Differential requirements for CD4+ T cells in the efficacy of the anti-PD-1+LAG-3 and anti-PD-1+CTLA-4 combinations in melanoma flank and brain metastasis models

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ABSTRACT
Background Although the anti-PD-1+LAG-3 and the anti-PD-1+CTLA-4 combinations are effective in advanced melanoma, it remains unclear whether their mechanisms of action overlap.

Methods We used single cell (sc) RNA-seq, flow cytometry and IHC analysis of responding SM1, D4M-UW2 and B16 melanoma flank tumors and SM1 brain metastases to explore the mechanism of action of the anti-PD-1+LAG-3 and the anti-PD-1+CTLA-4 combination. CD4+ and CD8+ T cell depletion, tetramer binding assays and ELISPOT assays were used to demonstrate the unique role of CD4+T cell help in the antitumor effects of the anti-PD-1+LAG-3 combination.

Results The anti-PD-1+CTLA-4 combination was associated with the infiltration of FOXP3+ regulatory CD4+ cells (Tregs), fewer activated CD4+T cells and the accumulation of a subset of IFNγ secreting cytotoxic CD8+ T cells, whereas the anti-PD-1+LAG-3 combination led to the accumulation of CD4+ T helper cells that expressed CXCR4, TNFSF8, IL21R and a subset of CD8+ T cells with reduced expression of cytotoxic markers. T cell depletion studies showed a requirement for CD4+T cells for the anti-PD-1+LAG-3 combination, but not the PD-1-CTLA-4 combination at both flank and brain tumor sites. In anti-PD-1+LAG-3 treated tumors, CD4+ T cell depletion was associated with fewer activated (CD69+) CD8+ T cells and impaired IFNγ release but, conversely, increased numbers of activated CD8+ T cells and IFNγ release in anti-PD-1+CTLA-4 treated tumors.

Conclusions Together these studies suggest that these two clinically relevant immune checkpoint inhibitor (ICI) combinations have differential effects on CD4+ T cell function. Further insights into the mechanisms of action/resistance of these clinically relevant ICI combinations will allow therapy to be further personalized.

INTRODUCTION
The development of immune checkpoint inhibitors (ICIs) has revolutionized the treatment of advanced melanoma.1 At this time, the most widely used ICIs are antibodies that block the function of programmed cell death protein (PD)-1, reversing the exhaustion of CD8+ cytotoxic T cells.2 These therapies, including nivolumab and pembrolizumab, are widely used for the treatment of melanoma and although successful, are frequently associated with both intrinsic and acquired resistance.3 4 Two of the most widely explored additional ICIs are antibodies that target cytotoxic lymphocyte associated protein (CTLA)-4 and lymphocyte-activation gene (LAG)-3, both of which are upregulated...
on T cell activation and associated with eventual T cell exhaustion. Inhibition of CTLA-4 is associated with low response rates in the single agent setting, with improved rates of response reported when CTLA-4 targeted therapies are used in combination with anti-PD-1, although at the cost of increased immune toxicity.

LAG-3 is a homolog of CD4 that binds to major histocompatibility complex (MHC) class 2. Preclinical studies demonstrated that co-targeting of LAG-3 and PD-1 frequently led to the regression of established tumors that were otherwise resistant to ICI monotherapy. In a recent randomized phase 2/3 clinical trial, the anti-PD-1+LAG-3 combination (nivolumab+relatlimab) significantly improved progression-free survival compared with single agent nivolumab. Significantly, the combination was well tolerated with far fewer adverse events reported than in patients treated with the anti-PD-1+CTLA-4 combination. The anti-PD-1+LAG-3 combination was subsequently explored in the neoadjuvant setting for patients with resectable stage III or stage IV oligometastatic melanoma where the combination therapy was associated with pathological complete response rate (pCR) of 57%. In light of these encouraging findings, the anti-PD-1+LAG-3 combination was FDA approved for advanced melanoma in the Spring of 2022.

The brain is one of the most common sites of melanoma metastasis, with 40%–60% of patients with advanced melanoma showing evidence of central nervous system (CNS) involvement. Although it was previously believed that the CNS exists as an immune-privileged site, it is now understood that the brain and the spinal cord are under continual immune surveillance. However, the mechanisms by which protumor and antitumor immune subsets traffic into the CNS and the impact these subsets have on CNS-metastases remains incompletely understood. A recent report demonstrated that melanoma brain metastases (MBMs) had a lower level of T cell infiltration and microvessel density than metastases from extracranial sites. A second histological study also confirmed the lower level of CD8+ T cell infiltrate in MBM compared with matched extracranial metastases. Although the mechanisms underlying the reduced infiltration of T cells in MBM are not clear, there is some evidence from neuroinflammation studies that have suggested activated microglia may limit T-cell accumulation. In mouse MBM models, immunotherapy responses in the brain are dependent on the peripheral expansion of T cells that traffic into the brain. Studies on matched cranial and extracranial melanoma metastases have demonstrated reduced T cell clonality in the brain compared with extracranial metastatic sites.

We recently used single cell (sc) RNA-Seq analyses of MBM and melanoma skin metastases to demonstrate that the extent of T cell infiltration into the brain was lower than of the skin metastases. Other work demonstrated that MBM had a higher proportion of monocyte-derived macrophages and TOX+CD8+ T cells with unique patterns of immune checkpoint expression compared with extracranial metastases.

