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CTLA-4: Challenges, limitations, and future perspective in cancer immunotherapy

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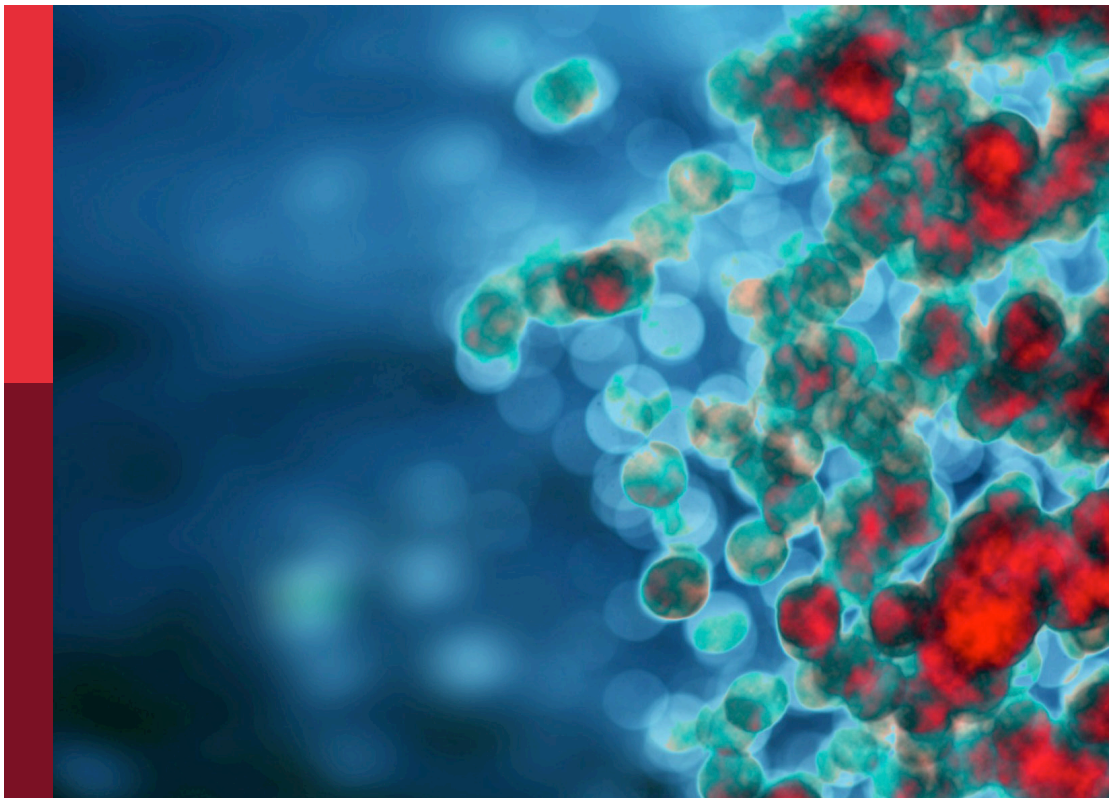

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CTLA-4: Challenges, limitations, and future perspective in cancer immunotherapy

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Chimeric CTLA4-CD28-CD3z T Cells Potentiate Antitumor Activity Against CD80/CD86–Positive B Cell Malignancies

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The adoptive transfer of chimeric antigen receptor T (CAR T) cells have been recognized as a promising therapeutic strategy for the treatment of hematological malignancies; however, clinical success using CAR T cells for the treatment of solid tumors are still limited since the T-cell function is inhibited by negative signals in the microenvironment of solid tumors. CTLA4 is a well-known immune checkpoint molecule, thus we developed a novel CAR by converting this negative signal to positive signal. The CAR developed consists of the extracellular and transmembrane domains of CTLA4 and the cytoplasmic domains of CD28 and CD3z (CTLA4-CAR T). CTLA4-CAR T cells exhibited superior cytokine secreting activities and cytotoxic to tumor cells *in vitro* and in xenograft models. CTLA4-CAR T cells were found to accumulate in tumors and are toxic to myeloid-derived suppressor cells (MDSCs) without signs of severe GVHD and CRS in preclinical models. Thus, this chimeric CTLA4-CAR can enhance the antitumor activity of CAR T cells and shed light on the strategy of using armed CAR T cells to target the immunomodulatory tumor microenvironment.

Keywords: immunotherapy, CAR-T, CTLA4, CD80, CD86, myeloid-derived suppressor cells

INTRODUCTION

T cell responses can be compromised by the presence of negative costimulatory signaling molecules, such as programmed death-1 (PD1), T cell Ig mucin-3 (TIM3), Lymphocyte activation gene-3 (LAG3), and cytotoxic T lymphocyte-associated antigen 4 (CTLA4) (1–3). The CTLA4-CD86/CD86 axis is a well-known immune checkpoint inhibitor pathway. CTLA4,

Abbreviations: CAR, chimeric antigen receptor; PD1, programmed death-1; TIM3, T cell Ig mucin-3; LAG3, lymphocyte activation gene-3; CTLA4, cytotoxic T lymphocyte-associated antigen 4; TME, tumor microenvironment; TAAs, tumor-associated antigens; MDSC: myeloid-derived suppressor cell.

which is up-regulated after T cell activation (2), is homologous to the T cell costimulatory protein CD28, and bind to its ligands CD80 and CD86 on many tumors and on cells within the tumor microenvironment (TME), such as antigen-presenting cells, B cells, macrophages, and the stromal cell subset (4). The CTLA4-CD80/CD86 pathway serves as a negative feedback mechanism to control the immune responses to inflammatory stimuli (5). CTLA4-CD80/CD86 signal initiates T cells anergy or exhaustion, which reduces the activities of T cells, whereas blockade of the interaction between CTLA4 and its ligands reverses effector T cells exhaustion, thereby reinforcing anti-tumor activities of T cells (6). Recent preclinical and clinical evidence has shown promise in treating cancer by utilizing anti-CTLA4 antibodies (7–10); however, the clinical trial for solid tumors achieved very limited success because of the weak anti-tumor T cell immune responses, it is very challenging to establish the effective anti-tumor response in solid tumors due to its immunosuppressive TME.

In recent years, chimeric antigen receptor T (CAR T) cell therapies have shown exciting therapeutic modalities for some difficult cancer (11–13). CARs generally comprise an extracellular ligand recognition domain, typically a single-chain variable fragment (ScFv) fused to the signaling domain of CD3z (14). Second-generation CARs contain another intracellular costimulatory domain, which may include CD28, 4-1BB, ICOS, CD40, and CD27 molecules, to enhance cytokine secretion and proliferation of CAR T cells (15–17). Many CARs have been developed to recognize multiple tumor-associated antigens (TAAs); however, currently available targets for CAR T cell therapy are still limited due to high heterogeneity among cancer patients (18–20). In addition, tumor relapse occurred in many patients who achieved disease remission after initial CAR T cell therapy, which may have been due to the loss of tumor antigen on tumor cells (21).

Targeting of CTLA4-CD86/CD86 interaction by the administration of blocking antibodies could enhance the potency of immunotherapy for cancers. However, partial or poor complete response (CR) was observed in patients, suggesting that the therapeutic effects of a naked antibody would not be potent enough for the curable treatment of cancer (22, 23). Previous studies had reported that CTLA4-CD28 chimera gene-modified T cell, which the intracellular signaling domain of CTLA4 was replaced with the CD28 signaling domain, showed significantly enhanced anti-tumor effect in murine tumor models (24, 25). Therefore, in this study, we attempted to convert the negative CTLA4-CD86/CD86 signal by generating a novel CTLA4 signaling pathway in T cells to disrupt the interaction of immune checkpoint inhibitors CTLA4 and CD80/CD86 to effectively treat CD80/CD86-expressing cancer. To this end, we developed a novel chimeric receptor that can recognize CD80 and CD86 as tumor antigens on malignant B cells by combining the extracellular and transmembrane domains of CTLA4 with the intracellular signaling domains of CD28 and CD3z and explored its potential in cancer treatment.

MATERIALS AND METHODS

Human Tissues

Cord blood samples and primary B lymphoma samples were obtained according to procedures approved by the institutional review boards at Nanfang Hospital (Guangdong, China). The use of human tissue samples in this study was approved by the Committee for the Ethical Review of Research Involving Human Subjects at Nanfang Hospital, Southern Medical University. Institutional guidelines regarding human experimentation were followed, according to the Helsinki Declaration of 1975. The protocol was approved by the Ethical Committee of The Third Affiliated Hospital of Guangzhou Medical University. Written informed consent was obtained from individual or guardian participants.

Mice

Animal experiments were performed in the Laboratory Animal Center of the Guangzhou Institutes of Biomedicine and Health (GIBH), and all animal procedures were approved by the Animal Welfare Committee of GIBH. NOD-*SCID-IL2Rg*$^{-/-}$ (NSI) mice were derived at the Laboratory Animal Center of GIBH. C57BL/6J mice were purchased from Vital River Laboratory Animal Technology Co. (Beijing, China). Mice were maintained in specific pathogen-free cages and provided with autoclaved food and water. Adult male mice aged 6 to 8 weeks were used in this study. Protocols were approved by the relevant Institutional Animal Care and Use Committee (IACUC).

Cell Cultures

HEK-293T, Platinum-E, and B16F10 cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, New York, NY, USA). All lymphoma cell lines (NALM6, RL, Raji, and K562 cells) were purchased from American Type Culture Collection (ATCC; Maryland, USA) and were labeled with green fluorescent protein (GFP) and luciferase. Cells were cultured in RPMI-1640 medium (Gibco, New York, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, New York, NY, USA). All cells were cultured at 37°C in an atmosphere of 5% carbon dioxide.

Flow Cytometry

Peripheral blood, spleen, liver, and bone marrow from mice were treated with red blood cell lysis buffer (Biolegend) before staining. Solid tissue samples were mechanically chopped with scalpels, placed in culture medium (DMEM with 5% FBS, 0.5 mg/ml collagenase A, 0.2 mg/ml hyaluronidase V, and 0.02 mg/ml DNase I), and digested for 45 min at 37°C. The resulting suspensions were resuspended in PBS, and the cells were pelleted at 300 r.c.f. for 3 min.

All antibodies were purchased from BD Biosciences unless otherwise noted. Flow cytometric analysis was performed using an Accuri C6 or LSRFortessa cell analyzer (BD Biosciences, San

Jose, CA), and data were analyzed using FlowJo software (FlowJo, LLC, Ashland, OR, USA). Anti-human CD80 PE-conjugated (560925), anti-human CD86 APC-conjugated (560956), anti-mouse CD11b FTTC-conjugated (561688), anti-mouse Ly6C PerCP-Cy5.5-conjugated (560525), and anti-mouse Ly6G PE-Cy7–conjugated (560601) antibodies were used for analyses. 4′,6-Diamidino-2-phenylindole (DAPI; 0.1 μg/ml final concentration; Invitrogen, D1306) was used to distinguish live and dead cells. Staining was performed on ice for 30 min, and cells were then washed with PBS containing 2% FBS before cytometry analysis.

Vector Design

The human CTLA4 CAR comprises the extracellular and transmembrane portions of human CTLA4, the cytoplasmic region of human CD28, and the intracellular domains of human CD3z (**Figure 1A**). All the domains were synthesized by GenScript (Nanjing) Co., Ltd. (Nanjing, China). The mouse chimeric CTLA4, comprising the extracellular and transmembrane domains of mouse CTLA4, the cytoplasmic region of mouse CD28 and the intracellular domain of mouse CD3z (**Figure 3A**), was synthesized by GenScript (Nanjing) Co., Ltd. (Nanjing, China). The human CTLA4-chimeric gene was

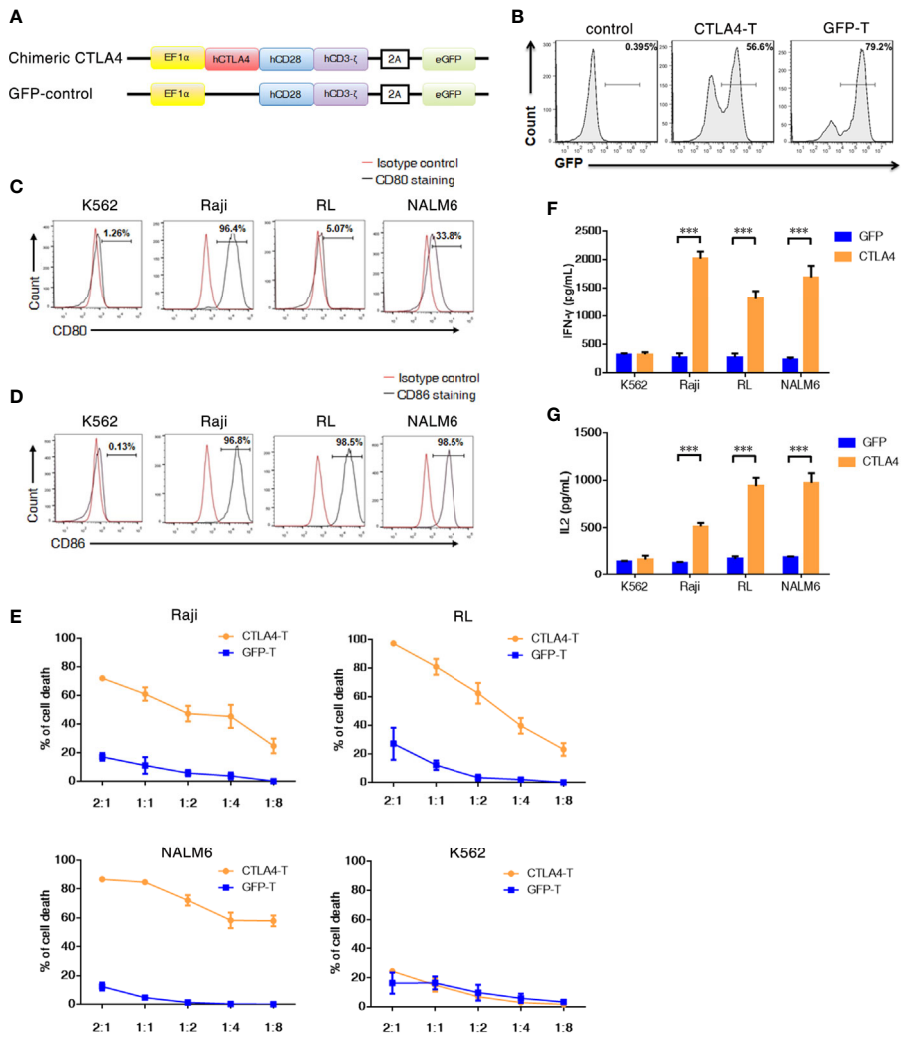

FIGURE 1 | T cells transduced with the CTLA4-CD28-CD3z chimeric gene showed enhanced *in Vitro* Cytotoxicity. **(A)** The chimeric CTLA4 molecule contains the extracellular and transmembrane domains of human CTLA4, the cytoplasmic signaling region of human CD28, and the intracellular domain of human CD3z. GFP was used to fluorescently label the cells. **(B)** Representative flow cytometric analysis of the transduction efficiency of chimeric CTLA4 or GFP (control) in human activated T cells that were transduced with a lentivirus. CTLA4-T: CTLA4 chimera-transduced T cells, GFP-T: GFP-transduced T cells. **(C, D)** Representative flow cytometric analyses of CD80/CD86 expression in K562, Raji, RL, and NALM6 cells. **(E)** Activated T cells transduced with either chimeric CTLA4 or GFP (control) and cocultured with the indicated cell lines for 18 h, mean ± SD. The levels of IFN-γ **(F)** and IL-2 **(G)** secreted into the culture supernatant were measured by ELISA with a 1:1 E:T ratio, mean ± SD, unpaired two-tailed t-test. Significance values: ***P < 0.001.

cloned into lentiviral pWPXLD expression vectors, the murine CTLA4-chimeric genes were cloned into pMX expression vectors. All the sequences used in experiments were confirmed by automated DNA sequencing.

Production of Lentivirus and Retrovirus

The lentivirus plasmids and another two packaging plasmid (psPAX2 and pMD2.G) were transduced to HEK-293T cells by using polyethyleneimine (Sigma-Aldrich, St. Louis, MO, USA). After 48 and 72 h, the supernatant containing lentivirus was harvested and filtered by using 0.45-μm syringe filter. The retrovirus plasmid was transduced to Platinum-E cells by using polyethyleneimine (Sigma-Aldrich, St. Louis, MO, USA). After 48, the supernatant containing retrovirus was harvested and filtered by using 0.45-μm syringe filter.

Isolation, Transduction, and Expansion of Primary Human T Lymphocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from cord blood using Lymphoprep (StemCell Technologies, Canada) according to the manufacturer's instructions. Primary human T cells were isolated from PBMCs *via* negative selection by using a pan-T Isolation Kit (Miltenyi Biotec, Germany). Isolated T cells were maintained in RPMI-1640 medium supplemented with 10% FBS (Biochrom, Australia), 10 mM HEPES, 100 IU/ml recombinant human IL-2, 2 mM glutamine, and 1% penicillin-streptomycin (Gibco, New York, USA). T cells were stimulated with an ImmunoCult™ Human CD3/CD28 T Cell Activator (StemCell Technologies, Canada) for 48 h. T cells were transfected with CAR vector lentiviral supernatants in the presence of 8 μg/ml polybrene at a multiplicity of infection of 2.0 (Sigma-Aldrich, St Louis, USA). Twelve hours after transfection, T cells were cultured in a fresh medium containing IL-2 (300 U/ml); subsequently, a fresh medium was added every 3 days to maintain cell density within the range of 0.5 to 1 × 10^6 cells/ml. CAR-T cells determined by flow cytometry at day 5, as GFP+, and then were included in the experiments.

