampered by a high rate of innate resistance and occasional severe immune-related adverse events (36). Understanding factors present within the tumor microenvironment that might help to predict patient response will be immensely important in helping to avoid "over-treating" patients and minimize adverse events without sacrificing response. For example, data from McQuade and colleagues show that obesity in males predicts for better overall response to therapy (19). In our studies, we investigated the role of aging on the immune component of the melanoma microenvironment. A very recent study by Elias and colleagues, compared HRs of older versus younger patients in response to anti-PD1, as compared with response to chemotherapy in multiple tumors (37). This study showed no difference in the HR across the broad range of tumor types. Because older melanoma patients often fare more

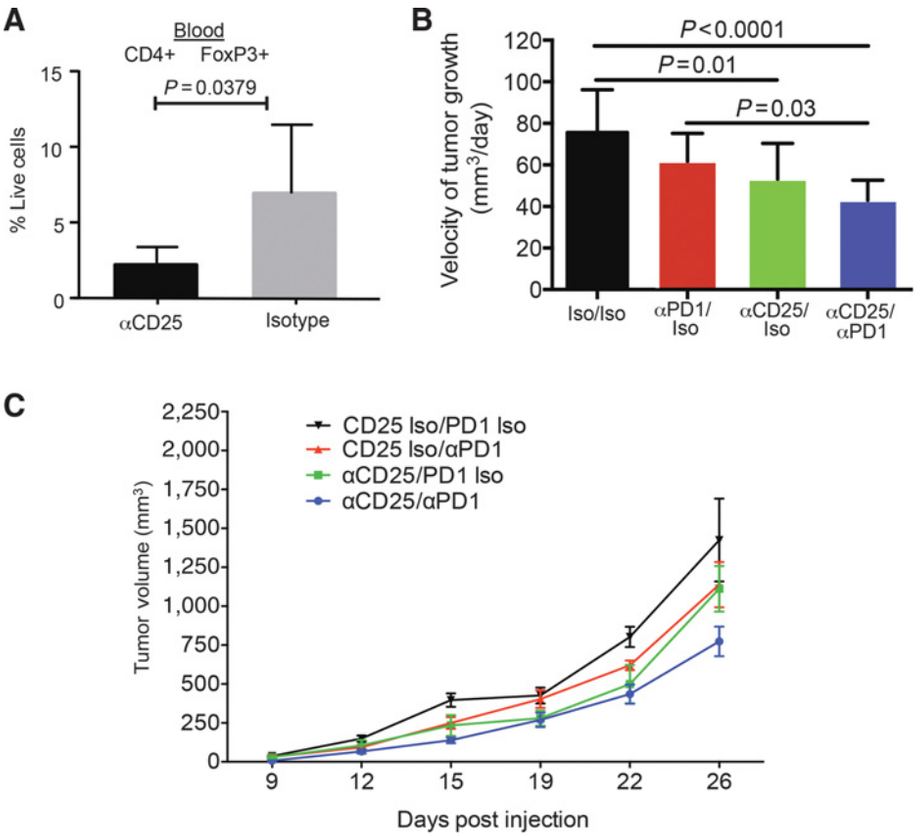

Figure 5.
Depletion of Tregs with anti-CD25 increases response to anti-PD1. Young female mice were treated with anti-CD25 or an IgG1A isotype 5 days prior to subdermal of BSC9AJ2 cells with treatments continuing every 5 days until sacrifice. Anti-PD1 was administered every 3–4 days, starting on day 9 posttumoral injection. **A,** Anti-CD25–depleted Tregs in the peripheral blood of mice. **B,** Statistical analysis of tumor growth curves comparing anti-CD25, anti-PD1, and the combination of both antibodies in young mice. **C,** Tumor growth curves for above. All experiments used a linear mixed-effect model to determine significance. Error bars, SEM.

poorly with chemotherapy, a similar HR could reflect a heightened response to anti-PD1 in older melanoma patients, as indicated by our data.

Increased numbers of Tregs have been identified in the skin of older individuals (38); however, little is known regarding age-dependent changes in Treg number and function at sites of inflammation, such as the tumor microenvironment. Here, we show that Tregs are specifically increased within the tumor microenvironment of young patients. In addition, $CD8^+$ T cells, which are the primary target cell type of anti-PD1 checkpoint blockade, are also decreased in melanoma tumors from younger patients. Given the suppressive role of Tregs on $CD8^+$ effector T cells (39, 40), and specifically on $CD8^+$ T-cell proliferation (39), it is unsurprising to find decreased $CD8^+$ T cells in younger tumors where increases of $FOXP3^+$ cells were detected. Moreover, we observed significant decreases in CD8:FoxP3 ratios in the tumors of young mice, and similar trends in patient samples. These age-related tumor immune signatures were predictive of response to anti-PD1 inhibition. Interesting data from Tietze and colleagues also suggest that the types of $CD8^+$ cells may change with age (41). Memory $CD8^+$ accumulate with age, and it is this population that expands in response to immunotherapy, and exhibits cytolytic activity, supporting our overall observations.