Recent studies from our group have shown that >50% of MBM patients exhibit significant clinical intracranial responses to the combination of the ICI that target CTLA-4 and PD-1 (ipilimumab+nivolumab). However, almost half of the treated population do not respond to treatment and the response rate and survival in patients with symptomatic MBM is significantly lower (~20%). The combination of relatlimab with nivolumab is currently being explored clinically in patients with MBM (NCT05704647). As little is known about its activity against MBM, or its mechanism of action in the brain, we here used dual flank/brain injection models of MBM to determine the mechanism of action of the anti-PD-1+LAG-3 and anti-PD-1+CTLA-4 combinations. Our studies demonstrated differential effects of the two combinations on CD8+ cytotoxic and CD4+ T cell populations, and the dependency of the anti-PD-1+LAG-3 combination on a CD4+ T helper phenotype.

**MATERIAL AND METHODS**

**Cell lines**
SM1 mouse BRAF-mutant melanoma cells were obtained from Dr. Eric Lau, Moffitt Cancer Center. D4M-UV2 mouse melanoma cells were from Dr. David Fisher, Massachusetts General Hospital. B16 mouse melanoma cells were obtained from Dr. Mary Jo Turk, Dartmouth University. The cells were maintained for maximum 10 passages in RPMI1640+10% FBS for SM1, RPMI1640+7.5% FBS for B16 cells and DMEM+10% FBS for D4M-UV2 cells. Cell lines were routinely tested for *Mycoplasma* (every 3 months).

**In vivo procedures**
Female 7-week-old immunocompetent C57BL/6J mice were used for the experiments. Mice were subcutaneously injected with 1.5×10⁶ SM1, D4M-UV2 or B16 cells in Matrigel (cat. #CB40234; Fisher Scientific) into the right flank. In some cases, SM1 flank tumor-bearing mice were also injected with 50,000 SM1 cells in PBS into the caudate nucleus of the right cerebral hemisphere of the brain using stereotactic surgical procedures. The flank tumors were allowed to grow approximately to 50–70 mm³ and brain tumors were allowed to grow until visible via MRI, before initiation of drug dosing for SM1 and D4M-UV2 models. For the B16 model, the drug dosing was initiated next day after the injections. Mice received intraperitoneal doses of anti-PD-1 antibody (200 µg/100 µL; clone RMP1-14; cat. #BE0146; Bio X Cell) or anti-CTLA-4 (200 µg/100 µL; clone 9D9; cat. #BE0164; Bio X Cell) or anti-LAG-3 (200 µg/100 µL; clone C9B7W; cat. #BE0174; Bio X Cell) as a single agent or as a combination every 5 days. For isotype control, a cocktail of IgG2a isotype control (200 µg/100 µL; clone 2A3; cat. #BE0089; Bio X Cell), IgG1 isotype control (200 µg/100 µL; clone HRPN; cat. #BE0888; Bio X Cell) and Syrian hamster IgG (200 µg/100 µL; cat. #BE087; Bio X Cell) were administered intraperitoneally every 5 days for SM1 and...
D4M-UV2 models. For the B16 model, mice received the antibodies every day. The flank tumors were measured using calipers and the brain tumors were monitored and measured by MRI scanning. The sequence used to generate MRIs was a standard T2-weighted Turbo Spring Echo. Brain tumors were manually contoured on all MRI slices using ImageJ. A custom program was written in MATLAB (MATLAB 2020b, The MathWorks, Natick, Massachusetts, USA, 2020) to extract voxels within the manually drawn contours and compute total volume burden, in mm³. During initial tumor development, mice were imaged twice per week. Following response treatment, mice were imaged once per week. The tumors were collected at the endpoint, weighed, and processed for sc RNA sequencing (scRNA-seq), flow cytometry analysis or immunohistochemistry.

**CD4+ and CD8+ T cell depletion**

CD4-specific antibody (clone YTS191; cat. #BE0003-1; Bio X Cell) and CD8a-specific antibody (clone YTS169.4; cat. #BE0117; Bio X Cell) from Bio X Cell were used to deplete CD4+ T cells and CD8+ T cells, respectively. The C57BL/6J mice were administered anti-CD4 and anti-CD8a (100 µg/100 µL) via intraperitoneal injections 3 days before injection with SM1 or B16 cells and then every 4 days thereafter. When the flank tumors reached 50–70 mm³ in size and brain tumors were visible by MRI, the mice were treated with either IgG control, anti-PD-1-LAG-3 or anti-PD-1-CTLA-4 every 5 days. Tumor size was measured twice weekly, with CD4+ T-cell or CD8+ T-cell depletion measured by flow cytometry at termination of the experiment.