Isolation, Transduction, and Expansion of Primary Mouse T Lymphocytes

Mouse spleens were removed from euthanized C57BL/6J mice, and mouse T cells were enriched by using a mouse pan-T Isolation Kit (Miltenyi Biotec, Germany). Isolated T cells were incubated in medium supplemented with human IL-2 (10 ng/ml, PeproTech, Rocky Hill, USA) and human IL-7 (2 ng/ml, PeproTech, Rocky Hill, USA) and were cocultured with mouse T cell activator beads (Miltenyi Biotec, Germany) for 48 h and then transduced with retrovirus by centrifuging at 1,200*g* for 90 min and then placed in a cell culture incubator (37°C, 5% CO_2) for 24 h. CAR-T cells determined by flow cytometry at day 5, as GFP+, and then were included in the experiments.

Cytotoxicity Assays

Target cells (NALM6-GL, RL-GL, and Raji-GL) were cocultured in triplicate wells U-bottomed 96-well plates with T cells that expressed either human CTLA4 CAR or GFP-CAR (control) at the indicated effector cell:target cell (E:T) ratios. Target cell viability was monitored 18 h later by adding 100 μl/well of the substrate D-luciferin (potassium salt) (Cayman Chemical, Michigan, USA) at 150 μg/ml. Background luminescence was negligible (<1% of the signal from wells containing viable target cells alone). Percent viable target cells (%) was calculated as (experimental signal − blank signal)/(targeted signal − blank signal) × 100, and percent cytotoxicity as 100 − percent viable target cells.

Cytokine Enzyme-Linked Immunosorbent Assay (ELISA)

After 18 h of co-culture cells at a 1:1 E:T ratio, the supernatants were collected, and the levels of interferon γ (IFN-γ) and interleukin-2 (IL-2) in the supernatant were detected using cytokine ELISA kits (e-Bioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Xenograft Models of Malignant B Cells

NOD/SCID/IL2Rg$^{-/-}$ mice were subcutaneously injected with NALM6, RL, or Raji cells (2 × 10^5) with 10% Matrigel (20 μl Matrigel in 180 μl PBS) on day 0. Two days after transplantation, the mice were randomly divided into three groups (five mice/group) and injected *via* the caudal vein with CTLA4-T cells, GFP-T cells, or non-transduced T cells (2×10^5 cells) in 200 μl PBS. Tumor volume was calculated every 7 days. The tumor length and width were measured every 5 days using a Vernier caliper, tumor volume was calculated using the following equation: (length × width^2)/2.

For primary lymphoma models, primary lymphoma tumors were resected and placed in RPMI-1640 medium in an ice bath. The tumors were diced into 3 × 3 × 3 mm cubes and subcutaneously transplanted into NOD/SCID/IL2Rg$^{-/-}$ mice. When the tumor volume in the PDX model mice reached 50 to 100 mm^3, the animals were intravenously injected with CTLA4-CAR T cells, GFP-CAR T cells, or non-transduced (2×10^5 cells). After 30 days, all the mice were sacrificed, and the tumors were assessed.

For autologous transplantation, 6- to 8-week-old CD45.2 B6 mice were used as recipients. Mice were radiated by 4.5 Gy and subcutaneously injected with B16F10 cells (2 × 10^5) with 10% Matrigel (20 μl Matrigel in 180 μl PBS) on day 0. A week after transplantation, the mice were randomly divided into three groups (five mice/group) and injected *via* the caudal vein with CTLA4-CAR T cells, GFP-CAR T cells (2 × 10^5 cells), or PBS, donor T cells were derived from CD45.1 B6 mice. Tumor volume was calculated every 7 days. After 35 days, all the mice were sacrificed, and the tumors were assessed. For long-term observation, the mice were raised until tumor volume exceeded 2,000 mm^3.

The severity of systemic graft-versus-host disease (GVHD) developed in the mice was assessed according to a mouse clinical GVHD scoring system as a previous report (26). Weight loss of <10% was scored 0, 10% to 25% was scored as 1, > 25% was scored as 2. For gastrointestinal symptoms, the scoring system

denoted 0 as normal and 1 as suffering from diarrhea. For posture and activity, the scoring system denoted 0 as normal, 1 for hunching at rest and a mild to moderate decrease in activity, and 2 for severe hunching and a severe decrease in activity. For fur texture and skin integrity, the scoring system denoted 0 as normal, 1 for mild to moderate fur ruffling and scaling of the paws and tails, and 2 for severe fur ruffling and an obviously denuded mouse. Each clinical GVHD score was measured twice a week.

For the CRS Model, 6- to 8-week-old female CB17.B6-Prkdc^{scid} Lyst^{bg}/Crl (SCID-beige) mice (Charles River) were intraperitoneally injected with 3 million Raji cells and tumors left to grow for 21 days. Mice were injected intraperitoneally with 30 million CAR T cells in PBS, weight change was measured every 8 h, the levels of the inflammatory cytokines were analyzed at 24 and 48 h, mice were euthanized and analyzed 3 days after T cell transplantation.

Immunohistochemical Staining and Analysis

Paraffin-embedded sections were deparaffinized and, after heat-mediated antigen retrieval, stained with an antibody against GFP protein (ab290, 1:500) overnight at 4°C. The sections were incubated with a peroxidase-labeled antibody at 37°C for 60 min. The slides were then stained with DAB and counterstained with hematoxylin. All slides were imaged with a microscope (DMI6000B; Leica Microsystems), results were analyzed using Image-Pro Plus 6.0 software.

Statistical Analysis

Samples and animals were random allocation and were unbiasedly included in the analysis unless specific mention. All cell culture experiments, real-time PCR, and ELISA were performed in triplicate at least three independent times. Statistical analysis to determine group differences was done by Student's t-test (two groups) or ANOVA analysis with Tukey's multiple comparison test (three or more groups) using GraphPad Prism, version 7.0 (GraphPad Software), all statistical analyses are described in the figure legends. All cell culture experiments were performed in triplicate at least three independent times. The p values are considered as follows: *p<0.05, **p<0.01, and ***p<0.001.

RESULTS

T Cells Transduced with the CTLA4-CD28-CD3z Chimeric Gene Showed Enhanced *In Vitro* Cytotoxicity

To alter the negative signals of CTLA4 in T cells, we attempted to convert the negative signals to the positive ones by linking the intracellular stimulatory domains from CD28 and CD3z with CTLA4 to generate a CTLA4-CD28-CD3z CAR (CTLA4-CAR). For the CTLA4-CAR plasmid, the extracellular and transmembrane domains from human CTLA4 were designed to be fused to the intracellular signaling domains from human CD28 and CD3z (**Figure 1A** and **Supplementary Figure 1A**). The corresponding cDNA was cloned into a lentiviral vector and transduced efficiently into activated human T cells, chimeric CTLA4 expression was analyzed in the GFP+ cell population. The transduction efficiencies of CTLA4-CAR were found to be approximately 50% to 60% (**Figure 1B** and **Supplementary Figure 1B**).

Studies have shown that the CD80 (B7.1) and CD86 (B7.2) ligands of CTLA4 are highly expressed in malignant B cells (27, 28). The expression of either CD80 or CD86 is likely to facilitate co-stimulation *via* CTLA4 chimeras. Therefore, we detected the surface expression levels of CD80 and CD86 in Pre-B acute lymphoblastic leukemia (ALL) (NALM6) and B cell lymphoma (Raji and RL) cell lines. Flow cytometry revealed that both CD80 and CD86 were highly expressed in Raji cells, CD86 was highly expressed in NALM6 and RL cells, which exhibited low levels of CD80 expression. Neither CD80 nor CD86 was expressed in an erythroid precursor cell line K562 cells (**Figures 1C, D**).

To determine whether CTLA4-chimera-transduced T cells (CTLA4-T) could specifically recognize and kill CD80-positive or CD86-positive tumor cells, we performed cytotoxicity assays by co-culturing CTLA4-T cells or genetically modified control T cells (GFP-T) with the tumor cells. CTLA4-T cells efficiently lysed CD80-positive or CD86-positive tumor cells (Raji, RL, and NALM6), but not CD80/CD86-negative K562 cells, whereas control effector cells (GFP-T) could not initiate specific lysis on either cell line (**Figure 1E** and **Supplementary Figure 1C**). In line with these observations, CTLA4-T cells secreted higher levels of IFN-γ and IL-2 than GFP-T cells when coculturing with Raji, RL, and NALM6 cells, no significant difference in cytokine secretion was observed in co-cultures of CAR-T cells and K562 cells (**Figures 1F, G**). These results suggested that modified CTLA4-T cells retained significant cytotoxic activities toward CD80/CD86-positive tumor cells specifically.

CAR T Cells Redirected to CD80/86 Significantly Suppress the Tumorigenesis of Subcutaneous Xenografts

To examine the *in vivo* anti-tumor activity of CTLA4-T cells toward CD80/CD86-expressing tumors, we developed subcutaneous xenograft models using NOD/SCID/*IL2Rg*$^{-/-}$ mice. Raji, RL, or NALM6 cells were subcutaneously implanted into the mice 2 days before initiating immunotherapy. Experimental mice received CTLA4-T cells, GFP-T cells, or non-transduced T cells (control group), and observation for 4 weeks. The potent anti-tumor effect was observed in mice treated with CTLA4-T cells, whereas GFP-T cells did not suppress tumor growth, at the end of the experiment, all mice treated with CTLA4-T cells had significantly decreased tumor volume and tumor weight (**Figures 2A–C**), whereas mice in GFP-T cells treated or the control groups developed large tumors.

The PDX model is an effective tool for preclinical research (29–32). To further assess the anti-tumor efficacy of CTLA4-T cells against primary tumors, we developed B cell lymphoma PDX mouse models by using primary tumor tissues with high CD80 and CD86 expression (**Figure 2D**). In line with the cell line

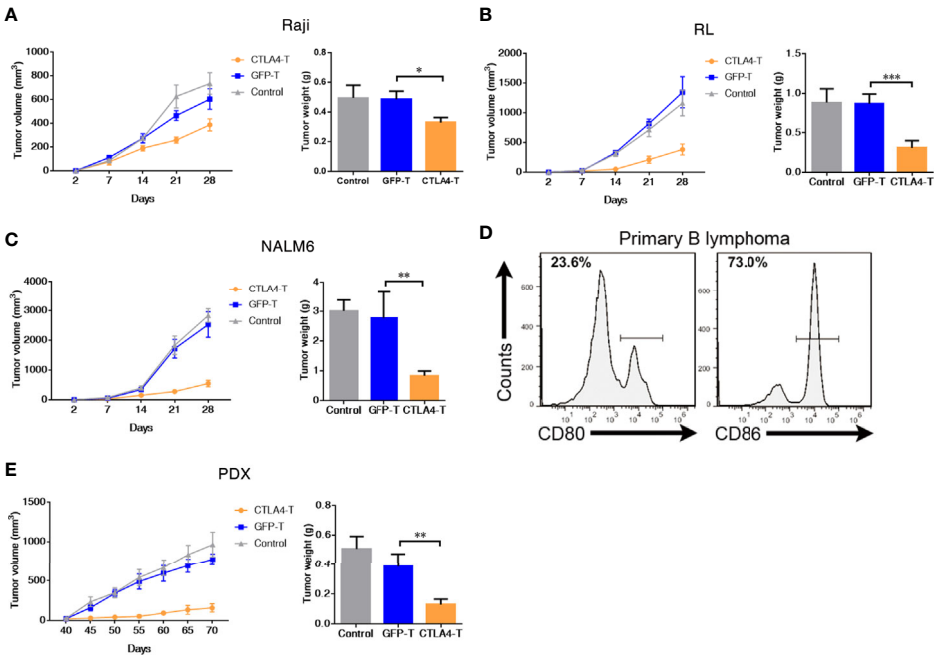

FIGURE 2 | CAR T cells redirected to CD80/86 significantly suppress the tumorigenesis of subcutaneous xenografts. **(A–C)** NOD/SCID/IL2Rg$^{-/-}$ mice were subcutaneously injected with 2 × 10^5 of Raji, RL, or NALM6 cells and were intravenously administered human T cells transduced with either chimeric CTLA4 or GFP. Blank control groups comprised mice intravenously administered non-transduced T cells (2 × 10^5 cells, five mice/group). The tumor weight of the Raji, RL, and NALM6 xenografts was weighed after 28 days. The tumor volumes in the CDX models were measured and calculated every 7 days. **(D)** Representative flow cytometric analyses of CD80 and CD86 expression in a xenograft comprising tumor cells from a B cell lymphoma patient. **(E)** NOD/SCID/IL2Rg$^{-/-}$ mice were subcutaneously transplanted with patient-derived xenografts (PDXs) of B cell lymphoma to create PDX mouse models, which were treated with CTLA4-T, GFP-T, or non-transduced T cells when the tumor volume reached 50 to 100 mm^3. The total number of GFP-positive T cells injected per mouse was 2 × 10^5. Tumors in the mice in all three groups (five mice/group) were weighed at the end of the experiment. The tumor volume was measured and calculated every 5 days. Data are shown as the mean ± SD from independent experiments. One-way ANOVA; significance values: *P < 0.05; **P < 0.01; ***P < 0.001.

experiments, the mice treated with CTLA4-T cells displayed more pronounced tumor regression than those treated with GFP-T cells or treated with PBS (**Figure 2E**). Taken together, these results suggested that CTLA4-T cells confer strong anti-tumor activities and could specifically suppress CD80/CD86-expressing tumors *in vivo*.

Murine CTLA4-Chimeric T Cells Are Toxic to MDSCs Within Tumors

We further investigated whether CTLA4-T cells targeting mouse CD80/CD86+ cells were safe and effective in an autologous transfer setting. We constructed a murine CTLA4-chimeric molecule, which comprised the extracellular and transmembrane domains of murine CTLA4, murine CD28 costimulatory domain, and murine CD3z signaling domain (**Figure 3A and Supplementary Figure 2A**). The transduction efficiencies of murine CTLA4-CAR were found to be approximately 55% (**Figure 3B**). Murine T cells expressing either the CTLA4 chimera or GFP were transplanted into C57BL/6J mice bearing B16F10 tumors. 4 weeks after transplantation, the mice were euthanized for analysis (**Figure 3C**). In autologous recipients, CTLA4-T cells significantly suppressed tumor growth (**Figure 3D**). Moreover, in peripheral blood the percentages of remaining CAR T cells in the CTLA4-T group were higher, compared to the GFP-T group (**Figure 3E**), suggesting that the converted CTLA4 signal improved the persistence of CAR T cells *in vivo*. At the experimental endpoint, mice treated with CTLA4-T cells had significantly decreased tumor weight (**Figure 3F**). The infiltration of T cells was validated in the tumor tissues, CTLA4-T cells were accumulated in residual tumors (**Figure 3G**). Also, fewer LAG3+TIM3+PD-1+ T cells were detected in the tumor tissue of the CTLA4-T group (**Figure 3G**, **Supplementary Figure 2B**). The expression of IL-2 and IFN-γ were up-regulated in the CTLA4-T cells (**Figure 3H**), which indicated advanced fitness of the CTLA4-T cells. Moreover, we found intratumoral infiltration of CTLA-4 T cells, whereas no specific infiltrated T cells were detected on staining in the sections of tumors treated with GFP-T cells (**Figure 3I**). CD4/CD8 ratio shifted after T cell transplantation, CD8+ CTLA4-T cells were growing faster *in vivo*, higher CD8+ T cell percentage was associated with higher anti-tumor efficacy (**Supplementary Figure 2C–D**).