In melanoma, increases in CD8:FOXP3 ratios in combination with lower levels of exhausted T cells were also predictive of complete responses to radiation, anti-CTLA4, and anti-PD1 triple therapy (4). Consistent with this, we show that young mice bearing BSC9AJ2 murine melanoma tumors were resistant to anti-PD1 inhibition *in vivo*, whereas aged mice saw a significant reduction in tumor growth following anti-PD1 treatment. Although the level of response to PD1 inhibition in aged mice was minor, we consistently observed significant decreases in tumor growth across multiple experiments. In addition, the lack of a strong response in the aged mice is likely due to the innate resistance of mouse melanoma models to PD1 inhibition (23, 24). An exception to this is the Yumm2.1 melanoma cells, which we have found to contain an overwhelming amount of immune cell infiltrate when implanted into young mice (Supplementary Fig. S3). This is corroborated by a published study that shows, of four murine melanoma lines tested, the Yumm2.1 line is the only one to respond to anti-PD1 therapy, and does so dramatically (23). It is therefore unlikely we would observe age-related differences in this model, given the robust response of these cells to anti-PD1 in young mice. Most importantly, despite the small (but consistent and significant) response in aged mice, our results still translated to patients where a significant OR indicated that for every 10 years older a patient was, their odds of responding to the FDA approved anti-PD1 therapy pembrolizumab improved by 13%. These differences were independent of both patient gender and treatment history with MAPK inhibitor therapies. Unfortunately, we were not able to account for somatic mutational load which has previously been shown to increase with age (42), and predict patient response to PD1 inhibition (20). However, the resistance to PD1 inhibition specifically in young mice, which harbor genetically identical tumors to aged mice, is independent of mutational burden. Thus, at least in mice, age-related differences in immune populations and response to anti-PD1 inhibition are independent of mutational burden.

Our data were quite surprising, especially given previous data from our laboratory demonstrating that older patients have more metastatic disease, and respond more poorly to targeted therapy (specifically, vemurafenib; ref. 16). In addition, we were surprised by the increased response of older patients to anti-PD1 because it is well known that as humans age, they undergo a process known as immunosenescence. As described earlier, this process is the result of multiple factors that result in a decrease in immune function in the elderly, including the involution of the thymus (9, 10). Balanced against this, however, is the fact that older individuals have increased chronic inflammation, which counterintuitively would suggest an increased immune function. It is thought that Tregs may play a role in this dichotomy. Treg populations that have been shown to change with aging include nTregs (naturally occurring Tregs) and memory Tregs. nTregs control autoimmunity by maintaining tolerance to self-antigens and decrease with aging, where memory Tregs increase in aged individuals (43). It may be that our observation that Treg numbers in aged spleens are much higher than those of young, where intratumoral Treg numbers are lower, could be in part due to these differences in Treg populations, or may reflect differences in the ability of Tregs to traffic to the tumor. As we explore this phenomenon further, we will be able to distinguish between the several different types of Tregs, and their distribution across a spectrum of aging and cancer.

Our study has obvious statistical limitations. We could not perform a meta-analysis of the data, because we did not have clinical trials with the same parameters from which to draw relative comparisons. Furthermore, we could not perform multivariate analyses on these data, as we did not initially know for which important confounders we would need to adjust. What this study did allow us to do was to generate and test in mice the hypothesis that age is an important factor to consider when administering immunotherapies for the treatment of metastatic melanoma. Overcoming this suppression by depleting Tregs helped to restore sensitivity to anti-PD1 inhibition in mice. There is a concern that this could lead to immune toxicity, as anti-CD25 was initially thought to interfere with effector T cells; however, in our mouse studies we did not observe either a significant weight loss nor any overt signs of toxicity. This is in keeping with data that show that CD25 is expressed largely on Tregs in the intratumoral microenvironment, as well as human data that suggest that daclizumab, which depletes Tregs, results in an increased response to immune therapy in the absence of autoimmunity (18). In that study, patients undergoing long-term Treg depletion with daclizumab had a consistent and sustained suppression of circulating Tregs, while effector T cells remained within baseline levels of healthy individuals. Taken together with the data we present here, this study suggests that a combination approach of depleting Tregs in combination with anti-PD1 might have great benefit in young patients, although immune toxicity would have to be taken into account.

Disclosure of Potential Conflicts of Interest

J.A. Sosman is a consultant/advisory board member for Bristol–Myers Squibb and Genentech. M.S. Carlino is a consultant/advisory board member for Bristol, Myers, Squibb, MSD, Novartis, and Amgen. G.V. Long is a consultant/advisory board member for MSD; Bristol, Myers, Squibb; Novartis; Roche; Amgen; Array; and Pierre Fabre. A. Ribas is a consultant/advisory board member for Merck and Bristol, Myers, Squibb. Z. Eroglu is a consultant/advisory board member for Compugen. M.A. Davies reports receiving commercial research grants from Roche/Genentech, AstraZeneca, GlaxoSmithKline, Sanofi-Aventis, Myriad, and Ocothyreon, and is a consultant/advisory board member for Novartis; Bristol, Myers, Squibb; Roche/Genentech; GlaxoSmithKline; Sanofi-Aventis; Vaccinex; and Syndax. A.M. Menzies is a consultant/advisory board member for Bristol, Myers, Squibb; MSD; Novartis; Roche; and Pierre-Fabre. D.B. Johnson reports receiving commercial research grants from Incyte, reports receiving other commercial research support from Bristol, Myers, Squibb, and is a consultant/advisory board member for Array, Bristol–Myers Squibb, Incyte, and Merck. No potential conflicts of interest were disclosed by the other authors.

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