**Flow cytometry**

Tumors were harvested at the endpoint or at listed time points for the kinetics experiments, under sterile conditions and weighed. Single-cell suspensions were prepared by enzymatic digestion, using a MACS tumor dissociation kit (cat. #130-095-929; Miltenyi Biotec) and the number of viable cells counted. To analyze immune-cell populations, 1×10⁶ cells were blocked with purified mouse CD16/32 antibody (1:100 dilution; cat. #101301; BioLegend) for 5 min on ice. The cells were then incubated with antibody cocktail of Live/Dead Near IR antibody (cat. #L10119; Thermo Fisher Scientific), anti-CD45-BUV395 (clone 30-F11; cat. #565967; BD Biosciences), anti-CD3-BUV737 (clone 17A2, cat. #564380; BD Biosciences), anti-CD4-BUV496 (clone GK1.5; cat. #564667; BD Biosciences), anti-CD8-BUV805 (clone 53-6.7; cat. #564920; BD Biosciences), anti-CD8-BV421 (clone 2G9; cat. #565254; BD Biosciences), anti-F4/80-BV785 (clone BM8; cat. #123141; BioLegend), and anti-CD103-PE (clone M290; cat. #561043; BD Biosciences) for myeloid cells analysis. Antibodies were used according to the manufacturer’s instructions. The cells were incubated with the antibody cocktail for 20 min at 4°C in dark. For FOXP3 staining, the cells were fixed/permeabilized overnight at 4°C in dark using e Biosci FOXP3 transcription factor staining buffer set (cat. #00-5523-00; Thermo Fisher) and FOXP3 monoclonal antibody (1:25 dilution; clone FJK-16s; cat. #17-5773-80; Thermo Fisher). All the washings were done with PBS (cat. #SH30256FS; Fisher Scientific)+2% FBS (cat. #F0926; Sigma). Flow cytometry acquisition was performed on the BD FACS Symphony or LSR II. The data analysis was carried out using FlowJo software. To detect melanoma antigen expression on tumor cells or cell lines treated with kinase inhibitors, the cells were incubated with anti-Tyrp1-APC (clone TA99; cat. #NBP2-34720APc; Novus Biologicals) at 1:50 dilution for 20 min at 4°C in dark. Flow cytometry acquisition was performed on the BD FACS LSR II. The data analysis was carried out using FlowJo software.

**gp-100 tetramer assay**

The number of gp-100 TCR positive cells in MBM samples from mice responding to anti-PD-1-LAG-3 and anti-PD-1-CTLA-4 therapy was determined by carrying out in vitro gp-100 tetramer assays. Prior to staining by gp-100 tetramer, a single cell suspension of cells from MBMs of responding and relapsing mice was prepared by enzymatic digestion, using a MACS tumor dissociation kit (cat. #130-095-929; Miltenyi Biotec). The number of viable cells was counted, and around 1×10⁶ cells were blocked with purified mouse CD16/32 antibody (1:100 dilution; cat. #101301; BioLegend) for 5 min on ice. The cells were then stained with antibody cocktail of Live/Dead Near IR antibody (cat. #L10119; Thermo Fisher Scientific), anti-CD45-BUV395 (clone 30-F11; cat. #565967; BD Biosciences), anti-CD3-BUV737 (clone 17A2, cat. #564380; BD Biosciences), anti-CD4-BUV496 (clone GK1.5; cat. #564667; BD Biosciences), anti-CD8-BUV805 (clone 53-6.7; cat. #564920; BD Biosciences), anti-CD8-BV421 (clone 2G9; cat. #565254; BD Biosciences), anti-F4/80-BV785 (clone BM8; cat. #123141; BioLegend), and anti-CD103-PE (clone M290; cat. #561043; BD Biosciences) for myeloid cells analysis. Flow cytometry acquisition was performed on the BD FACS LSR II. The data analysis was carried out using FlowJo software.

**IFNγ ELISPOT assay**

IFNγ ELISPOT assay was performed using Mouse IFN-gamma ELISpot kit (cat. # EL485; R&D systems)
according to the manufacturer’s instructions. Around 1 million cells from SM1 and B16 tumors were stimulated with eBioscience Cell Stimulation Cocktail (500X) (cat. #00-4970-03; Thermo Fischer Scientific) and plated in the 96-well plate provided overnight at 37°C in a 5% CO2 incubator. The assay was performed as per the manufacturer’s instructions.

sc-RNA seq
Individual tumors from mice treated with IgG (collected at day 9), individual ICIs (all collected at day 11) or each of the ICI combinations (day 19 for PD-1+CTLA-4 and day 25 for PD-1+LAG-3) were harvested under sterile conditions and weighed. Single-cell suspensions were prepared by enzymatic digestion, using a MACS tumor dissociation kit (cat. #130-095-929; Miltenyi Biotec). Cells were strained through MACS strainer (cat. #130-098-458; Miltenyi Biotec). The cell count and viability were analyzed by staining the cells with AO/PI stain on the Nexcelom Cellometer K2. The cells were then resuspended at a concentration of 500 cells/µL in PBS (cat. #SH30256PS; Fisher Scientific) +0.4% nonacetylated BSA (cat. #BP1605100; Fisher Scientific). The samples were then loaded onto 10X Genomics Chromium Single-Cell Controller (10X Genomics) to prepare scRNA-seq libraries. Around 50,000–1,000,000 mean sequencing reads per cell were generated on Illumina NextSeq 500 flow cells. 10X Genomics Cell-Ranger software was used for demultiplexing, barcode processing, alignment, and gene counting. Finally, the analysis of single-cell data set was performed using interactive single-cell visual analytics (ISCVA), which was previously outlined in. 22 23 Cells with high mitochondria content were not filtered as these may reflect cell populations going through apoptosis. Data are available through Gene Expression Omnibus (GEO: GSE243281).

Statistical analysis
One-way ANOVA in Microsoft Excel V.15.40 was used to compare the results between different groups with a single independent variable. The mean of three independent experiments SEM is shown for each data set. Results with values of p≤0.05 were considered statistically significant.