Myeloid-derived suppressor cell (MDSC) is a population of immature myeloid cells with immune suppressive function, these cells are also expressing CD80 and CD86 (33, 34). We further explored the anti-MDSCs potential of CTLA4-T cells, both granulocytic MDSCs (G-MDSCs, CD11b+Ly6G+) and monocytic MDSCs (M-MDSCs, CD11b+Ly6C+) were

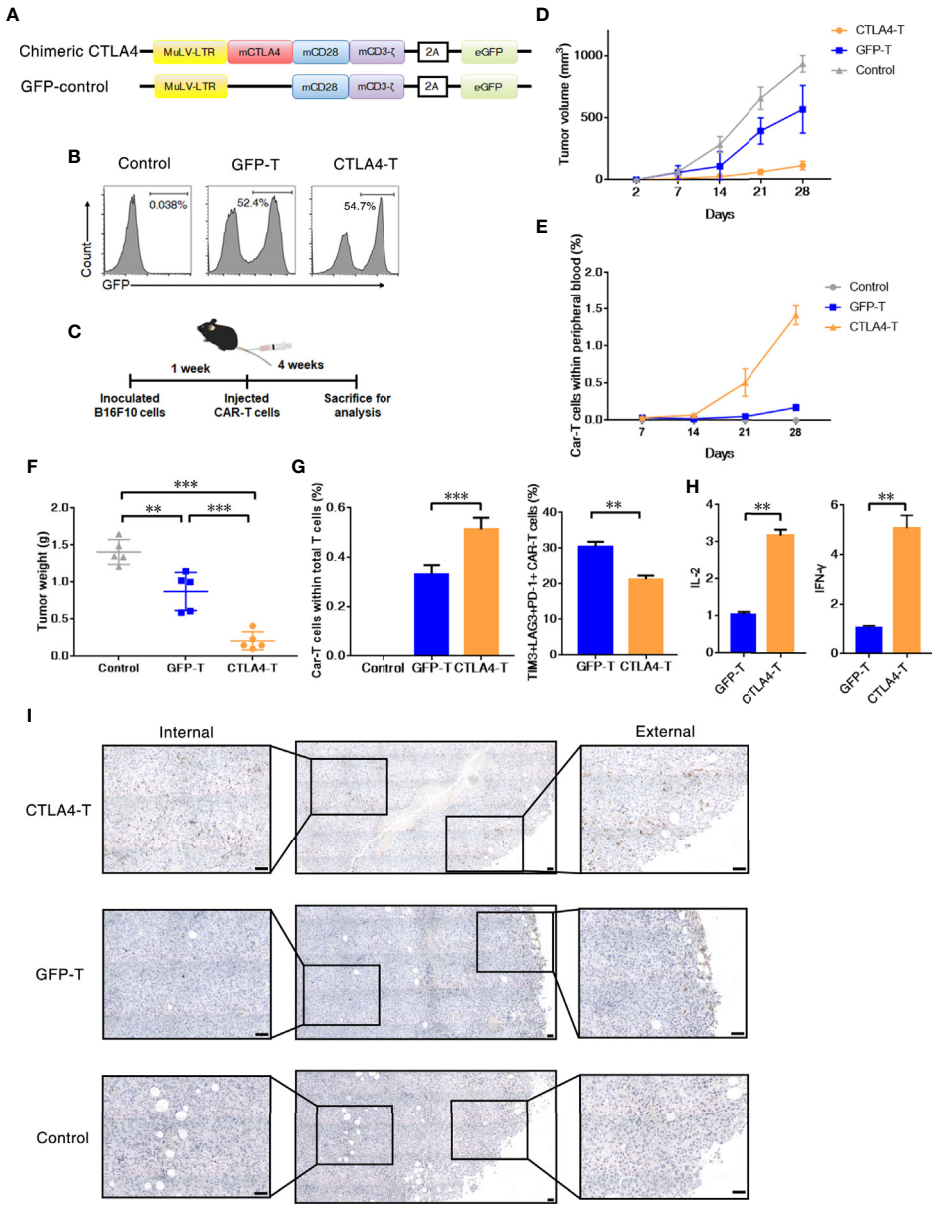

FIGURE 3 | T cells expressing the CTLA4-CD28-CD3z chimera had effective tumor infiltration. **(A)** Murine chimeric CTLA4 molecules contained the extracellular and transmembrane domains of mouse CTLA4, the cytoplasmic region of mouse CD28, and the intracellular domains of mouse CD3z. T cells expressing GFP were constructed as the control group. **(B)** Representative flow cytometric analysis of murine chimeric CTLA4 or GFP expression in mouse T cells. **(C)** Experimental scheme for evaluating murine CTLA4-CAR T cells efficacy, 2×10^5 of B16F10 cells were subcutaneously transplanted, and mice were intravenously administered T cells transduced with either chimeric CTLA4 or GFP or PBS (Control), five mice/group. **(D)** The tumor volumes in the mice were measured and calculated every 7 days. **(E)** The percentages of CAR T cells in peripheral blood of the mice were measured and calculated every 7 days. **(F)** The B16F10 tumor weight was weighed after 35 days, mean ± SD, one-way ANOVA. **(G)** The percentages of CAR T cells in total infiltrated T cells within the tumor tissues, and the percentages of TIM3+LAG+PD-1+ CAR T cells, mean ± SD, one-way ANOVA. **(H)** qRT-PCR analysis of the mRNA expression of the indicated genes. The results were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels and are presented as the mean ± SEM (n = 3), unpaired two-tailed t-test. **(I)** Immunohistochemical staining identified the infiltrated CAR T cells in resected tumors, GFP+ cells were stained. Significance values: $^{**}P < 0.01$; $^{***}P < 0.001$.

decreased within tumor tissues following CTLA4-T cells administration (**Figure 4A**). CD80/CD86-positive MDSCs were targeted by CTLA4-T cells (**Figure 4B**). These observations indicate that targeting MDSCs is responsible for some, if not all, of the enhanced intratumoral infiltration and anti-tumor activity of CTLA4-T cells.

Although no significant weight loss was observed in autologous recipients (**Figure 4C**), we found the T cell

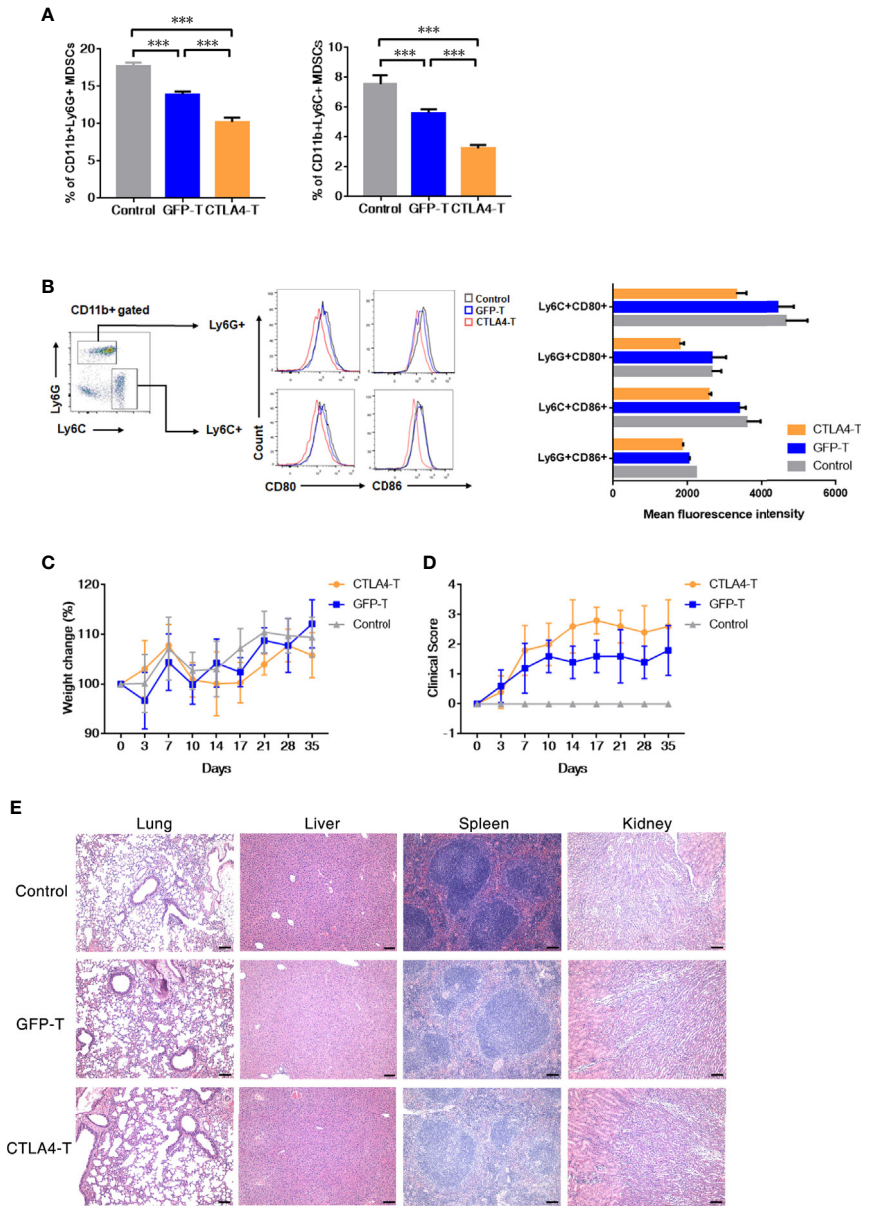

FIGURE 4 | Murine CTLA4-chimeric T cells show toxicity against MDSCs. **(A)** Statistical analysis of MDSCs percentage with tumors by flow cytometry, mean ± SD, one-way ANOVA. **(B)** Representative flow cytometric analysis of the expression of CD80 and CD86 on MDSCs after CTLA4-CAR T cell therapy. **(C)** Weight change of autologous mice (n = 5) after T cell transfer. **(D)** Mice were monitored for GVHD pathology score twice a week. **(E)** H&E staining of organs, scale bar, 100 μm. Significance values: ***$P < 0.001$.

inoculated mice showed signs of few Graft-versus-host disease (GVHD). Using the GVHD clinical scoring system, the clinical status of the T cell inoculated mice were scored. Assessing the clinical GVHD manifestations, the mice with CTLA4-T cells transfusion showed a mild hunching posture and a ruffling fur texture, suggesting the occurrence of GVHD (**Figure 4D**). The histopathology of lung, liver, spleen, and kidney tissue was assessed, no significant morphological changes were observed (**Figure 4E**).

To evaluate the potential for CTLA4-T cells to elicit the cytokine release syndrome (CRS) in mice, we established high tumor burden xenograft models as reported in prior studies (35–37). We found that human CTLA4-T cells elicited acute inflammatory responses associated with piloerection, malaise and, weight loss in recipients (**Figure 5A**). No death due to CRS happened in mice infused with CTLA4-T cells (**Figure 5B**), though high levels of mouse IL-6, an indicator of CRS (38), were detected with CTLA4-T cells and GFP-T cells (**Figure 5C**). The

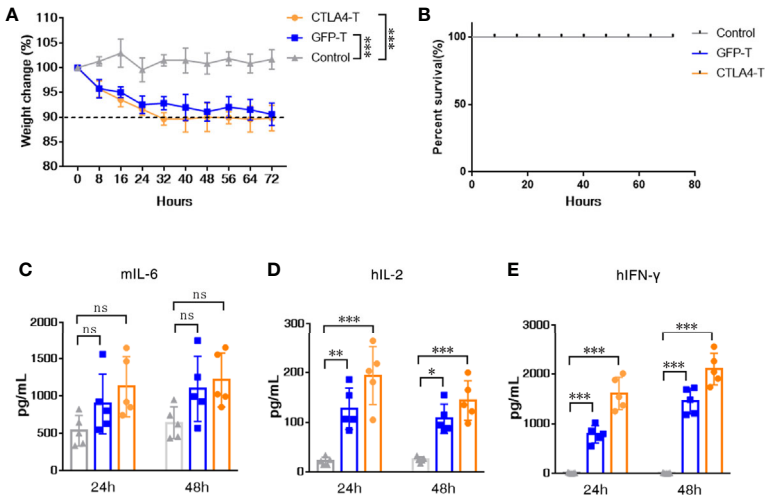

FIGURE 5 | CTLA4-chimeric T cells elicited mild CRS *in vivo*. **(A)** Weight change of tumor-bearing mice (n = 5) after T cell transfer, two-way ANOVA with Tukey's multiple comparison test. **(B)** Percent survival of mice after CAR T cell transfer (n=5 mice for each group). **(C–E)** Serum levels of mIL-6, hIL-2, and hIFN-γ 24 and 48 h post CAR T cell transfer were measured by ELISA. Significance values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, non-significance.

levels of the inflammatory cytokines hIL-2 and hIFN-γ in the serum were higher in the mice with CTLA4-T cells transfusion (**Figures 5D, E**). Taken together, these results suggest that CTLA4-T cells may have the potential to elicit CRS or GVHD.

DISCUSSION

The expression of CD80/CD86 on tumor cells and immunosuppressive cells with the TME, such as MDSCs, makes the CTLA4-CD80/CD86 axis a remarkable target for cancer immunotherapies. In this study, we generated CD80/CD86-targeted CAR T cells to destroy CD80/CD86-associated tumor cells in culture and in the tumor xenograft mouse models. Our findings revealed that the cytotoxic activities of CTLA4-T cells against tumor cells were associated with their targeting specificity. CTLA4-T cells had enhanced abilities of TME infiltration, tumor suppression, and targeted MDSCs in tumor xenograft mouse models. These results rationalized the extension of our CD80/CD86-targeted CAR T cell-based immunotherapy for human malignant B cell lymphoma treatment.

CD80 and CD86 are highly expressed in approximately two-thirds of a cohort of 70 malignant B cell samples from patients with non-Hodgkin lymphoma. Thus, CTLA4-chimeric T cell therapy could be a promising approach to treat B cell malignancies. In clinical reports, B cell lymphoma patients who have received standard therapy may suffer from a relapse concomitant with increased expression of the costimulatory molecule CD86 (28). Currently, Patel et al. observed the expansion of CTLA4+PD-1− T cells close to CD86+ tumor cells and tumor-associated macrophages (39), which suggests that the interaction between CD86 and CTLA4 might be a key negative regulator in Hodgkin lymphoma. However, CTLA4 blockade using ipilimumab (anti-CTLA4) failed to show clinical benefits in high-risk cancer patients (40).

Immunotherapy using CAR T cells is in its infancy, its efficacy against leukemia has been widely recognized (41). However, clinical success using CAR T cells for the treatment of solid tumors was still limited. Clinical trials based on CAR targeting single antigens, such as human epidermal growth factor receptor 2 (HER2) (42), mesothelin (MSLN) (43), prostate stem cell antigen (PSCA) (44) are found mixed results due to the loss of tumor antigen on tumor cells, which confirms a fundamental issue with CAR T cell therapies: a lack of ideal single antigen targets. Another major barrier for CAR T cell therapies in solid tumors is the immunosuppressive TME (45). Thus, reports recommended that combining CAR T cell therapies with immune checkpoint inhibitors, such as CTLA4 and PD-1 inhibitors. Whereas our strategy of utilizing CTLA4-targeted CAR T cells has the advantages of not only suppressing cancer cells but also blocking immune checkpoint. For other CD80/CD86-negative solid tumors, it is worthy of consideration to utilize CAR construct targeting the tumor-associated antigens as well as converting CTLA4 signals. Besides, adoptive transfer of CTLA4-chimeric CAR T cells can avoid the loss of the negative regulation of the entire body's immune system caused by anti-CTLA4 antibodies (25). This response may reduce the risk of developing an autoimmune disease for tumor patients (46, 47). Therefore, treating with CTLA4-CAR T cells as an alternative therapeutic regimen may be a promising option for these patients.

Our study showed the effects of CTLA4-CAR T cells on MDSCs cytotoxicity. Although it shed light on the strategy of using armed CAR T cells to target immunomodulatory TME, it also indicated that CTLA4-CAR T cells showed toxicity against non-malignant CD80/CD86 expressing cells. We observed mild GVHD in autologous recipients of CTLA4-T cells, high tumor

burden xenograft models of CAR T cell-induced CRS suggested that CTLA4-T cells have the potential to elicit CRS. Therefore, CTLA4-T cells may present a risk for clinical development even in the autologous setting. Additional preclinical and clinical studies would be needed to validate the safety of CTLA4-T cells.

The current proof-of-concept study provides support of using armed CAR T cells for targeting immunomodulatory TME not only for CD80/CD86-CTLA4 axis but also for PD-1/PD-L1 (48), TIM3 (49), LAG3 (50). Our results indicate that converting the negative CTLA4-CD86/CD86 signal leads to improved antitumor activity without eliciting severe GVHD and CRS, suggesting that CTLA4-CAR could be employed to improve CAR T cell efficacy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences.

AUTHOR CONTRIBUTIONS

SHL, LC, and WY contributed to the conception and design, the collection and/or assembly of data, data analysis and interpretation, and manuscript writing. SLL, DZ, GH, and LQ contributed to the provision of study material or patient samples and the collection and/or assembly of data. QW, YL, SiL, and SW provided animal care and administrative support. PL, YY, and XS contributed to the conception and design of the study, data analysis and interpretation, manuscript writing, and the final approval of the manuscript and provided financial support. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.642528/full#supplementary-material