RESULTS

Anti-PD-1+LAG-3 and anti-PD-1+CTLA-4 combinations are both effective against mouse models of melanoma
As the relative effectiveness of the anti-PD-1+LAG-3 and anti-PD-1+CTLA-4 combinations have never been directly compared, we undertook studies in which SM1 melanoma cells (BRAFV600E mutation and deletion in CDKN2A) were injected into the flanks of mice. Animals were treated with either IgG control, anti-PD-1, anti-LAG-3, anti-CTLA-4, anti-PD-1+CTLA-4 or anti-PD-1+LAG-3 (figure 1A). Combination ICI therapies were more effective than each single agent ICI; notably 100% of flank tumors exhibited full regression in response to the anti-PD-1+LAG-3 and anti-PD-1+CTLA-4 combination therapy. Next, mice exhibiting tumor regression were rechallenged with an additional SM1 flank tumor (figure 1B,C). After 63 days, SM1 rechallenged mice did not develop new tumors, indicating an antitumor immunological memory response (figure 1D). Long-term evaluation of mouse weights did not demonstrate any significant change in response to either anti-PD-1+LAG-3 or anti-PD-1+CTLA-4 therapy (online supplemental figure 1). We next evaluated the two ICI combinations in a more resistant B16 melanoma flank model and noted that although both combinations were initially effective at reducing the volume of established tumors (figure 1D, E and F), 3/10 tumors relapsed on anti-PD-1+LAG-3 therapy. The anti-PD-1+CTLA-4 combination was also not curative in every mouse with 1/10 initially failing and 2/10 mice relapsing after cessation of treatment (figure 1F). Of 10, 7 mice treated with anti-PD-1+CTLA-4 and 6/10 tumors on anti-PD-1+LAG-3 therapy did not relapse (figure 1F). Mice responding to each of the ICI combinations were later rechallenged with B16 tumor at day 97, with no regrowth detected (figure 1F). It, therefore, seemed that although the B16 model exhibited greater resistance to combination ICI therapy, some mice did have prolonged antitumor responses to both anti-PD-1+LAG-3 and anti-PD-1+CTLA-4, associated with the development of immunological memory. Although B16 cells are a useful model of immunotherapy resistance, they lack the driver oncogenes that characterize human melanoma. To confirm our findings in a further clinically relevant model, we treated established D4M-UV2 (BRAFV600E mutant, PTEN-null) mouse melanomas with IgG control, anti-PD-1+LAG-3 or anti-PD-1+CTLA-4 and once again saw durable levels of tumor regression (figure 1G, H1). It was noted that the anti-PD-1+LAG-3 combination had similar efficacy to the anti-PD-1+CTLA-4 combination.

We also collected samples from responding SM1 flank tumors over multiple time points (2, 4, 6 and 14 days) and analyzed the dynamics of immune infiltrate (online supplemental figure 2). It was noted that both the combination of anti-PD-1+LAG-3 and anti-PD-1+CTLA-4 led to increased infiltration of CD4+ and CD8+ T cells. In contrast, the IgG treated controls showed initially higher levels of CD4+ and CD8+ T cells that declined by day 14. An analysis of myeloid cells did not reveal a clear trend over the 14 day time period (online supplemental figure 2: Gating strategy for myeloid-derived suppressor cells (MDSCs) shown in online supplemental figure 3). Tumors from IgG treated mice saw an increased accumulation of macrophages over time, whereas treatment of melanomas with either anti-PD-1+CTLA-4 or anti-PD-1+LAG-3 led to a decrease in macrophage numbers.

The combination of anti-PD-1+LAG-3 and anti-PD-1+CTLA-4 are effective against MBMs
We next compared the efficacy of the anti-PD-1+LAG-3 and anti-PD-1+CTLA-4 combinations in mouse MBM models by injecting SM1 cells stereotactically into the...
Figure 1  Anti-PD-1+LAG-3 and Anti-PD-1+CTLA-4 combinations suppress melanoma flank models. (A) Survival curves of the SM1 mouse model treated with IgG, single agent anti-PD-1, anti-LAG-3, anti-CTLA-4, the anti-PD-1+LAG-3 combination or the anti-PD-1+CTLA-4 doublet combination. (B) Dosing schema for the rechallenge experiment in the SM1 mouse model. (C) The ICI doublets were more effective than single agents ICI in the flank tumors of SM1 mouse model with no tumor regrowth observed. Mice received 200 µg/100 µL i.p. doses of single agents and doublets every 5 days. (D) Survival curves for the B16 mouse model following treatment with IgG, or the anti-PD-1+LAG-3 and anti-PD-1+CTLA-4 combinations. (E) Dosing schema for the rechallenge experiment in the B16 mouse model. (F) The doublets showed initial response in the flank tumors of B16 melanoma model with 3/10 tumors relapsed on anti-PD-1+LAG-3 and 2/10 tumors relapsed on anti-PD-1+CTLA-4 after cessation of treatment. Mice received 200 µg/100 µL i.p. doses of combinations every day. (G) Survival curves of the D4M-UV2 mouse melanoma model treated with IgG or the doublets of the anti-PD-1+LAG-3 or the anti-PD-1+CTLA-4 combination. (H) Dosing schema for the experiment using the D4M-UV2 mouse melanoma model. (I) The doublets showed similar response to the anti-PD-1+LAG-3 and the anti-PD-1+CTLA-4 combination in the flank tumors of D4M-UV2 mouse model (p=0.22). Mice received 200 µg/100 µL i.p. doses of combinations every 5 days for 22 days.
brain. A dual injection model (eg, concurrent flank and brain tumors) was used as our preliminary studies had indicated that mice with only brain metastases (and no flank tumors) had poor immune responses and showed little response to ICI therapy (data not shown). It was noted that both combination ICI therapies were effective against brain metastases (figure 2A–C). In mice who responded to each combination ICI therapy, responses were durable with no evidence of tumor recurrence when therapy was stopped for 28 days (MRI images in figure 2B).