REFERENCES

1. Yokosuka T, Takamatsu M, Kobayashi-Imanishi W, Hashimoto-Tane A, Azuma M, Saito T. Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. *J Exp Med* (2012) 209(6):1201–17. doi: 10.1084/jem.20112741
2. Nagai S, Azuma M. The CD28-B7 Family of Co-signaling Molecules. *Adv Exp Med Biol* (2019) 1189:25–51. doi: 10.1007/978-981-32-9717-3_2
3. Shin DS, Ribas A. The evolution of checkpoint blockade as a cancer therapy: what's here, what's next? *Curr Opin Immunol* (2015) 33:23–35. doi: 10.1016/j.coi.2015.01.006
4. Dai ZS, Chen QF, Lu HZ, Xie Y. Defective expression and modulation of B7-2/CD86 on B cells in B cell chronic lymphocytic leukemia. *Int J Hematol* (2009) 89(5):656–63. doi: 10.1007/s12185-009-0320-7
5. Chikuma S. CTLA-4, an Essential Immune-Checkpoint for T-Cell Activation. *Curr Top Microbiol Immunol* (2017) 410:99–126. doi: 10.1007/82_2017_61
6. Zarour HM. Reversing T-cell Dysfunction and Exhaustion in Cancer. *Clin Cancer Res* (2016) 22(8):1856–64. doi: 10.1158/1078-0432.CCR-15-1849
7. Calabro L, Morra A, Giannarelli D, Amato G, D'Incecco A, Covre A, et al. Tremelimumab combined with durvalumab in patients with mesothelioma (NIBIT-MESO-1): an open-label, non-randomised, phase 2 study. *Lancet Respir Med* (2018) 6(6):451–60. doi: 10.1016/S2213-2600(18)30151-6
8. Duffy AG, Ulahannan SV, Makorova-Rusher O, Rahma O, Wedemeyer H, Pratt D, et al. Tremelimumab in combination with ablation in patients with advanced hepatocellular carcinoma. *J Hepatol* (2017) 66(3):545–51. doi: 10.1016/j.jhep.2016.10.029
9. Ji D, Song C, Li Y, Xia J, Wu Y, Jia J, et al. Combination of radiotherapy and suppression of Tregs enhances abscopal antitumor effect and inhibits metastasis in rectal cancer. *J Immunother Cancer* (2020) 8(2):e000826. doi: 10.1136/jitc-2020-000826
10. van Dijk N, Gil-Jimenez A, Silina K, Hendricksen K, Smit LA, de Feijter JM, et al. Preoperative ipilimumab plus nivolumab in locoregionally advanced

urothelial cancer: the NABUCCO trial. *Nat Med* (2020) 26(12):1839–44. doi: 10.1038/s41591-020-1085-z
11. Somalwar AR, Shelkar GP, Subhedar NK, Kokare DM. The role of neuropeptide CART in the lateral hypothalamic-ventral tegmental area (LH-VTA) circuit in motivation. *Behav Brain Res* (2017) 317:340–9. doi: 10.1016/j.bbr.2016.09.054
12. Mikkilineni L, Kochenderfer JN. Chimeric Antigen Receptor T-cell Therapies for Multiple Myeloma. *Blood* (2017) 130(24):2594–602. doi: 10.1182/blood-2017-06-793869
13. Wei X, Lai Y, Li J, Qin L, Xu Y, Zhao R, et al. PSCA and MUC1 in non-small-cell lung cancer as targets of chimeric antigen receptor T cells. *Oncoimmunology* (2017) 6(3):e1284722. doi: 10.1080/2162402X.2017.1284722
14. Algarra I, Cabrera T, Garrido F. The HLA crossroad in tumor immunology. *Hum Immunol* (2000) 61(1):65–73. doi: 10.1016/S0198-8859(99)00156-1
15. Sadelain M, Brentjens R, Riviere I. The basic principles of chimeric antigen receptor design. *Cancer Discov* (2013) 3(4):388–98. doi: 10.1158/2159-8290.CD-12-0548
16. Long AH, Haso WM, Shern JF, Wanhainen KM, Murgai M, Ingaramo M, et al. 4-1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors. *Nat Med* (2015) 21(6):581–90. doi: 10.1038/nm.3838
17. Kowolik CM, Topp MS, Gonzalez S, Pfeiffer T, Olivares S, Gonzalez N, et al. CD28 costimulation provided through a CD19-specific chimeric antigen receptor enhances in vivo persistence and antitumor efficacy of adoptively transferred T cells. *Cancer Res* (2006) 66(22):10995–1004. doi: 10.1158/0008-5472.CAN-06-0160
18. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LAJr, Kinzler KW. Cancer genome landscapes. *Science* (2013) 339(6127):1546–58. doi: 10.1126/science.1235122
19. Gubin MM, Artyomov MN, Mardis ER, Schreiber RD. Tumor neoantigens: building a framework for personalized cancer immunotherapy. *J Clin Invest* (2015) 125(9):3413–21. doi: 10.1172/JCI80008
20. Alcantara M, Du Rusquec P, Romano E. Current Clinical Evidence and Potential Solutions to Increase Benefit of CAR T-Cell Therapy for Patients with Solid Tumors. *Oncoimmunology* (2020) 9(1):1777064. doi: 10.1080/2162402X.2020.1777064
21. Gulley JL, Madan RA, Pachynski R, Mulders P, Sheikh NA, Trager J, et al. Role of Antigen Spread and Distinctive Characteristics of Immunotherapy in Cancer Treatment. *J Natl Cancer Inst* (2017) 109(4):djw261. doi: 10.1093/jnci/djw261
22. Li J, Kan H, Zhao L, Sun Z, Bai C. Immune checkpoint inhibitors in advanced or metastatic mucosal melanoma: a systematic review. *Ther Adv Med Oncol* (2020) 12:1758835920922028. doi: 10.1177/1758835920922028
23. Witkowska M, Smolewski P. Immune Checkpoint Inhibitors to Treat Malignant Lymphomas. *J Immunol Res* (2018) 2018:1982423. doi: 10.1155/2018/1982423
24. Park HB, Lee JE, Oh YM, Lee SJ, Eom HS, Choi K. CTLA4-CD28 chimera gene modification of T cells enhances the therapeutic efficacy of donor lymphocyte infusion for hematological malignancy. *Exp Mol Med* (2017) 49 (7):e360. doi: 10.1038/emm.2017.104
25. Shin JH, Park HB, Oh YM, Lim DP, Lee JE, Seo HH, et al. Positive conversion of negative signaling of CTLA4 potentiates antitumor efficacy of adoptive T-cell therapy in murine tumor models. *Blood* (2012) 119(24):5678–87. doi: 10.1182/blood-2011-09-380519
26. Lai HY, Chou TY, Tzeng CH, Lee OKS. Cytokine Profiles in Various Graft-Versus-Host Disease Target Organs Following Hematopoietic Stem Cell Transplantation. *Cell Transplant* (2012) 21(9):2033–45. doi: 10.3727/096368912X653110
27. Yang ZZ, Novak AJ, Ziesmer SC, Witzig TE, Ansell SM. Malignant B cells skew the balance of regulatory T cells and TH17 cells in B-cell non-Hodgkin's lymphoma. *Cancer Res* (2009) 69(13):5522–30. doi: 10.1158/0008-5472.CAN-09-0266
28. Mansour A, Elkhodary T, Darwish A, Mabed M. Increased expression of costimulatory molecules CD86 and sCTLA-4 in patients with acute lymphoblastic leukemia. *Leuk Lymphoma* (2014) 55(9):2120–4. doi: 10.3109/10428194.2013.869328
29. Lai Y, Wei X, Lin S, Qin L, Cheng L, Li P. Current status and perspectives of patient-derived xenograft models in cancer research. *J Hematol Oncol* (2017) 10(1):106. doi: 10.1186/s13045-017-0470-7
30. Lin S, Huang G, Cheng L, Li Z, Xiao Y, Deng Q, et al. Establishment of peripheral blood mononuclear cell-derived humanized lung cancer mouse models for studying efficacy of PD-L1/PD-1 targeted immunotherapy. *MAbs* (2018) 10(8):1301–11. doi: 10.1080/19420862.2018.1518948
31. Lin S, Huang G, Xiao Y, Sun W, Jiang Y, Deng Q, et al. CD215+ Myeloid Cells Respond to Interleukin 15 Stimulation and Promote Tumor Progression. *Front Immunol* (2017) 8:1713:1713. doi: 10.3389/fimmu.2017.01713
32. Lin S, Zhang X, Huang G, Cheng L, Lv J, Zheng D, et al. Myeloid-derived suppressor cells promote lung cancer metastasis by CCL11 to activate ERK and AKT signaling and induce epithelial-mesenchymal transition in tumor cells. *Oncogene* (2021) 40(8):1476–89. doi: 10.1038/s41388-020-01605-4
33. Dugast AS, Haudebourg T, Coulon F, Heslan M, Haspot F, Poirier N, et al. Myeloid-derived suppressor cells accumulate in kidney allograft tolerance and specifically suppress effector T cell expansion. *J Immunol* (2008) 180 (12):7898–906. doi: 10.4049/jimmunol.180.12.7898
34. Liu Y, Zeng B, Zhang Z, Zhang Y, Yang R. B7-H1 on myeloid-derived suppressor cells in immune suppression by a mouse model of ovarian cancer. *Clin Immunol* (2008) 129(3):471–81. doi: 10.1016/j.clim.2008.07.030
35. Giavridis T, van der Stegen SJC, Eyquem J, Hamieh M, Piersigilli A, Sadelain M. CAR T cell-induced cytokine release syndrome is mediated by macrophages and abated by IL-1 blockade. *Nat Med* (2018) 24(6):731–8. doi: 10.1038/s41591-018-0041-7
36. Mestermann K, Giavridis T, Weber J, Rydzek J, Frenz S, Nerreter T, et al. The tyrosine kinase inhibitor dasatinib acts as a pharmacologic on/off switch for CAR T cells. *Sci Trans Med* (2019) 11(499):eaau5907. doi: 10.1126/scitranslmed.aau5907
37. Norelli M, Camisa B, Barbiera G, Falcone L, Purevdorj A, Genua M, et al. Monocyte-derived IL-1 and IL-6 are differentially required for cytokine-release syndrome and neurotoxicity due to CAR T cells. *Nat Med* (2018) 24 (6):739–48. doi: 10.1038/s41591-018-0036-4
38. Davila ML, Riviere I, Wang XY, Bartido S, Park J, Curran K, et al. Efficacy and Toxicity Management of 19-28z CAR T Cell Therapy in B Cell Acute Lymphoblastic Leukemia. *Sci Trans Med* (2014) 6(224):224ra25. doi: 10.1126/scitranslmed.3008226
39. Patel SS, Weirather JL, Lipschitz M, Lako A, Chen PH, Griffin GK, et al. The microenvironmental niche in classic Hodgkin lymphoma is enriched for CTLA-4-positive T cells that are PD-1-negative. *Blood* (2019) 134 (23):2059–69. doi: 10.1182/blood.2019002206
40. Zeidan AM, Knaus HA, Robinson TM, Towlerton AMH, Warren EH, Zeidner JF, et al. A Multi-center Phase I Trial of Ipilimumab in Patients with Myelodysplastic Syndromes following Hypomethylating Agent Failure. *Clin Cancer Res* (2018) 24(15):3519–27. doi: 10.1158/1078-0432.CCR-17-3763
41. Fesnak AD, June CH, Levine BL. Engineered T cells: the promise and challenges of cancer immunotherapy. *Nat Rev Cancer* (2016) 16(9):566–81. doi: 10.1038/nrc.2016.97
42. Feng K, Liu Y, Guo Y, Qiu J, Wu Z, Dai H, et al. Phase I study of chimeric antigen receptor modified T cells in treating HER2-positive advanced biliary tract cancers and pancreatic cancers. *Protein Cell* (2018) 9(10):838–47. doi: 10.1007/s13238-017-0440-4
43. Beatty GL, O'Hara MH, Lacey SF, Torigian DA, Nazimuddin F, Chen F, et al. Activity of Mesothelin-Specific Chimeric Antigen Receptor T Cells Against Pancreatic Carcinoma Metastases in a Phase 1 Trial. *Gastroenterology* (2018) 155(1):29–32. doi: 10.1053/j.gastro.2018.03.029
44. Abate-Daga D, Lagisetty KH, Tran E, Zheng Z, Gattinoni L, Yu Z, et al. A novel chimeric antigen receptor against prostate stem cell antigen mediates tumor destruction in a humanized mouse model of pancreatic cancer. *Hum Gene Ther* (2014) 25(12):1003–12. doi: 10.1089/hum.2013.209
45. Oliver AJ, Lau PKH, Unsworth AS, Loi S, Darcy PK, Kershaw MH, et al. Tissue-Dependent Tumor Microenvironments and Their Impact on Immunotherapy Responses. *Front Immunol* (2018) 9:70. doi: 10.3389/fimmu.2018.00070
46. Geraud A, Gougis P, Vozy A, Anquetil C, Allenbach Y, Romano E, et al. Clinical Pharmacology and Interplay of Immune Checkpoint Agents: A Yin-Yang Balance. *Annu Rev Pharmacol Toxicol* (2020) 61:85–112. doi: 10.1146/annurev-pharmtox-022820-093805
47. Kahler KC, Hassel JC, Heinzerling L, Loquai C, Thoms KM, Ugurel S, et al. Side effect management during immune checkpoint blockade using CTLA-4 and PD-1 antibodies for metastatic melanoma - an update. *J Dtsch Dermatol Ges* (2020) 18(6):582–609. doi: 10.1111/ddg.14128

48. Yang CY, Fan MH, Miao CH, Liao YJ, Yuan RH, Liu CL. Engineering Chimeric Antigen Receptor T Cells against Immune Checkpoint Inhibitors PD-1/PD-L1 for Treating Pancreatic Cancer. *Mol Ther Oncolytics* (2020) 17:571–85. doi: 10.1016/j.omto.2020.05.009
49. Zou F, Lu L, Liu J, Xia B, Zhang W, Hu Q, et al. Engineered triple inhibitory receptor resistance improves anti-tumor CAR-T cell performance via CD56. *Nat Commun* (2019) 10(1):4109. doi: 10.1038/s41467-019-11893-4
50. Zhang Y, Zhang X, Cheng C, Mu W, Liu X, Li N, et al. CRISPR-Cas9 mediated LAG-3 disruption in CAR-T cells. *Front Med* (2017) 11(4):554–62. doi: 10.1007/s11684-017-0543-6

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CTLA-4 Synergizes With PD1/PD-L1 in the Inhibitory Tumor Microenvironment of Intrahepatic Cholangiocarcinoma

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Intrahepatic cholangiocarcinoma (ICC) is highly invasive and carries high mortality due to limited therapeutic strategies. In other solid tumors, immune checkpoint inhibitors (ICIs) target cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and programmed death 1 (PD1), and the PD1 ligand PD-L1 has revolutionized treatment and improved outcomes. However, the relationship and clinical significance of CTLA-4 and PD-L1 expression in ICC remains to be addressed. Deciphering CTLA-4 and PD-L1 interactions in ICC enable targeted therapy for this disease. In this study, immunohistochemistry (IHC) was used to detect and quantify CTLA-4, forkhead box protein P3 (FOXP3), and PD-L1 in samples from 290 patients with ICC. The prognostic capabilities of CTLA-4, FOXP3, and PD-L1 expression in ICC were investigated with the Kaplan–Meier method. Independent risk factors related to ICC survival and recurrence were assessed by the Cox proportional hazards models. Here, we identified that CTLA-4$^+$ lymphocyte density was elevated in ICC tumors compared with peritumoral hepatic tissues (P <.001), and patients with a high density of CTLA-4$^+$ tumor-infiltrating lymphocytes (TILs$^{CTLA\text{-}4\ High}$) showed a reduced overall survival (OS) rate and increased cumulative recurrence rate compared with patients with TILs$^{CTLA\text{-}4\ Low}$ (P <.001 and P = .024, respectively). Similarly, patients with high FOXP3$^+$ TILs (TILs$^{FOXP3\ High}$) had poorer prognoses than patients with low FOXP3$^+$ TILs (P = .021, P = .034, respectively), and the density of CTLA-4$^+$ TILs was positively correlated with FOXP3$^+$ TILs (Pearson r = .31, P <.001). Furthermore, patients with high PD-L1 expression in tumors (Tumor$^{PD\text{-}L1\ High}$) and/or TILs$^{CTLA\text{-}4\ High}$ presented worse OS and a higher recurrence rate than patients with TILs$^{CTLA\text{-}4\ Low}$Tumor$^{PD\text{-}L1\ Low}$. Moreover, multiple tumors, lymph node metastasis, and high Tumor$^{PD\text{-}L1}$/TILs$^{CTLA\text{-}4}$ were

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independent risk factors of cumulative recurrence and OS for patients after ICC tumor resection. Furthermore, among ICC patients, those with hepatolithiasis had a higher expression of CTLA-4 and worse OS compared with patients with HBV infection or undefined risk factors ($P = .018$). In conclusion, CTLA-4 is increased in TILs in ICC and has an expression profile distinct from PD1/PD-L1. $Tumor^{PD\text{-}L1}/TILs^{CTLA\text{-}4}$ is a predictive factor of OS and ICC recurrence, suggesting that combined therapy targeting PD1/PD-L1 and CTLA-4 may be useful in treating patients with ICC.

Keywords: intrahepatic cholangiocarcinoma, cytotoxic T-lymphocyte-associated antigen-4, programmed death ligand-1, prognosis, hepatolithiasis

INTRODUCTION

Evasion of immune destruction is a hallmark of cancer and results in immune tolerance (1). Immune tolerance can be mediated through multiple pathways, including the immune checkpoint receptors cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD1) (2). A costimulatory signal exerted by CD28:B7 binding is necessary for T cell maturation; CTLA-4 belongs to the CD28 family of immunoglobulins and competitively binds to B7 to produce inhibitory signals that counteract stimulatory signals from CD28:B7 and TCR: MHC binding (3). CTLA-4: B7-1/2 (CD80/B7-1 and CD86/B7-2) binding suppresses several signaling cascades in T cells, including differentiation, proliferation, and survival through inhibits IL-2 accumulation and cell cycle progression etc. (4, 5). CTLA-4 maintains peripheral tolerance by enhancing regulatory T cell (Treg) functions; thus, undirected control of the effector T cells (6) and overexpression of CTLA-4 in tumor samples implicates poor prognosis in patients with melanoma (7). Because CTLA-4 inhibition results in increased activation of the immune system, Ipilimumab, an inhibitor of CTLA-4, was approved for the treatment of advanced or unresectable melanoma (8).

PD1 regulates the activation of T cells by binding to programmed death-ligand 1/2 (PD-L1/2). Activation of the PD1/PD-L1/L2 pathway inhibits T cell proliferation and secretion of interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and IL-2, sustaining the immune inhibitory state of the tumor microenvironment (9). Clinical evidence supports aberrant PD-L1 expression in tumor cells, which aids in their escape from T cell immune attack in non-small-cell lung cancer (NSCLC), renal cell carcinoma, Hodgkin's lymphoma, hepatocellular carcinoma (HCC), and intrahepatic cholangiocarcinoma (ICC) (10). Based on these findings, immune checkpoint inhibitors (ICIs) targeting PD1/PD-L1 are approved or being evaluated as a malignant tumor treatment in various tumors (11).

Although CTLA-4 and PD1/PD-L1 exert similar negative effects on T cell activity, their timing and mechanisms differ. CTLA-4 acts in the initial stage of the immune response, typically in lymph nodes, and the PD1/PD-L1 pathway regulates previously activated T cells at later stages primarily in peripheral tissues (12). Recent evidence reveals interactions between PD1/PD-L1 and CTLA-4 signals. For example, the tumor cell glycolytic rate is depressed by anti-PD-L1 therapy, and patients with low glucometabolic levels in tumors may benefit from CTLA-4 blockade (13, 14). [A] combination of anti-CTLA-4 and anti-PD1/PD-L1 therapies may have an additive or synergistic effect in the treatment of advanced malignancies. Preliminary results of a clinical study report that the combination of the anti-CTLA-4 antibody ipilimumab and anti-PD1 antibody nivolumab elevated the objective response rate (ORR) and the progression-free survival of patients with $BRAF^{WT}$ metastatic or unresectable melanoma (15).