To explore the kinetics of T cell accumulation in MBM following anti-PD-1+LAG-3 or anti-PD-1+CTLA-4 therapy we collected samples of responding SM1 brain tumors over time (days 7, 14, 21 and 28) (figure 2D) and observed that levels of CD4+ and CD8+ T cell infiltration increased following treatment with each of the ICI combinations. Of note, T cell infiltration was more rapid in response to anti-PD-1+LAG-3 (>14 days) whereas slower dynamics (>21 days) were noted to the anti-PD-1+CTLA-4 combination (figure 2D). We next performed tetramer assays to measure the numbers of gp100+ (an antigen expressed by the SM1 cells) tumor-reactive T cells in the brain tumors, with increased numbers of gp100 recognizing CD8+ T cells being noted in response to both anti-PD-1+LAG-3 and the anti-PD-1+CTLA-4 combination (online supplemental figure 4). In the brain, initial levels of MDSC-like cells were high and then declined in response to both ICI combinations (figure 2E). Treatment with either the anti-PD-1+LAG-3 or anti-PD-1+CTLA-4 combination was associated with increased accumulation of macrophages (figure 2F).

**Single cell analysis of the immune landscape of flank and brain tumors treated with different combination immunotherapies**

Droplet-based scRNA-seq was used to interrogate the immune landscape of SM1 tumors treated with either single agent or combination ICI. Analysis of samples from responding flank tumors along with detailed cell curation revealed a diverse cellular landscape composed of tumor cells, stromal cells (including fibroblasts, endothelial cells, microglial cells) and multiple immune cell populations (T cells, NK cells, B cells, dendritic cells, monocytes, granulocytes, macrophages/MDSCs) (figure 3A). The immune landscape of tumors treated with the anti-PD-1+LAG-3 combination had a large proportion of T cells, the greatest accumulation of B cells, granulocytes and endothelial cells and the lowest proportion of macrophages. The anti-PD-1+CTLA-4 combination was also associated with an increased proportion of T cells, and the highest number of regulatory T cells (Tregs) (figure 3B).

We next defined the transcriptional makeup of the T cells in tumors responding to single or combination immunotherapy (figure 3C) and identified six major clusters including four sub-groups of CD8+T cells (T1, 3, 4 and 5), one major subgroup of CD4+T cells (T2) and NK

**Figure 2** The anti-PD-1+LAG-3 and the anti-PD-1+CTLA-4 combinations suppress melanoma brain metastases in the SM1 mouse melanoma model. (A) The ICI doublets were more effective than single agent ICI in the brain tumors of SM1 mouse melanoma model with 2/10 mice relapsing in each combination group. Mice received 200 mg/kg anti-PD1 treatment (n=10 mice per group for all the growth curve experiments). The results were represented as average ±SEM of 3 mice per group for all the growth curve experiments. The results were represented as average ±SEM of 3 mice per group for all the growth curve experiments. The results were represented as average ±SEM of 3 mice per group for all the growth curve experiments. The results were represented as average ±SEM of 3 mice per group for all the growth curve experiments.
Figure 3  Immune landscape of flank and brain tumors in SM1 mouse model following the treatment with single agent and combination ICI therapy. (A) t-SNE plots showing major cell types identified in relapsed brain tumors and responding flank SM1 tumors following treatment with single agent and combination ICI. (B) Proportion of each cell type in the responding flank tumors from the indicated treatment groups. (C) Proportion of different T-cell clusters identified in the responding flank tumors from the indicated treatment groups. (D) Heatmap showing expression of activation/exhaustion markers across the identified T-cell clusters. (E) Violin plots showing expression of T cell activation markers and immune checkpoints in each T-cell cluster. (F) IHC analysis of tumors treated with anti-PD-1+LAG-3 or anti-PD-1+CTLA-4 demonstrates increased CD4+ and CD8+ T cell infiltration in responding flank tumors. The samples were stained with anti-CD4 and anti-CD8 by IHC. Tumors were harvested at day 9 for IgG and single agents and at day 19 for anti-PD-1+LAG-3 and day 25 for anti-PD-1+CTLA-4 following the initiation of treatment. ICI, immune checkpoint inhibitor.
cells. It was noted that the anti-PD-1+CTLA-4 combination was associated with the largest accumulation of T1 CD8+T cells, whereas the anti-PD-1+LAG-3 combination was associated with T3 CD8+T cells and the CD4+T cell cluster T2. Analysis of differentially expressed markers in the CD8+T cell subsets identified T1 to express higher levels of cytotoxicity markers such as IFNγ, GZMB and PRF1 as well as multiple checkpoints including PDCD1 (PD-1), LAG-3, TIM3, TIGIT and CTLA-4 (figure 3D,E). By contrast, the T3 subset, associated with anti-PD-1+LAG-3 responding tumors, had lower levels of cytotoxicity markers, decreased expression levels of LAG-3 and TIM3 (HAVCR2) and increased expression of TNFSF8, CXCR4 and IL21R (figure 3E). IHC analysis confirmed the infiltration of CD4+ and CD8+ T cells (figure 3F).

The anti-PD-1+LAG-3 and anti-PD-1+CTLA-4 combinations differentially polarize CD4+ T cells

Of particular interest were the CD4+T cells, which comprised both T helper subsets and regulatory T cells (Tregs). Clustering analysis of the initial T2 cluster identified two further major subsets of CD4+T cells (and a series of very minor clusters) in addition to Tregs (figure 4A). The major CD4+T cell cluster found in anti-PD-1+CTLA-4 responding tumors expressed markers consistent with Tregs such as FOXP3 and IL-2RA (CD25). It additionally expressed multiple checkpoints, including those not expressed in the other CD4+T cell subsets, such as LAG-3. The T6 CD4+T cell cluster was primarily found in anti-PD-1+LAG-3 treated tumors and expressed the highest levels of CXCR4, TNFSF8 and IL21R. This cluster also expressed genes known to be characteristic of Th1 cells (STAT1, STAT4, T-bet, IFNγ), Th2 (GATA3, STAT5, STAT6, IL4Ra), Th9 cells (TGFB2, IRF4, IL4Ra) and Th17 cells (IL21R, BATF, RORα, TGFBR2, STAT3) (online supplemental figure 5). These genes had a heterogeneous expression, with some being found only in limited numbers of cells—suggesting that multiple phenotypes of helper CD4+T cells were located within this cluster. Cytokine expression, such as IL-17A, IL-9, IL-4, among others was poorly represented in the scRNA-Seq data, as others have previously reported.24 The final cluster T7 was composed of a subset CD4+T cells predicted to be activated that expressed CCR7, IL7R and TCF7 (figure 4A,B). Flow cytometry demonstrated the anti-PD-1+LAG-3 combination to be associated with increased levels of CD69+ effector CD4+ T cells and reduced numbers of CD62L- CD4+ memory cells (figure 4C,D). The increased levels of Tregs in the anti-PD-1+CTLA-4 treated SM1 flank tumors was confirmed both by flow cytometry and IHC staining for FOXP3 (figure 4C,E).

Antitumor responses to anti-PD-1+LAG-3 and anti-PD-1+CTLA-4 therapy show differential requirements for CD4+ T cells

As our scRNA-seq analyses suggested that the anti-PD-1+CTLA-4 and anti-PD-1+LAG-3 combinations had differential effects on T cell polarization we next determined the dependency of each ICI combination on CD4+ and CD8+ T cell activity. It was noted that whereas depletion of CD8+T cells abrogated responses to both the anti-PD-1+LAG-3 and anti-PD-1+CTLA-4 combination, CD4+T cell depletion only impacted responses to the anti-PD-1+LAG-3 combination in the SM1 model (figure 5A and B: online supplemental figure 6). These effects were noted in both the SM1 brain and flank tumors, with pronounced effects of CD4+T cell depletion being noted in the brain metastases (figure 5A,B and online supplemental figure 7).

There is evidence that CD4+T cells exert antitumor effects through helper activity that increases CD8+T cell function. Depletion of CD4+T cells in the anti-PD-1+LAG-3 treated SM1 tumors reduced the accumulation of activated CD69+CD8+ T cells (54% decrease) accompanied by a more modest decrease in total CD8+T cell numbers (figure 5C). Conversely, depletion of CD4+ T cells in anti-PD-1+CTLA-4 treated SM1 tumors led to an increase in CD69+CD8+ T cells (figure 5C, online supplemental figure 8). Similar findings were noted in the B16 melanoma model, with depletion of CD4+T cells impacting antitumor responses to the anti-PD-1+LAG-3 combination, but not the anti-PD-1+CTLA-4 combination (figure 5D,E). In the B16 model, depletion of CD4+T cells was associated with decreased total numbers of CD8+T cells in anti-PD-1+LAG-3 treated tumors and an increase (37%) in the total CD8+T cells in anti-PD-1+CTLA-4 treated tumors (figure 5E). Depletion of CD4+T cells also diminished responses to the anti-PD-1+LAG-3 combination, but not the anti-PD-1+CTLA-4 combination, in the D4M-UV2 model (figure 5F). As with the other models, depletion of CD4+T cells led to a significant decrease in the accumulation of both total CD8+T cells and activated CD69+CD8+ T cells in D4M-UV2 tumors (figure 5G).