ICC accounts for 10%–15% of primary liver cancer cases, but its incidence has rapidly increased worldwide, and major ICC risk factors are hepatitis virus B (HBV) and C (HCV) infection along with hepatolithiasis (16, 17). ICC has a poor prognosis owing to local invasion and distal metastasis at first diagnosis (18). First-line therapy for ICC is gemcitabine-based chemotherapy as it is for other advanced biliary tract tumors, but even with treatment, the median overall survival (OS) is 11.7 months (19). Our previous study revealed elevated PD1/PD-L1 signals in tumor samples and distinct profiles of PD-1/PD-L1 in ICC patients with different risk factors (20), and the PD1 inhibitor Toripalimab, in combination with GEMOX (oxaliplatin and gemcitabine) chemotherapy and Lenvatinib, showed an ORR of 80% (24/30) and a 93.3% (28/30) disease control rate in treating advanced ICC (21). On the other hand, CTLA-4 expression and its relationship with tumor-infiltrating Tregs has not been characterized in ICC, and little is known about CTLA-4 and PD1/PD-L1 expression and interaction in ICC. This information could guide both diagnosis and treatment. To address this knowledge gap, we investigated the expression and interaction of CTLA-4 and PD1/PD-L1 in ICC and assessed their value as prognostic indicators in ICC.

Abbreviations: ICC, intrahepatic cholangiocarcinoma; HCC, hepatocellular carcinoma; ICIs, immune checkpoint inhibitors; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4; FOXP3, forkhead box protein P3; PD1, programmed death 1; PD-L1, programmed death-ligand L1; $TILs^{CTLA\text{-}4\ High}$, high density of $CTLA\text{-}4^+$ tumor-infiltrating lymphocytes; $TILs^{CTLA\text{-}4\ Low}$, low density of $CTLA\text{-}4^+$ tumor-infiltrating lymphocytes; $TILs^{FOXP3\ High}$, high density of $FOXP3^+$ tumor-infiltrating lymphocytes; $TILs^{FOXP3\ Low}$, low density of $FOXP3^+$ tumor-infiltrating lymphocytes; $Tumor^{PD\text{-}L1\ High}$, high PD-L1 expression in tumors; $Tumor^{PD\text{-}L1\ Low}$, low PD-L1 expression in tumors; TMA, tissue microarray; TME, tumor microenvironment.

MATERIALS AND METHODS

Patients and Clinical Samples

Study participants consisted of 290 patients with ICC who underwent curative resection between May 2002 and December 2011 at Zhongshan Hospital, Fudan University. Enrolled patients met the following criteria: (1) pathologically confirmed ICC; (2) ≥3 months of disease-free survival (DFS) after resection; (3) had not undergone antitumor treatment before surgery; and (4) had complete medical records and follow-up data available. Patients were stratified by a tumor-node-metastases (TNM) stage system according to the American Joint Committee on Cancer (AJCC) 8th edition (22). The histological grade of ICC was based on World Health Organization criteria (23). Tumor samples and adjacent liver tissue samples were collected, formalin-fixed, and paraffin-embedded. The last follow-up was on April 30, 2016. The study was approved by the institutional review board of Zhongshan Hospital (Y2017-130), and all related procedures conformed to the Declaration of Helsinki.

Tissue Microarrays and Immunohistochemistry

We previously described methods for the construction of tissue microarrays (TMAs) and immunohistochemistry (IHC) (24). Briefly, antihuman rabbit monoclonal antibodies for FOXP3 (1:50; #98377S, CST, Massachusetts, USA) and antihuman mouse monoclonal antibodies for CTLA-4 (1:100; #ab19792, Abcam, Cambridge, UK) were used as primary antibodies to detect the expression of FOXP3 and CTLA-4. An automated digital pathological slice scanner, KF-PRO-120 (KONFOONG Biotech International Co. Ltd., Ningbo, China), was used to scan images of IHC slides, and slides were photographed by digital slices view software K-Viewer (KONFOONG). IHC for PD-L1 was performed as described (20).

Evaluation of CTLA-4 and FOXP3 Expression

The previous study in extrahepatic bile duct cancer revealed CTLA-4 expressed on both tumor cells and TILs (25); here, we found that CTLA-4 positively stained both tumor cells and interstitial cells as well, and CTLA-4$^+$ TILs were distinguished by their topographic localization, cell nucleus volume, and other morphological characteristics. Two independent pathologists evaluated the expression of CTLA-4, and FOXP3 as a marker of Tregs was also evaluated to reveal the relationship between CTLA-4 and tumor-infiltrating Tregs (26). Lymphocytes with positive staining for CTLA-4 and FOXP3 were manually counted in five high-power fields that were randomly selected under 200× magnification for each TMA core, and the mean density (the number of positively stained cells per field) was determined to represent the expression level for each patient. Positive expression of CTLA-4 in tumor cells was scored 0–5 (0, <5% of the tissue section; 1, 5%–40%; 2, 40%–75%; 3, 75%–85%; 4, 85%–95%; 5, ≥95%). The median number of CTLA-4 and FOXP3-positive infiltrating lymphocytes was defined as the cutoff value for high or low expression levels. By calculating the Youden index, patients with CTLA-4 expression on tumor cells were divided into high (score >2) and low (score ≤2) score subgroups. PD-L1 expression was evaluated as described (20).

Statistical Analyses

Statistical analyses were performed with SPSS 25.0 (Chicago, IL, USA), R (version 4.0.2, R foundation for statistical, Vienna, Austria), and GraphPad Prism 8 software (La Jolla, CA, USA). Values are presented as median (range) or mean ± standard deviation (SD). Paired Student's *t*-test, χ^2 tests, one-factor analysis of variance (one-way ANOVA), Pearson correlation analysis, Spearman rank correlation analysis, and the Wilcoxon rank-sum test were used to compare differences between groups. The Kaplan–Meier method was used to construct the survival and recurrence curves. Cox proportional hazards model analysis was used to analyze the correlation between variables and ICC patient prognosis. Statistical tests were two-tailed, and P-values <.05 were considered significant.

RESULTS

Expression and Prognostic Implication of CTLA-4 in ICC

The distribution of positive CTLA-4 expression in ICC was highly heterogeneous (**Figure 1A**). CTLA-4 positive staining is mainly localized in lymphocytes, the tumor cell membrane, and the hepatocyte cytoplasm in adjacent liver tissues. CTLA-4 is transferred from intracellular vesicles to the cell surface after environmental stimulation and plays a role in sustaining the inhibitory tumor environment (27, 28). Here, we investigated the expression of CTLA-4 in the membrane of T cells and tumor cells. The density of positively stained CTLA-4$^+$-infiltrating lymphocytes in the tumor tissue was 22.0 ± 19.1/field, which was significantly higher than in para-tumor hepatic tissue (7.5 ± 7.8/field, P <.001, **Figure 1B**). The different expression levels of CTLA-4 in tumor cells are presented in **Supplementary Material**, **Figure S1**.

By final follow-up, 177 patients experienced relapsed disease, and median DFS was 14 months (range, 3–122 months). Postoperative 2-, 5-, and 10-year recurrence rates were 53.8%, 64.5%, and 79.4%, respectively. Two hundred patients died, and the median OS was 24.5 months (range, 3–122 months). The 2-, 5-, and 10-year postoperative survival rates were 55.5%, 34.0%, and 19.1%, respectively. Analysis of the relationship between CTLA-4 expression and patient prognosis revealed that patients with low CTLA-4 density in TILs (TILs$^{\text{CTLA-4 Low}}$) had a much longer OS (P <.001) and a lower recurrence rate (P = .024) compared with patients with high density (TILs$^{\text{CTLA-4 High}}$) (**Figures 1C, D**). However, the density of CTLA-4$^+$ lymphocytes in adjacent hepatic tissues was not related to patient prognosis in terms of OS (P = .111) or recurrence rates (P = .057) (**Supplementary Material**, **Figures S2A, B**). We also analyzed the prognostic role of CTLA-4 expression in tumor cells (Tumor$^{\text{CTLA-4}}$) in patients with ICC. No statistical difference in

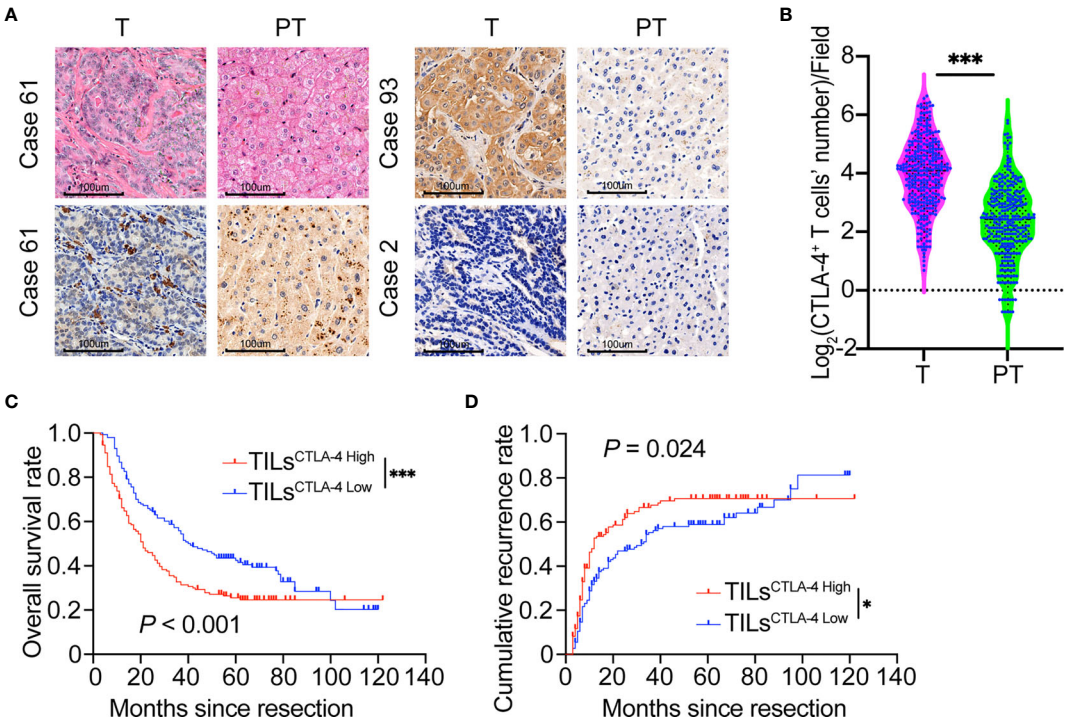

FIGURE 1 | Prognostic implications of CTLA-4 expression in ICC tumors versus paired adjacent normal liver tissues. **(A)** Representative H&E staining of CTLA-4 in ICC tumor and paired adjacent normal liver tissues (Case 61, CTLA-4 positively stained on tumor-infiltrating lymphocytes; Case 93, CTLA-4 positively stained on ICC tumor cells; Case 2, Negative staining on tumor cells or lymphocytes of CTLA-4; T, Tumor; PT, Paired adjacent normal liver tissues). Magnification 200x. **(B)** Density of CTLA-4$^+$ infiltrating lymphocytes was higher in ICC tissues than paired adjacent normal liver tissues in the whole ICC cohort (P <0.001, paired Student's t-test). **(C, D)** The Kaplan–Meier curve of OS and cumulative recurrence shows that patients with TILs$^{CTLA\text{-}4\ High}$ were associated with worse OS and a higher cumulative recurrence rate compared with patients with TILs$^{CTLA\text{-}4\ Low}$. *P < 0.05 and ***P < 0.001.

OS (P = .402) or recurrence rate (P = .080) was observed between Tumor$^{CTLA\text{-}4\ High}$ and Tumor$^{CTLA\text{-}4\ Low}$ subgroups (**Supplementary Material**, **Figures S2C, D**).

Moreover, a higher density of TILs$^{CTLA\text{-}4}$ was related to malignant characteristics in ICC, including a higher level of preoperative serum CA19-9 (P = .003), larger tumor size (P = .014), lymph node metastasis (P = .019), and high TNM stage (P = .036). Other parameters were not related to CTLA-4 expression; detailed information is listed in **Table 1**.

Expression Pattern of FOXP3 in ICC

Because Treg cells maintain an inhibitory immune state in malignancies (29) and CTLA-4 inhibition could reduce Treg-mediated suppression of T cell responses (30), we further investigated FOXP3 expression and its relationship with CTLA-4 in patients with ICC. FOXP3 exhibited nuclear localization in lymphocytes (**Figure 2A**). The density of FOXP3$^+$ TILs (TILs^{FOXP3}) in ICC tumor samples was 15.7 ± 14.8/field, which is significantly higher than that in para-tumor liver tissues (4.8 ± 5.3/field, P <.001, **Figure 2B**) and lower than CTLA-4 (P <.001, **Figure S3A**). Pearson correlation analysis revealed a positive relationship between the density of CTLA-4$^+$ TILs and FOXP3$^+$ TILs (r = .31, P <.001, **Figure 2C**).

We also evaluated the prognostic potential of FOXP3 expression in ICC. Similar to studies in gastric and lymph node-positive breast cancer (31, 32), ICC patients with a high density of FOXP3$^+$ TILs showed poor prognosis in terms of shorter OS (P = .021) and higher recurrence rates (P = .034, **Figures 2D, E**).

Interaction Between CTLA-4/PD-L1 and Prognostic Implication in ICC

PD-1/PD-L1 and CTLA-4 had distinct modes of inhibitory T responses. Our previous studies (20) show that patients with high PD-L1 expression in ICC tumor cells (Tumor$^{PD\text{-}L1\ High}$) had a shorter OS and higher recurrence rates than patients with low tumor PD-L1.

Here, in the whole cohort, Spearman rank correlation analysis revealed no correlation between the expression of TILs$^{CTLA\text{-}4}$ and Tumor$^{PD\text{-}L1}$ (r = .015, P = .802), suggesting that the expression of TILs$^{CTLA\text{-}4}$ and Tumor$^{PD\text{-}L1}$ in ICC was relatively independent. We next divided the cohort into four subgroups according to Tumor$^{PD\text{-}L1}$ and TILs$^{CTLA\text{-}4}$ expression (G I refers to Tumor$^{PD\text{-}L1\ High}$ and TILs$^{CTLA\text{-}4\ High}$ patients; G II refers to Tumor$^{PD\text{-}L1\ High}$ and TILs$^{CTLA\text{-}4\ Low}$ patients; G III refers to Tumor$^{PD\text{-}L1\ Low}$ and TILs$^{CTLA\text{-}4\ High}$ patients; G IV

TABLE 1 | Correlation between CTLA-4 and clinicopathological features in 290 patients with ICC.

Features	$TILs^{CTLA-4}$		
	Low	High	*P* value
Age (y)			
<58	72	67	.484
≥58	72	79	
Sex			
Female	56	58	.884
Male	88	88	
Hepatolithiasis			
Negative	138	136	.317
Positive	6	10	
HBV infection			
Negative	40	37	.639
Positive	104	109	
Liver cirrhosis			
Negative	106	108	.944
Positive	38	38	
ALT(U/L)			
<75	136	135	.496
≥75	8	11	
AFP (ng/mL)			
<20	127	128	.891
≥20	17	18	
CA19-9(U/L)			
<37	89	65	.003
≥37	55	81	
Tumor size(cm)			
≤5	78	58	.014
>5	66	88	
Tumor number			
Single	116	110	.284
Multiple	28	36	
Tumor differentiation			
I/II	92	85	.322
III/IV	52	61	
Lymph node metastasis			
Negative	128	115	.019
Positive	16	31	
Nerve invasion			
Negative	137	136	
Positive	7	10	.471
Microvascular invasion			
Negative	131	122	
Positive	13	24	.059
TNM stage			
I/II	121	108	
III	23	38	.036

HBV, hepatitis B virus; ALT, Alanine aminotransferase; AFP, alpha-fetoprotein; CA19-9, Carbohydrate antigen 19-9; $TILs^{CTLA-4}$, the density of CTLA-4$^+$ TILs.

refers to $Tumor^{PD-L1\ Low}$ and $TILs^{CTLA-4\ Low}$ patients). Representative pictures are presented in **Figure 3A**. Commonly hyperactivated PD-L1 and CTLA-4 expression (G I) was observed in 44 patients, overexpression of PD-L1 alone (G II) in 49 patients, and overexpression of CTLA-4 alone (G III) in 102 patients. Low expression of PD-L1 and CTLA-4(G IV) was observed in 95 patients (**Figure 3B**).

Given the different activated states of PD-L1 and CTLA-4 pathways in tumor tissues, we further investigated the synthesized effect of $Tumor^{PD-L1}$ and $TILs^{CTLA-4}$ expression on prognosis in patients with ICC. Survival analysis showed that patients with $Tumor^{PD-L1\ High}$ and/or $TILs^{CTLA-4\ High}$ subgroups (G I, II, and III) had poorer prognoses in terms of shorter OS (G I *vs.* G IV, P <.001; G II *vs.* G IV, P = .017; G III *vs.* G IV, P = .001) and higher recurrence rates (G I *vs.* G IV, P <.001; G II *vs.* G IV, P = .008; G III *vs.* G IV, P = .046, log-rank test) compared with patients with $Tumor^{PD-L1\ Low}$ $TILs^{CTLA-4\ Low}$ (**Figures 3C, D**).