We next performed IFNγ ELISPOP assays on tumor infiltrating immune cells from the IgG and ICI treated SM1 tumors and demonstrated that the anti-PD-1+CTLA-4 combination led to greater increases in IFNγ release compared with the anti-PD-1+LAG-3 combination (figure 5H). These findings confirmed the scRNA-seq data from figure 3E and suggested that the T1 CD8+ T cells associated with anti-PD-1+CTLA-4 treatment exhibited enhanced effector functions relative to the T3 subset of CD8+T cells enriched for by the anti-PD-1+LAG-3 combination. Consistent with the anti-PD-1+LAG-3 combination being reliant on CD4+ T cell help, it was noted that depletion of the CD4+ cells led to an 89% decrease in IFNγ release from immune cells infiltrating anti-PD-1+LAG-3 treated SM1 and B16 tumors (figure 5H1), suggesting a role for T helper function. In contrast, depletion of CD4+T cells from B16 tumor bearing mice treated with anti-PD-1+CTLA-4 led to a 110% increase in IFNγ release, suggesting a suppression of Treg activity (figure 5I). A scheme outlining the likely mechanism of action is shown in (figure 6).
Figure 4  Anti-PD-1+LAG-3 and anti-PD-1+CTLA-4 differentially polarize CD4+ T cells in the SM1 mouse melanoma model.
(A) Proportion of T cell clusters and three major subsets of CD4+T cells identified in the responding flank tumors from the indicated treatment groups. (B) Expression of immune checkpoints and markers for T helper subsets and T regulatory cells in the three major subsets of CD4+T cells identified by violin plots. (C) Percentage of CD4+ T cells and activated CD69+CD4+ T cells in the responding flank tumors treated with combination of anti-PD-1+LAG-3 and anti-PD-1+CTLA-4 identified by flow cytometry. Tumors were harvested at the endpoint following the treatment initiation. (D) Flow cytometry histogram plot showing activated CD4+T cells in tumors treated with IgG, anti-PD-1+LAG-3 or anti-PD-1+CTLA-4 as evidenced by cell surface CD69 and CD4 staining. (E) Treatment with anti-PD-1+CTLA-4 increased Tregs infiltration in the responding flank tumors and relapsing brain tumors. The samples were stained with anti-FOXP3 by IHC. Tumors were harvested at day 9 for IgG and single agents and at day 19 for anti-PD-1+LAG-3 and day 25 for anti-PD-1+CTLA-4 following the initiation of treatment for sc-RNAseq and IHC. The results were represented as average±SEM of 3 mice per group for panels C and D. Statistical significance was assessed with one-way ANOVA test (**p<0.001). ANOVA, analysis of variance; IHC, immunohistochemistry.
Figure 5  Antitumor responses to anti-PD-1+LAG-3 are dependent on CD4+T helper function. (A, B) Depletion of CD4+T cells demonstrates that responses to anti-PD-1+LAG-3 but not anti-PD-1+CTLA-4 are dependent on CD4+T cells in both SM1 flank and brain tumor models. The results were represented as an average±SEM of 5 mice per group. (C) Decreased percentage of tumor-infiltrating CD69+CD8+ T cells and CD8+T cells in SM1 flank tumors, respectively, following CD4+T cell depletion and anti-PD-1+LAG-3 treatment. Increased percentages of CD69+CD8+ T cells and total CD8+T cells were seen in SM1 flank tumors following CD4+T cell depletion and anti-PD-1+CTLA-4 treatment. Tumors were collected at day 10 after the treatment initiation. (D) Antitumor responses of the PD-1+LAG-3 combination but not the PD-1+CTLA-4 combination are dependent on CD4+T cells in B16 flank tumors. Results show the average±SEM of 5 mice per group. (E) Decreased percentage of tumor-infiltrating CD69+CD8+ T cells and CD8+T cells in B16 flank tumors, respectively, following CD4+T cell depletion and anti-PD-1+LAG-3 treatment. Increased percentages of CD69+CD8+ T cells and total CD8+T cells were seen in B16 flank tumors following CD4+T cell depletion and anti-PD-1+LAG-3 treatment. Tumors were harvested for flow cytometry analysis at day 5 following the start of the treatment. (F) Responses to Anti-PD-1+LAG-3 but not Anti-PD-1+CTLA-4 are dependent on CD4+T cells in the flank tumors of D4M-UV2 mouse melanoma model. The results were represented as an average±SEM of 5 mice per group. (G) Percentage of CD69+CD8+ and CD8+ T cells decrease in the D4M-UV2 flank tumors significantly, following CD4+T cell depletion and Anti-PD-1+LAG-3 treatment. Tumors were collected for flow cytometry analysis at day 16 of treatment for IgG and day 19 of treatment for the doublets. (H, I) ELISPOT assays showing decreased levels of IFNγ production following CD4+T cell depletion and treatment with the PD-1+LAG-3 combination in both the SM1 and B16 mouse melanoma models. The results were represented as average±SEM of 3 mice per group for panels D-G. Statistical significance was assessed with one-way ANOVA test (*p<0.05, **p<0.01, ***p<0.001). ANOVA, analysis of variance.
DISCUSSION

CTLA-4, LAG-3 and PD-1 are all known to be expressed on T cell activation and serve as biomarkers of T cell exhaustion. Despite these similarities, the mechanisms of action of the anti-PD-1+CTLA-4 and the anti-PD-1+LAG-3 combinations have never been directly compared. In the current study, we used three different mouse melanoma models and high dimensional scRNA-seq analysis to identify a unique dependency of the anti-PD-1+LAG-3 combination on CD4+T helper cell function that was not seen in the tumors from mice treated with the anti-PD-1+CTLA-4 combination. The CD4+T cell dependency of the anti-PD-1+LAG-3 effects were noted in flank tumor models as well as in a dual flank-brain injection model. It was additionally found that the anti-PD-1+LAG-3 combination enriched for T cell transcriptional states that were very different from those associated with anti-PD-1 or anti-LAG-3 alone. Although the reactivation of CD8+T-cell activity has been the major focus of ICI research there is growing evidence that CD4+T cells may also be critical. CD4+T cells can differentiate into multiple T helper (Th) states including Th1, Th2, Th9, Th17 and Tregs that subserve different functions. Th1 CD4+T cells with cytotoxic function can mediate direct regression of established tumors in a MHC class II (MHC II)-restricted manner. Cytotoxic CD4+T cells have been identified in melanoma patients undergoing ICI treatment and there is evidence that increased levels of circulating CD4+T cells correlate with better outcomes in melanoma patients treated with anti-PD-1 therapy. They also have an important helper function that can improve B cell responses and CD8+T cell responses directly (leading to memory) and indirectly through DC function. Recent studies have also suggested roles for Th9 and Th17 T helper cells in antitumor responses. Th9 cells have been reported to have superior antitumor activity compared with other T helper subsets, and have the ability to activate both innate (NK cells, dendritic cells) and cytotoxic CD8+T cell activity. Th17 cells also have antitumor activity, with studies showing that adoptively transferred Th17 cells can eradicate melanomas in mice. There is also evidence that IL-17 deficient mice are more susceptible to melanoma development in the lung. Differentiation of Th17 cells requires TGF-β, IL-6 and IL-1β and then IL-21 and IL-23 for their maintenance.

An analysis of the tumor infiltrating CD4+T cell phenotypes identified key differences between the two ICI combinations. scRNA-Seq analysis demonstrated that CD4+T cells from anti-PD-1+CTLA-4 treated tumors expressed multiple Treg markers including FOXP3 and IL2RA, whereas those from anti-PD-1+LAG-3 treated tumors were more characteristic of T helper CD4+T cells. These T helper cells did not have one predicted phenotype and comprised cells with gene expression profiles previously associated with Th1, Th9 and Th17 helper cells. CD4+T cell depletion studies confirmed this likely T helper phenotype, demonstrating a diminished response to anti-PD-1+LAG-3 therapy in three independent melanoma models, associated with reduced CD8+T cell activation. The results of CD4+T cell depletion were particularly striking in the SM1 brain metastasis model. Further evidence for a role for T helper activity came from the scRNA-seq based cell type curation data which identified increased numbers of B cells and dendritic cells in tumors treated with the anti-PD-1+LAG-3 combination. In contrast, depletion of CD4+T cells followed by anti-PD-1+CTLA-4 treatment had little effect on antitumor activity and was instead associated with increased CD8+T cell activity. This apparent paradoxical effect of CD4+T cell depletion on CD8+T cell activation is likely consequence of the higher levels of Tregs in the PD-1+CTLA-4 treated tumors. It is known that Tregs express high levels of CTLA-4, and that CTLA-4 inhibition leads to Treg expansion. These effects are mediated through disruption of a CTLA-4 dependent feedback loop that leads to a CD28-mediated expansion of tumor-associated Tregs, increased tumor tolerance and decreased cytotoxic CD8+T cell activity. Tregs limit cytotoxic CD8+T cell antitumor activity through multiple mechanisms including the modulation of antigen presentation (through downregulation of CD80 and CD86 on DCs), the release of inhibitory cytokines (such as TGF-β and IL-10) and through depletion of important T cell growth factors in the environment (such as IL-2).

LAG-3 has also been implicated in the regulation of Treg function, with prior work demonstrating that LAG-3 blockade inhibits the suppressive activity of Tregs in vitro and in vivo models of pulmonary vasculitis. Other studies have suggested that high levels of LAG-3 expression are critical for the maintenance of Treg suppressive function in models of mucosal immunity. In addition to its expression on T cells, LAG-3 is also expressed on multiple other immune cell types including B cells and γδ-T cells and it is known that mice who are LAG-3 deficient have increased numbers of T cells, B cells, macrophages,
granulocytes and dendritic cells.42 Our scRNA-seq analysis of the T cell repertoire in melanomas treated with either anti-PD-1+CTLA-4 or anti-PD-1+LAG-3 led to the accumulation of distinct populations of both CD8+ and CD4+ T cells. The anti-PD-1+CTLA-4 combination enriched for a T1 population of CD8+ T cells, which expressed increased levels of cytotoxic markers such as IFN, GZMB and PRF1, whereas anti-PD-1+LAG-3 treatment was more associated with greater numbers of T3 cluster CD8+ T cells which expressed lower levels of cytotoxic markers. ELISPOT assays confirmed these findings and showed that anti-PD-1+CTLA-4 treatment led to an increased infiltration of tumor-infiltrating IFNγ secreting CD8+ T cells compared with mice receiving anti-PD-1+LAG-3 therapy. It thus seemed that the anti-PD-1+CTLA-4 combination directly improved CD8+ T cell function, boosting their cytotoxic activity whereas the anti-PD-1+LAG-3 combination was associated with decreased Treg activity and enhanced CD4+ helper-mediated CD8+ T cell activation. The anti-PD-1+LAG-3 activated CD8+ T cells appeared to be less cytotoxic than those from tumors treated with the anti-PD-1+CTLA-4 combination. Although relatively little is known about the mechanism of action of the anti-PD-1+LAG-3 combination, analysis of samples from the recent neoadjuvant phase 2 clinical trial of anti-PD-1+LAG-3 treated melanoma patients identified increased numbers of memory CD4+ T cells and CD8+ T cells in responding lesions, with responding patients additionally showing a reduction in M2-like macrophages.11

The observation that these two different, Food and Drug Administration (FDA)-approved ICI combinations have different mechanisms of action is highly significant. Many patients exhibit upfront or acquired resistance to standard-of-care anti-PD-1 or the anti-PD-1+CTLA-4 combination, suggesting there could still be responses to second line ICI therapy with a different mechanism of action. These observations are of particular interest in the context of MBM, where additional therapeutic strategies are urgently needed. The resulting outcome in trial relitlimab with nivolumab (NCT05704647) are the context of MBM, where additional therapeutic strategies, suggesting there could still be responses to MBM. Where additional therapeutic strategies could be added, selecting the optimal treatment regimen will become more critical.

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MSP: investigation, conceptualization, formal analysis, data curation, writing—original draft. JL: Formal analysis, data curation. ZC: Formal analysis, data curation. PCR: supervision, writing—review and editing. JKM: investigation, writing—review and editing. LK: Formal analysis, writing—review and editing. PF: investigation, writing—review and editing. AC: Formal analysis, investigation, supervision, writing—review and editing. KS: conceptualization, supervision, funding acquisition, visualization, writing—original draft, guarantor of the content.

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Competing interests
KS reports research funding from Revolution Medicines, unrelated to the current study. PF serves as a Consultant to Abbvie, Pfizer, Novartis, BMS, BTG, GSK, Zopharm, Tocagen, Boehringer Ingelheim, National Brain Tumor Society, Midatech Pharma, Inovio, NCCN unrelated to the current study. All other authors report no conflicts of interest.

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Data are available in a public, open access repository. scRNA-seq data are freely available through Gene Expression Omnibus https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE243281 (GSE243281)

Supplemental material
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