As for the FOXP3 expression level in the four groups' patients, $Tumor^{PD-L1\ High}/TILs^{CTLA-4\ Low}$ patients have a higher level of Tregs compared with $Tumor^{PD-L1\ Low}/TILs^{CTLA-4\ High}$ patients (P <.001, **Figure S3B**), and integrally, $Tumor^{PD-L1\ High}$ patients are prone to have more Tregs infiltrating into the ICC tumor than $Tumor^{PD-L1\ Low}$ patients (P <.001, **Figure S3C**).

Cox regression analysis showed that clinicopathological characters, including tumor size and number, lymph node metastases, nerve invasion, $TILs^{FOXP3}$, $TILs^{CTLA-4}$, $Tumor^{PD-L1}$, and $Tumor^{PD-L1}/TILs^{CTLA-4}$ were related to OS and recurrence rate of ICC. Hepatolithiasis was only related to patients' survival (**Table 2**). Individual clinicopathological features that showed significance in univariate analysis, including $tumor^{PD-L1}$ and $TILs^{CTLA-4}$, were adopted as covariates in a multivariate Cox proportional hazards model (**Supplementary Material, Table S1**), and then combined variables of $tumor^{PD-L1}/TILs^{CTLA-4}$ were further analyzed (**Table 2**). Multiple tumors, lymph node metastasis, and $tumor^{PD-L1\ High}$ were determined as independent risk factors of cumulative recurrence for patients with ICC, and hepatolithiasis, large tumor, multiple tumors, lymph node metastasis, nerve invasion, and high $TILs^{CTLA-4}$ were independent risk factors of OS. Interestingly, $tumor^{PD-L1}/TILs^{CTLA-4}$ was an independent risk factor of patient prognosis for ICC in terms of recurrence and OS (**Table 2**).

Distinct CTLA-4 Expression and Prognostic Role in ICC With Different Risk Factors

HBV/HCV infection and hepatolithiasis are risk factors for ICC (16). Our previous studies show that hepatolithiasis is an independent risk factor for ICC, and patients with hepatolithiasis had worse survival than patients with HBV infection or undefined risk factors (20). In the present study, we classified 290 patients with ICC into four subgroups according to HBV infection ($HBV^{+/-}$) and hepatolithiasis ($Stone^{+/-}$): 206 patients had HBV infection only ($HBV^+/Stone^-$), nine patients had hepatolithiasis only ($HBV^-/Stone^+$), seven patients had both hepatolithiasis and HBV infection ($HBV^+/Stone^+$), and 68 patients had undefined risk factors ($HBV^-/Stone^-$).

Our previous data also showed a PD1/PD-L1 signal in distinct expression mode and prognostic implication in different risk factor–related ICCs (20). Here, we further investigated CTLA-4 expression and prognostic significance in different risk factor–related ICCs. One-way ANOVA analysis showed that the density of CTLA-4$^+$ TILs in tumor tissues from the four subgroups was significantly different (P = .031, **Figure 4A**). The density of $TILs^{CTLA-4}$ in samples from patients with $HBV^-/Stone^+$ (37.4 ± 22.3/field) was higher than in patients with $HBV^+/Stone^-$ (21.2 ± 17.9/field, P = .013) and patients with

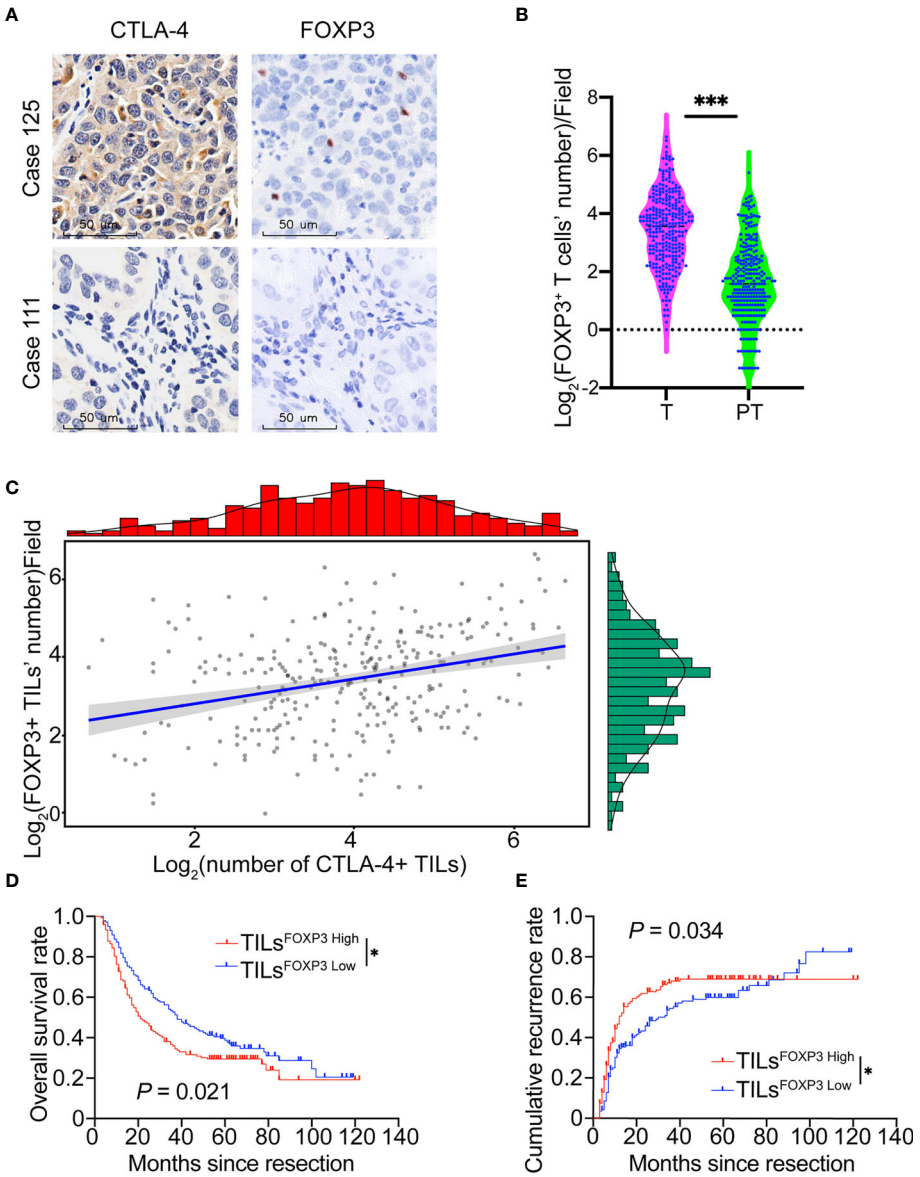

FIGURE 2 | FOXP3 expression and relationship with CTLA-4 in patients with ICC. **(A)** Representative staining of CTLA-4 and FOXP3 in ICC tumor samples (Case 125: patient with a high density of CTLA-4$^+$ TILs and FOXP3$^+$ TILs; Case 111: patient with low density of CTLA-4$^+$ TILs and FOXP3$^+$ TILs). Magnification 400x. **(B)** Density of FOXP3$^+$ infiltrating lymphocytes was higher in ICC tissues than paired adjacent normal liver tissues in the whole ICC cohort. **(C)** Positive correlation between the density of FOXP3$^+$ TILs and CTLA-4$^+$ TILs. **(D, E)** The Kaplan-Meier curve of OS and cumulative recurrence shows that patients with TILs$^{FOXP3\ High}$ were associated with worse OS and a higher cumulative recurrence rate compared with patients with TILs$^{FOXP3\ Low}$. *P < 0.05 and ***P < 0.001.

HBV$^-$/Stone$^-$ (21.0 ± 20.3/field, P = .007). Interestingly, tumor samples from patients with HBV$^-$/Stone$^+$ showed a lower expression of PD-L1 than patients with HBV$^+$/Stone$^-$ as described in our previous study (20). We further investigated the prognostic influence of the density of TILs$^{CTLA\text{-}4}$ in patients with HBV$^-$/Stone$^+$. The OS of the nine patients with HBV$^-$/Stone$^+$ was limited with a median of 7 months (from 4 to 12 months), and the TILs$^{CTLA\text{-}4\ High}$ patients with HBV$^-$/Stone$^+$ had poor survival (P = .018, **Figure 4B**).

DISCUSSION

We determined a profile of CTLA-4 expression with prognostic implication in a large cohort of patients with ICC. CTLA-4 was hyperactivated in tumor samples from patients with ICC, and the high density of CTLA-4$^+$ TILs (TILs$^{CTLA\text{-}4\ High}$) was significantly correlated with malignant characteristics. Clinically, the density of CTLA-4$^+$ TILs was an independent risk factor for OS in patients with ICC, and we found that patients with TILs$^{CTLA\text{-}4\ High}$ showed

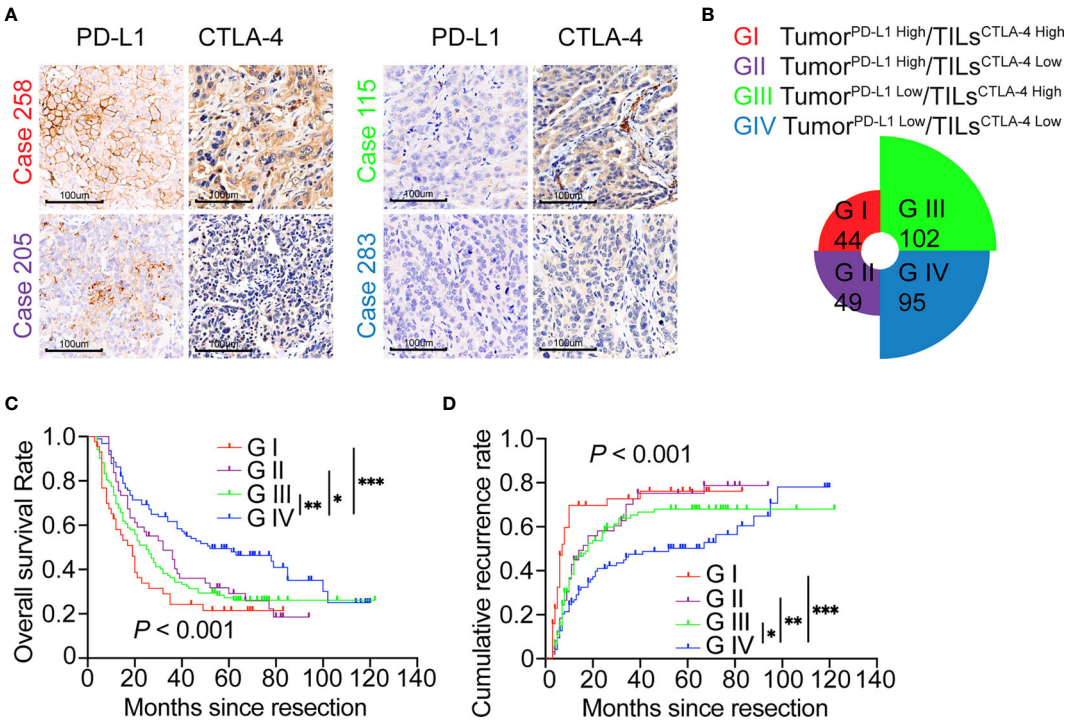

FIGURE 3 | ICC classification and combined prognostic implications of PD-L1 and CTLA-4 expression. **(A, B)** Classification of patients with ICC according to PD-L1 expression in tumor cells and density of CTLA-4$^+$ TILs along with representative staining pictures for each subgroup. Magnification 200x. **(C)** The Kaplan–Meier curve of OS shows that patients with Tumor$^{PD\text{-}L1\ High}$ or TILs$^{CTLA\text{-}4\ High}$ (GI/GII/GIII) are associated with worse OS compared with patients with Tumor$^{PD\text{-}L1\ Low}$ plus TILs$^{CTLA\text{-}4\ Low}$ (GIV). **(D)** The Kaplan–Meier curve of cumulative recurrence shows that patients with Tumor$^{PD\text{-}L1\ High}$ or TILs$^{CTLA\text{-}4\ High}$ (GI/GII/GIII) are associated with higher cumulative recurrence rates compared with patients with Tumor$^{PD\text{-}L1\ Low}$ plus TILs$^{CTLA\text{-}4\ Low}$ (GIV). *P < 0.05, **P < 0.01, and ***P < 0.001.

an unfavorable prognosis. These data indicate that CTLA-4 expression in TILs is an important factor for sustaining the inhibitory immune microenvironment in the clinical setting of ICCs. Thus, this provides a rationale for anti-CTLA-4 therapy in ICCs, at least in a subset of patients. Treg cells are considered the strongest inhibitor of antitumor activity, and CTLA-4 expression is essential for the activation of FOXP3$^+$ T cells. Our data also show a positive relationship between the density of CTLA-4$^+$ TILs and FOXP3$^+$ TILs, which provides indirect evidence to support a role for CTLA-4 in the inhibitory immune microenvironment of ICCs. Furthermore, plenty of studies suggest that CTLA-4 on both activated conventional T cells and FoxP3+ Tregs is important for immunology suppression (33, 34), and it is demonstrated that Treg CTLA-4 blockade alone could not induce antitumor immunity, but it could augment the antitumor responses induced by CTLA-4 blockade of conventional T cells by using selective blockade of CTLA-4 on Treg or conventional T cell (35). Here, we show that both hyperactivated CTLA-4 and FOXP3 are related to an unfavorable prognosis, and the amount of CTLA-4+ TILs is higher than FOXP3+ Tregs, which indicates that besides FOXP3+ Tregs, other CTLA-4+ TILs may be involved in antitumor immune disorders. Thus, the overexpression of CTLA-4 reflects a more global immunomodulatory effect, not just Treg infiltration.

CTLA-4 maintains immune homeostasis through complex mechanisms; the cell-intrinsic model of CTLA-4 function describes that the cytoplasmic tail of CTLA-4 affects intracellular posttranslational modifications and regulates cellular localization of CTLA-4, and the cell-extrinsic model describes CTLA-4 acting through Tregs to exert its function (5). CD28 can costimulate T cell functions by affecting cytokine production, reducing the TCR signaling threshold for T cell activation and enhancing T cell proliferation and survival (36). CTLA-4 may act as an antagonist of CD28–ligand interaction by competing for ligand binding. Recent studies show that CTLA-4 expression was overactivated in several malignant tumors, such as melanoma and spinal chordoma (7, 37). Here, we also demonstrate that the CTLA-4 signal was activated in tumor tissues of ICCs. Patients with early recurrence of ICC had a higher density of CTLA-4 expression than patients without early recurrence. Moreover, the density of CTLA-4$^+$ TILs is related to the density of FOXP3$^+$ TILs. Therefore, CTLA-4 acts as a central element of immunologic tolerance to lessen the immune response in the tumor microenvironment (38). Further, the density of CTLA-4$^+$ TILs in ICC tissues is related to aggressive clinicopathologic features, such as preoperative serum CA19-9, larger tumor size, lymph node metastasis, and high TNM stage. The augmentation of CTLA-4 expression in T cells could reduce the secretion of IFN-γ (39) and then facilitate malignant phenotypes, such as tumorigenesis and metastasis (40). Hence, CTLA-4$^+$ TILs may be involved in the invasive behavior of ICC

TABLE 2 | Univariate and multivariate analyses of characteristics associated with prognosis in 290 patients with ICC.

Characteristics	Univariate analysis				Multivariate analysis			
	Cumulative recurrence		OS		Cumulative recurrence		OS	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age, years (>58 *vs* ≤58)	0.823 (0.612-1.106)	.196	0.894 (0.677-1.18)	.429	NA	NA	NA	NA
Sex (male *vs* female)	1.174 (0.865-1.594)	.302	1.187 (0.89-1.583)	.239	NA	NA	NA	NA
Hepatolithiasis (positive *vs* negative)	1.923 (0.979-3.777)	.058	3.932 (2.303-6.713)	<.001	NA	NA	4.326 (2.497-7.494)	<.001
HBV infection (positive *vs* negative)	1 (0.715-1.399)	.999	0.885 (0.649-1.205)	.436	NA	NA	NA	NA
Liver cirrhosis (positive *vs* negative)	1.173 (0.839-1.640)	.350	1.224 (0.896-1.670)	.204	NA	NA	NA	NA
Tumor differentiation (III/IV *vs* I/II)	1.283 (0.95-1.731)	.104	1.174 (0.884-1.559)	.267	NA	NA	NA	NA
Tumor size (>5 *vs* ≤5)	1.420 (1.053-1.913)	.021	1.585 (1.195-2.102)	.001	1.264 (0.930-1.717)	.134	1.399 (1.049-1.864)	.022
Tumor number (multiple *vs* single)	1.719 (1.219-2.425)	.002	1.510 (1.090-2.092)	.013	1.638 (1.156-2.322)	.006	1.604 (1.147-2.244)	.006
Lymph node metastasis (positive *vs* negative)	2.183 (1.500-3.177)	<.001	2.419 (1.709-3.424)	<.001	1.839 (1.234-2.741)	.003	1.973 (1.364-2.853)	<.001
Microvascular invasion (positive *vs* negative)	1.294 (0.847-1.977)	.233	1.341 (0.899-2.002)	.150	NA	NA	NA	NA
Nerve invasion (positive *vs* negative)	1.906 (1.055-3.44)	.033	2.766 (1.669-4.582)	<.001	1.459 (0.795-2.677)	.222	2.358 (1.394-3.988)	.001
$TILs^{FOXP3}$ (high *vs* low)	1.365 (1.016-1.835)	.039	1.383 (1.047-1.828)	.023	1.116 (0.819-1.521)	.487	1.116 (0.831-1.499)	.467
$Tumor^{CTLA-4}$ (high *vs* low)	1.296 (0.963-1.745)	.088	1.125 (0.852-1.485)	.407	NA	NA	NA	NA
$TILs^{CTLA-4}$ (high *vs* low)	1.393 (1.036-1.873)	.028	1.617 (1.222-2.141)	.001	NA	NA	NA	NA
$Tumor^{PD-L1}$ (high *vs* low)	1.655 (1.219-2.247)	.001	1.394 (1.04-1.867)	.026	NA	NA	NA	NA
$Tumor^{PD-L1}/TILs^{CTLA-4}$ (G I/II/III *vs* G IV)	1.683 (1.210-2.341)	.002	1.806 (1.319-2.472)	<.001	1.566 (1.110-2.210)	.011	1.587 (1.141-2.206)	.006

Cox proportional hazards regression model. OS, overall survival; NA, not applicable; HBV, hepatitis B virus; TILsFOXP3, density of FOXP3+ TILs; TILsCTLA-4, density of CTLA-4+ TILs; TumorCTLA-4, expression level of CTLA-4+ tumor cells; TumorPD-L1, expression level of PD-L1+ tumor cells; G I, Patients with TumorPD-L1 High plus TILsCTLA-4 High; G II, Patients with TumorPD-L1 High plus TILsCTLA-4 Low; G III, Patients with TumorPD-L1 Low plus TILsCTLA-4 High; G IV, Patients with TumorPD-L1 Low plus TILsCTLA-4 Low; 95%CI, 95% confidence interval; HR, Hazard ratio.

cells. Our data indicate that overexpression of CTLA-4 in TILs promotes the invasion and metastasis of ICC and may be a prognostic indicator in patients with ICC.

However, CTLA-4 expressed in tumor cells was not related to the prognosis of ICC. The role of CTLA-4 in tumor cells is controversial, and a previous study suggests that elevated CTLA-4 expression in tumor cells of NSCLC is predictive of a good outcome (41). CTLA-4 is constitutively expressed in a variety of tumor cell lines, such as breast, colon, kidney, lung, ovarian, and uterine cancers and in melanoma cell lines, and elevated CTLA-4 expression is associated with the induction of apoptosis through sequential activation of caspase-8 and caspase-3 (42), but the exact role and mechanism of CTLA-4 in ICC remain to be fully elucidated.

Furthermore, we determined a distinct expression profile of CTLA-4 and PD1/PD-L1 in ICC. PD-L1 was overexpressed in tumor cells, and CTLA-4 was activated in TILs but not tumor cells. CTLA-4 acts as an antagonist of CD28–ligand interactions by competing for ligand binding with CD80. Meanwhile, a large amount of CD80 expressed in antigen-presenting cells (APCs) directly competes with PD1 on the overlapping interface on PD-L1 to disrupt the combination of PD-L1/PD-1 and its inhibitory function in T cell activation. Further, PD-L1 inhibition reduces the expression of CD80 on APCs, and the effect could be offset by the blockage of CTLA-4. This molecular basis has implications for the combination of anti-PD-L1 and anti-CTLA-4 in treating ICC (43, 44). In the present study, patients with coactivation of PD1/PD-L1 and CTLA-4 signals presented the worst prognosis among patients with ICC. Moreover, the density of $CTLA\text{-}4^+$ TILs was determined as an independent predictor of OS, and PD-L1 expression in tumor cells was an independent predictor of cumulative recurrence. Interestingly, combined $CTLA\text{-}4^+$ TILs and $PD\text{-}L1^+$ in tumor cells showed better sensitivity for predicting prognosis of ICCs in terms of OS and cumulative recurrence than that of overexpression of either CTLA-4 or PD-L1 alone. These data indicate that CTLA-4 is a good assistant of PD1/PD-L1 in the inhibitory TEM of ICC.

Moreover, we found that $Tumor^{PD\text{-}L1\ High}$ patients have more Tregs infiltrating into the ICC tumor than $Tumor^{PD\text{-}L1\ Low}$

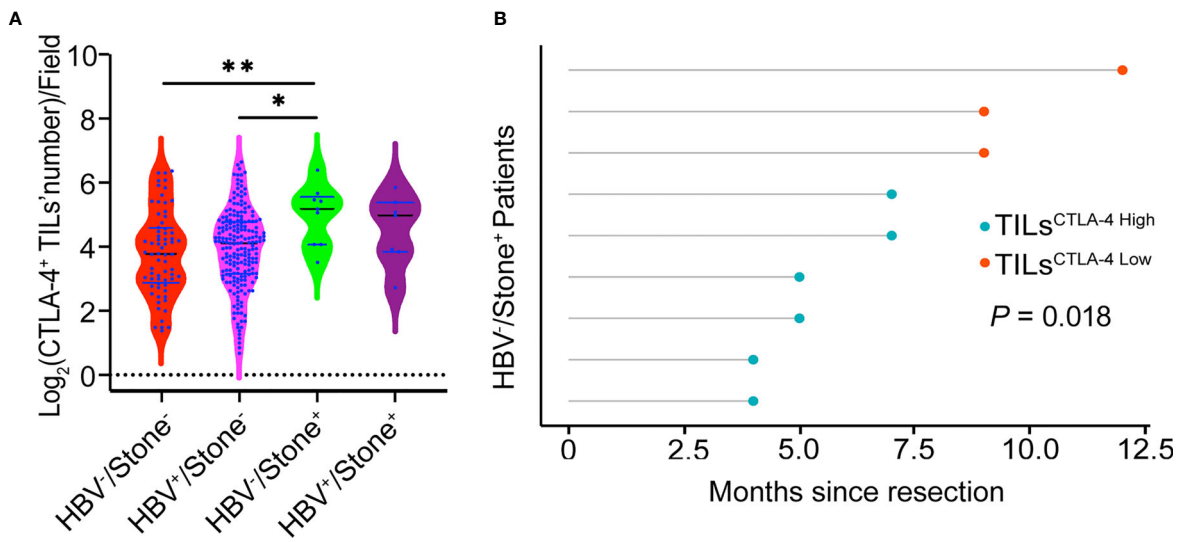

FIGURE 4 | Relationship of the density of CTLA-4$^+$ TILs to risk factors and prognosis. **(A)** Patients with HBV$^-$/Stone$^+$ ICC had a higher density of CTLA-4$^+$ TILs in tumor samples compared with patients with HBV$^+$/Stone$^-$ ICC and HBV$^-$/Stone$^-$ ICC. **(B)** Patients with TILs$^{CTLA\text{-}4\ High}$ show a reduced OS compared with patients with TILs$^{CTLA\text{-}4\ Low}$ among nine patients with HBV$^-$/Stone$^+$ ICC. *P < 0.05 and **P < 0.01.

patients. A previous study revealed that PD-L1 could promote Treg development and enhance Treg function (45), which provides implications in the synergistic use of anti-PD-L1 and anti-CTLA-4 therapies.

Additionally, distinct expression of CTLA-4 and PD1/PD-L1 was observed among different risk factors in ICC. Our data suggest that CTLA-4 overactivation in hepatolithiasis-related ICC is likely the predominant factor involved in sustaining the inhibitory immune environment, providing a promising therapeutic target for such patients.

In conclusion, our findings reveal elevated CTLA-4 and FOXP3 in ICC; the combined overexpression of CTLA-4 and PD-L1 is a good marker for predicting poor prognosis in ICCs and presents a potential target for ICI treatment strategies. These findings will be further evaluated in our clinical trial (**NCT**04634058) about the combination of anti-PD-L1 and anti-CTLA-4 in treating ICC patients, which is already in progress.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Zhongshan Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Concept and design: X-YH, G-MS. Data collection: L-XY, YC, X-JG, J-CL, H-YZ, Q-MS, G-HY, A-WK, Y-HShi, JZ, and JF. Experiments: X-JG, J-CL, H-YZ, Y-ZP, X-LM, P-FZ, and P-XH. Data analysis and visualization: X-JG, G-MS, X-YH, J-CL, Q-MS, G-HY, Y-HShi, J-BC, and RZ. Writing article: X-JG, X-YH, and G-MS. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.705378/full#supplementary-material

Supplementary Figure 1 | Six-point scale for scoring CTAL-4 expression level on ICC tumor cells.

Supplementary Figure 2 | Prognostic implications of CTLA-4 expression on ICC tumor cells or adjacent normal liver tissues. **(A, B)** Kaplan-Meier curves of OS and cumulative recurrence for patients with CTLA-4^{high} against patients with CTLA-4^{low},

grouped by the density of CTLA-4+ lymphocytes in adjacent normal liver tissues. **(C, D)** Kaplan-Meier curves of OS and cumulative recurrence for patients with $Tumor^{CTLA\text{-}4\ High}$ against patients with $Tumor^{CTLA\text{-}4\ Low}$, grouped by expression of CTLA-4 in ICC tumor samples. "ns" refers to no significance.

Supplementary Figure 3 | FOXP3 expression level in ICC patients with different characteristics. **(A)** Density of $CTLA\text{-}4^+$ tumor infiltrating lymphocytes was higher than paired $FOXP3^+$ infiltrating lymphocytes in the whole ICC cohort ($P < 0.001$, paired Student's t-test). **(B)** Density of $FOXP3^+$ tumor infiltrating lymphocytes was higher in $Tumor^{PD\text{-}L1\ High}/TILs^{CTLA\text{-}4\ Low}$ patients than $Tumor^{PD\text{-}L1\ Low}/TILS^{CTLA\text{-}4\ High}$ patients ($P < 0.001$, paired Student's t-test). **(C)** Density of $FOXP3^+$ tumor infiltrating lymphocytes was higher in $Tumor^{PD\text{-}L1\ High}$ patients than $Tumor^{PD\text{-}L1\ Low}$ patients ($P < 0.001$, paired Student's t-test). $^{***}P < 0.001$.

REFERENCES

1. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. *Cell* (2011) 144(5):646–74. doi: 10.1016/j.cell.2011.02.013
2. Topalian SL, Drake CG, Pardoll DM. Immune Checkpoint Blockade: A Common Denominator Approach to Cancer Therapy. *Cancer Cell* (2015) 27(4):450–61. doi: 10.1016/j.ccell.2015.03.001
3. Leach DR, Krummel MF, Allison JP. Enhancement of Antitumor Immunity by CTLA-4 Blockade. *Science* (1996) 271(5256):1734–6. doi: 10.1126/science.271.5256.1734
4. Krummel MF, Allison JP. CTLA-4 Engagement Inhibits IL-2 Accumulation and Cell Cycle Progression Upon Activation of Resting T Cells. *J Exp Med* (1996) 183(6):2533–40. doi: 10.1084/jem.183.6.2533
5. Van Coillie S, Wiernicki B, Xu J. Molecular and Cellular Functions of CTLA-4. *Adv Exp Med Biol* (2020) 1248:7–32. doi: 10.1007/978-981-15-3266-5_2
6. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4 Control Over Foxp3+ Regulatory T Cell Function. *Science* (2008) 322(5899):271–5. doi: 10.1126/science.1160062
7. Chakravarti N, Ivan D, Trinh VA, Glitza IC, Curry JL, Torres-Cabala C, et al. High Cytotoxic T-Lymphocyte-Associated Antigen 4 and Phospho-Akt Expression in Tumor Samples Predicts Poor Clinical Outcomes in Ipilimumab-Treated Melanoma Patients. *Melanoma Res* (2017) 27(1):24–31. doi: 10.1097/CMR.0000000000000305
8. Hall CJ, Doss S, Robertson J, Adam J. NICE Guidance on Ipilimumab for Treating Previously Untreated Advanced (Unresectable or Metastatic) Melanoma. *Lancet Oncol* (2014) 15(10):1056–7. doi: 10.1016/s1470-2045(14)70341-9
9. Jiang X, Wang J, Deng X, Xiong F, Ge J, Xiang B, et al. Role of the Tumor Microenvironment in PD-L1/PD-1-Mediated Tumor Immune Escape. *Mol Cancer* (2019) 18(1):10. doi: 10.1186/s12943-018-0928-4
10. Yi M, Niu M, Xu L, Luo S, Wu K. Regulation of PD-L1 Expression in the Tumor Microenvironment. *J Hematol Oncol* (2021) 14(1):10. doi: 10.1186/s13045-020-01027-5
11. Zeng Z, Yang B, Liao Z. Biomarkers in Immunotherapy-Based Precision Treatments of Digestive System Tumors. *Front Oncol* (2021) 11:650481. doi: 10.3389/fonc.2021.650481
12. Buchbinder EI, Desai A. CTLA-4 and PD-1 Pathways: Similarities, Differences, and Implications of Their Inhibition. *Am J Clin Oncol* (2016) 39(1):98–106. doi: 10.1097/COC.0000000000000239
13. Chang CH, Qiu J, O'Sullivan D, Buck MD, Noguchi T, Curtis JD, et al. Metabolic Competition in the Tumor Microenvironment Is a Driver of Cancer Progression. *Cell* (2015) 162(6):1229–41. doi: 10.1016/j.cell.2015.08.016
14. Zappasodi R, Serganova I, Cohen IJ, Maeda M, Shindo M, Senbabaoglu Y, et al. CTLA-4 Blockade Drives Loss of Treg Stability in Glycolysis-Low Tumours. *Nature* (2021) 591(7851):652–8. doi: 10.1038/s41586-021-03326-4
15. Postow MA, Chesney J, Pavlick AC, Robert C, Grossmann K, McDermott D, et al. Nivolumab and Ipilimumab Versus Ipilimumab in Untreated Melanoma. *N Engl J Med* (2015) 372(21):2006–17. doi: 10.1056/NEJMoa1414428
16. Zhang H, Yang T, Wu M, Shen F. Intrahepatic Cholangiocarcinoma: Epidemiology, Risk Factors, Diagnosis and Surgical Management. *Cancer Lett* (2016) 379(2):198–205. doi: 10.1016/j.canlet.2015.09.008
17. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *Ca Cancer J Clin* (2018) 68(6):394–424. doi: 10.3322/caac.21492
18. Koh KC, Lee H, Choi MS, Lee JH, Paik SW, Yoo BC, et al. Clinicopathologic Features and Prognosis of Combined Hepatocellular Cholangiocarcinoma. *Am J Surg* (2005) 189(1):120–5. doi: 10.1016/j.amjsurg.2004.03.018
19. Shroff RT, Javle MM, Xiao L, Kaseb AO, Varadhachary GR, Wolff RA, et al. Gemcitabine, Cisplatin, and Nab-Paclitaxel for the Treatment of Advanced Biliary Tract Cancers: A Phase 2 Clinical Trial. *JAMA Oncol* (2019) 5(6):824–30. doi: 10.1001/jamaoncol.2019.0270
20. Lu JC, Zeng HY, Sun QM, Meng QN, Huang XY, Zhang PF, et al. Distinct PD-L1/PD1 Profiles and Clinical Implications in Intrahepatic Cholangiocarcinoma Patients With Different Risk Factors. *Theranostics* (2019) 9(16):4678–87. doi: 10.7150/thno.36276
21. Zhou J, Fan J, Shi G, Huang X, Wu D, Yang G, et al. Anti-PD1 Antibody Toripalimab, Lenvatinib and Gemox Chemotherapy as First-Line Treatment of Advanced and Unresectable Intrahepatic Cholangiocarcinoma: A Phase II Clinical Trial. *Ann Oncol* (2020) 31:S262–3. doi: 10.1016/j.annonc.2020.08.034
22. Amin MBAmerican Joint Committee on Cancer and American Cancer Society. *AJCC Cancer Staging Manual. Eight edition*. MB Amin;, SB Edge, DM Gress, LR Meyer, editors. Chicago IL: American Joint Committee on Cancer, Springer (2017).
23. Ishak KG, Anthony PP, Sobin LH, Gibson JB. *Histological Typing of Tumours of the Liver. 2nd ed*. Berlin; New York: Springer-Verlag (1994).
24. Shi GM, Ke AW, Zhou JA, Wang XY, Xu Y, Ding ZB, et al. CD151 Modulates Expression of Matrix Metalloproteinase 9 and Promotes Neoangiogenesis and Progression of Hepatocellular Carcinoma. *Hepatology* (2010) 52(1):183–96. doi: 10.1002/hep.23661
25. Lim YJ, Koh J, Kim K, Chie EK, Kim S, Lee KB, et al. Clinical Implications of Cytotoxic T Lymphocyte Antigen-4 Expression on Tumor Cells and Tumor-Infiltrating Lymphocytes in Extrahepatic Bile Duct Cancer Patients Undergoing Surgery Plus Adjuvant Chemoradiotherapy. *Target Oncol* (2017) 12(2):211–18. doi: 10.1007/s11523-016-0474-1
26. Hori S, Nomura T, Sakaguchi S. Control of Regulatory T Cell Development by the Transcription Factor Foxp3. *Science* (2003) 299(5609):1057–61. doi: 10.1126/science.1079490
27. Linsley PS, Bradshaw J, Greene J, Peach R, Bennett KL, Mittler RS. Intracellular Trafficking of CTLA-4 and Focal Localization Towards Sites of TCR Engagement. *Immunity* (1996) 4(6):535–43. doi: 10.1016/s1074-7613(00)80480-x
28. Walker LSK, Sansom DM. Confusing Signals: Recent Progress in CTLA-4 Biology. *Trends Immunol* (2015) 36(2):63–70. doi: 10.1016/j.it.2014.12.001
29. Togashi Y, Shitara K, Nishikawa H. Regulatory T Cells in Cancer Immunosuppression - Implications for Anticancer Therapy. *Nat Rev Clin Oncol* (2019) 16(6):356–71. doi: 10.1038/s41571-019-0175-7
30. Selby MJ, Engelhardt JJ, Quigley M, Henning KA, Chen T, Srinivasan M, et al. Anti-CTLA-4 Antibodies of IgG2a Isotype Enhance Antitumor Activity Through Reduction of Intratumoral Regulatory T Cells. *Cancer Immunol Res* (2013) 1(1):32–42. doi: 10.1158/2326-6066.CIR-13-0013
31. Li F, Sun Y, Huang J, Xu W, Liu J, Yuan Z. CD4/CD8 + T Cells, DC Subsets, Foxp3, and IDO Expression are Predictive Indictors of Gastric Cancer Prognosis. *Cancer Med* (2019) 8(17):7330–44. doi: 10.1002/cam4.2596
32. Kim MH, Koo JS, Lee S. FOXP3 Expression is Related to High Ki-67 Index and Poor Prognosis in Lymph Node-Positive Breast Cancer Patients. *Oncology* (2013) 85(2):128–36. doi: 10.1159/000351473
33. Chikuma S. CTLA-4, an Essential Immune-Checkpoint for T-Cell Activation. *Curr Top Microbiol Immunol* (2017) 410:99–126. doi: 10.1007/82_2017_61
34. Krummey SM, Ford ML. Braking Bad: Novel Mechanisms of CTLA-4 Inhibition of T Cell Responses. *Am J Transplant* (2014) 14(12):2685–90. doi: 10.1111/ajt.12938
35. Peggs KS, Quezada SA, Chambers CA, Korman AJ, Allison JP. Blockade of CTLA-4 on Both Effector and Regulatory T Cell Compartments Contributes to the Antitumor Activity of Anti-CTLA-4 Antibodies. *J Exp Med* (2009) 206(8):1717–25. doi: 10.1084/jem.20082492

36. Esensten JH, Helou YA, Chopra G, Weiss A, Bluestone JA. CD28 Costimulation: From Mechanism to Therapy. *Immunity* (2016) 44(5):973–88. doi: 10.1016/j.immuni.2016.04.020
37. He G, Liu X, Pan X, Ma Y, Liu X. Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) Expression in Chordoma and Tumor-Infiltrating Lymphocytes (TILs) Predicts Prognosis of Spinal Chordoma. *Clin Transl Oncol* (2020) 22 (12):2324–32. doi: 10.1007/s12094-020-02387-7
38. Van Allen EM, Miao D, Schilling B, Shukla SA, Blank C, Zimmer L, et al. Genomic Correlates of Response to CTLA-4 Blockade in Metastatic Melanoma. *Science* (2015) 350(6257):207–11. doi: 10.1126/science.aad0095
39. Cui J, Yu J, Xu H, Zou Y, Zhang H, Chen S, et al. Autophagy-Lysosome Inhibitor Chloroquine Prevents CTLA-4 Degradation of T Cells and Attenuates Acute Rejection in Murine Skin and Heart Transplantation. *Theranostics* (2020) 10(18):8051–60. doi: 10.7150/thno.43507
40. Street SE, Cretney E, Smyth MJ. Perforin and Interferon-Gamma Activities Independently Control Tumor Initiation, Growth, and Metastasis. *Blood* (2001) 97(1):192–7. doi: 10.1182/blood.v97.1.192
41. Salvi S, Fontana V, Boccardo S, Merlo DF, Margallo E, Laurent S, et al. Evaluation of CTLA-4 Expression and Relevance as a Novel Prognostic Factor in Patients With Non-Small Cell Lung Cancer. *Cancer Immunol Immun* (2012) 61(9):1463–72. doi: 10.1007/s00262-012-1211-y
42. Contardi E, Palmisano GL, Tazzari PL, Martelli AM, Fala F, Fabbi M, et al. CTLA-4 is Constitutively Expressed on Tumor Cells and can Trigger Apoptosis Upon Ligand Interaction. *Int J Cancer* (2005) 117(4):538–50. doi: 10.1002/ijc.21155
43. Zhao Y, Lee CK, Lin CH, Gassen RB, Xu X, Huang Z, et al. PD-L1:CD80 Cis-Heterodimer Triggers the Co-Stimulatory Receptor CD28 While Repressing the Inhibitory PD-1 and CTLA-4 Pathways. *Immunity* (2019) 51(6):1059–73.e9. doi: 10.1016/j.immuni.2019.11.003
44. Sugiura D, Maruhashi T, Okazaki IM, Shimizu K, Maeda TK, Takemoto T, et al. Restriction of PD-1 Function by Cis-PD-L1/CD80 Interactions is Required for Optimal T Cell Responses. *Science* (2019) 364(6440):558–66. doi: 10.1126/science.aav7062
45. Francisco LM, Sage PT, Sharpe AH. The PD-1 Pathway in Tolerance and Autoimmunity. *Immunol Rev* (2010) 236:219–42. doi: 10.1111/j.1600-065X.2010.00923.x

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TGFβ2 Induces the Soluble Isoform of CTLA-4 – Implications for CTLA-4 Based Checkpoint Inhibitor Antibodies in Malignant Melanoma

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Malignant melanoma is an aggressive form of cancer, which can be treated with anti-CTLA-4 and anti-PD-1 checkpoint inhibitor antibodies but while anti-CTLA-4 antibodies have clear benefits for some patients with melanoma, productive responses are difficult to predict and often associated with serious immune related adverse events. Antibodies specific to CTLA-4 bind two major isoforms of CTLA-4 in humans, the receptor isoform and a second naturally secretable, soluble isoform - sCTLA-4. The primary aim here was to examine the effect of selectively blocking the function of sCTLA-4 on *in vitro* immune responses from volunteer healthy or melanoma patient PBMC samples. Addition of recombinant sCTLA-4 to healthy PBMC samples demonstrated sCTLA-4 to have immunosuppressive capacity comparable to recombinant CTLA4-Ig, partially reversible upon antibody blockade. Further, we identified a mechanistic relationship where melanoma patient TGFβ2 serum levels correlated with sCTLA-4 levels and provided the basis for a novel protocol to enhance sCTLA-4 production and secretion by T cells with TGFβ2. Finally, a comparison of selective antibody blockade of sCTLA-4 demonstrated that both healthy and melanoma patient effector cytokine responses can be significantly increased. Overall, the data support the notion that sCTLA-4 is a contributory factor in cancer immune evasion.

Keywords: checkpoint inhibitor, CTLA-4 (cytotoxic T lymphocyte-associated antigen 4), sCTLA-4, melanoma, TGFβ2, T cells

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INTRODUCTION

Malignant melanoma is a dangerous form of skin cancer, whose incidence has risen over the last thirty years. In 2020, global incidence of melanoma reached 324,635 cases with 57,043 deaths (1). Treatment of primary lesions, which emerge from hyperproliferation of melanocytes, is usually performed by resection and offers a favourable prognostic outcome, whereas metastatic malignant melanoma is very difficult to treat effectively and has a very poor prognosis with a 5-year survival rate of only 22.5% compared with >98% for stage I disease. Recently though, the outcome for such patients has been improved by the introduction of novel checkpoint inhibitor therapies that target immune checkpoint receptors including CTLA-4, PD-1 and PD-L1, to promote anti-tumor immunity (2).

The inhibitory CTLA-4 receptor (3, 4), expressed primarily on regulatory (Treg) and activated effector T cells, was the first target for checkpoint inhibitor immunotherapy (5) and antibodies that target the CTLA-4 receptor allow priming and expansion of tumor-specific T cell responses by promoting CD28 mediated T cell costimulation (6). Ipilimumab, a fully human IgG_1 anti-CTLA-4 antibody, has been approved as a monotherapy for the treatment of advanced malignant melanoma (7) and together with anti-PD-1 mAb, nivolumab, for the treatment of metastatic melanoma, renal cell carcinoma and metastatic colorectal cancer (8–10).

The introduction of ipilimumab in 2011 (7) provided a tangible improvement in metastatic melanoma patient outcomes, and for some individuals an enduring remission from disease, but there remains scope for improving the therapy further. Currently, there is no biomarker for effective stratification of patient responders, or as a guide for severe immune-related adverse effects associated with the therapy. Retrospective analyses of patients treated with ipilimumab as a monotherapy also indicate that approximately only 22% of patients benefit from long term disease remission (11). In contrast, more recent checkpoint inhibitor antibodies that disrupt interaction between PD-1 on T cells (12, 13) and PD-L1 (14–16) on tumor cells have been approved for a broader range of cancers but also demonstrate improved patient response frequency and safety compared with ipilimumab (17). This latter approach is aided by using PD-L1 tumor expression levels as a stratification biomarker to identify patients most likely to respond to the therapy (18).

While there is no doubt about the potential therapeutic benefits of ipilimumab, its precise mechanism of action remains to be fully resolved, as does the biology of CTLA-4 more generally. CTLA-4 is crucial at the priming stage of naïve anti-tumor T cell responses by professional antigen presenting cells (APC), and therefore its blockade has the potential to generate *de novo* powerful $CD8^+$ cytotoxic and $CD4^+$ helper T cell anti-tumor effector responses. Despite its fundamental importance to naïve T cell differentiation and activation, the clinical response variation between patients receiving anti-CTLA-4 therapy hints that other processes such as differences in tumour immunogenicity, the manifestation of tumour neoantigens and other immune factors may also be important for the generation of effective responses (19, 20). Anti-CTLA-4 antibodies may also function by directly binding to and depleting Treg *via* macrophage dependent antibody-dependent cell-mediated cytotoxicity (ADCC) within the tumor microenvironment (21, 22). This uncertainty of mechanism is reflected in the unusual kinetics of anti-CTLA-4 mAb therapy, in which for some patients, tumors may grow for weeks or months after therapy commences, before eventually stabilising, shrinking and ultimately disappearing altogether. No one hypothesis adequately explains this extended hiatus between therapeutic intervention and productive anti-tumor immunity.

Despite a primary focus on the CTLA-4 receptor, anti-CTLA-4 antibodies bind two isoforms of CTLA-4 in humans, the full length CTLA-4 receptor and a second secretable form of CTLA-4, soluble CTLA-4 (sCTLA-4) (23, 24). Soluble CTLA-4 is an alternatively spliced variant of full length CTLA-4 in which the transmembrane domain, encoded by exon 3, is not utilised and a frameshift mismatch during post-transcriptional splicing of exon 2 (extracellular domain) to exon 4 (cytoplasmic domain) gives rise to a unique C terminal amino acid sequence that replaces the cytoplasmic domain of full length CTLA-4 (24). Soluble CTLA-4 like its full-length counterpart can bind to B7 (CD80/CD86) ligands on APC (24).

The secretory isoform of CTLA-4 was identified after the receptor isoform and less is known regarding any immunoregulatory properties it may possess. Few studies that aim to explain response diversity in patients receiving anti-CTLA-4 therapy have examined sCTLA-4 as a significant influential factor (25, 26). Analysis of sCTLA-4 serum levels in patients with acute B lymphoblastic leukaemia (B-ALL) (27), malignant melanoma (28) or mesothelioma (29), however, revealed increased production together with good evidence that tumour cells may actually be secreting this potentially immunosuppressive molecule. Retrospective analyses of patients treated with ipilimumab also discovered that patients with higher serum levels of sCTLA-4 were significantly more likely to respond to the therapy than those with low levels (25). Further support for the clinical relevance of sCTLA-4 to cancers in particular, has emerged with the discovery that the repulsive guidance molecule B (RGMB) acts as a ligand for sCTLA-4 and greatly strengthens its immunosuppressive activity through enhanced CD80 binding (30).

The TGF-β family includes three closely related molecules, TGFβ1, 2 and 3 whose roles in maintaining immune homeostasis are crucial, but which also play a complex role in tumour development (31, 32). Despite initial anti-tumor growth effects, during tumorigenesis TGFβ can both assist the metastatic EMT transition process and promote evasion from anti-tumour immunity (33–35). Thus TGFβ represents another potential target for anti-tumour immunotherapy and could be used in combination with anti-CTLA-4/PD-1/PD-L1 antibodies (31).

Previously, we demonstrated that selectively blocking sCTLA-4 boosted antigen-specific immune responses and further, generated effective anti-tumour immunity in the B16F10 model of metastatic melanoma, which was comparable to that achieved with pan-specific anti-CTLA-4 mAb (36). Our initial

analysis of sCTLA-4 production identified Tregs to produce and secrete this immunoregulatory molecule. Here, we demonstrate recombinant sCTLA-4 to be directly immunosuppressive with regard to T cell responses and identify a novel functional association between sCTLA-4 production and the immunoregulatory cytokine, TGFβ2. Further, by selectively targeting this particular isoform, melanoma patient derived PBMC response activity *in vitro* was increased significantly with higher production of the T cell effector cytokines IFN-γ and IL-17A when compared with non-selective ipilimumab. We propose a new mechanism through which tumor cells utilise TGFβ2 to promote production of sCTLA-4 as a novel mechanism of immune evasion and further, highlight the urgent need to examine the relevance of sCTLA-4 to immune checkpoint inhibitor therapy.

METHODS

Donors, Ethics and PBMC Sample Preparation

Blood samples were collected by venepuncture from patients at the dermatology, oncology or plastic surgery clinics at Aberdeen Royal Infirmary (see **Table 1** for demographic summary) and compared with age and sex-matched healthy volunteer donors (n=27). Sera from the Lupus patient cohort was provided from a previous study, which was age but not sex matched (n=40) (37). PBMC were prepared using Lymphoprep 1.077 (Axis Shield, Dundee, UK) density gradient centrifugation and cultured essentially in RPMI 1640 medium (Thermofisher Scientific, Paisley, UK) supplemented with 5% autologous human serum in an atmosphere of 37°C, 5% CO_2 as previously described (38). 1×10^6 PBMC were cultured for 5 days in 1 mL wells unless otherwise stated. Jurkat T cells (1×10^6 per well) were stimulated for 48 hours with PHA-L according to manufacturer's instructions and B7.1/2Ig at 1 μg/mL (Peprotech EC, London, UK) in an atmosphere of 37°C, 5% CO_2. Spleens from Balb/c mice were obtained from the University of Aberdeen, Medical research facility and stimulated in the presence of anti-CD3 antibody (Clone: 2C11, BD Biosciences, Oxford, UK), murine TGFβ2 (R&D Systems, Abingdon, UK) and IL-2 (Peprotech EC) for 8 days.

Cytokine and sCTLA-4 ELISA

ELISA for cytokines in cell culture supernatants or sera was based on previously published methods. Antibody pairs used for the human IL-10 ELISA were clones JES3-19F1 and JES3-12G8 from BD Biosciences), for anti-IFN-γ (clones NIB42 and 4S.B3 BD Biosciences, Oxford, UK), and for anti-IL-17A (clones eBio64CAP17 and eBio64DEC17, Thermofisher Scientific). Cytokine standards were from Peprotech EC Ltd. Bound antibody was detected using streptavidin-labelled alkaline phosphatase with a phosphate substrate (both Sigma Aldrich, Gillingham, UK), and absorbance measured at 450nm (corrected with a reference reading at 492nm) with a Multiskan MS microplate photometer (Life and Laboratory Sciences, Basingstoke, UK). IL-2 was measured using an IL-2 Human Uncoated ELISA Kit with Plates (Thermofisher) according to manufacturer's instructions.

The selective ELISA for human sCTLA-4 used the anti-CTLA-4 murine mAb clone BNI3 (2 μg/ml) as a capture reagent and biotinylated mAb clone 73-B1 (IgG1κ) as the sCTLA-4 specific detection reagent using the same protocol described for the cytokine ELISA above. The alternatively spliced recombinant human sCTLA-4 (MRC PPU Services, University of Dundee, Dundee) was used to construct standard curves. Biotinylated anti-sCTLA-4 clone 73-B1 cross-reacts with murine sCTLA-4 and was used in ELISA to detect the presence of murine sCTLA-4 together with a capture anti-murine CTLA-4 mAb (clone: 4F10, BD Biosciences).

TABLE 1 | Summary of the melanoma patient demographics.

Demographic and clinical characteristics	Melanoma patients
Age, years	57.3 ± 36.1
Sex, no. female/male	18/12
Average disease duration (months)	36 ± 2
Recruiting clinic	
- Oncology	9
- Dermatology	22
- Plastic surgery	1
Average Breslow thickness (range mm)	2.0 (0 – 10.0)

Cancer Cell Lines

Adherent malignant melanoma epithelial cell lines G-361 (CRL-1424) and A-375 (CRL-1619) were obtained from the American Tissue culture collection (LGC standards, London, UK) and cultured in McCoy's 5a Medium Modified Medium supplemented with 10% Foetal bovine serum (FBS; G-361) or Dulbecco's Modified Eagle's Medium +10% FBS (A-375) according to protocols provided by ATCC.

Confocal Microscopy

For intracellular analysis of CTLA-4 and sCTLA-4 by confocal microscopy, G-361 and A-375 cell lines were prepared according to ATCC protocols and seeded into 24 well plates containing sterile coverslips coated with poly-l-lysine to aid adherence. Cells were allowed to grow to 80-90% confluence before fixing with 4% paraformaldehyde (BD Cytofix) for 10 min at RT, treatment with 0.3M glycine in PBS to prevent background fluorescence (10 min at RT), permeabilization with 0.2% Triton X-100 in PBS (5 min at RT) and blocking with 1% BSA in PBS. Cells were washed three times (2 minutes per wash) between each step. The cell lines were then incubated with biotinylated anti-sCTLA-4 mAb, JMW-3B3 or a non-specific biotinylated IgG1 antibody (Thermofisher; both 20 μg/mL) and incubated O/N at 4°C. The cell lines were subsequently washed and incubated with rabbit polyclonal anti-CTLA-4 antibody before staining with Streptavidin-AF555 to selectively reveal sCTLA-4 and Goat anti-Rabbit IgG AF488 to reveal total CTLA-4 (CTLA-4 receptor and sCTLA-4), each according to manufacturer's instructions. Finally, coverslips were placed on glass slides and mounted with Prolong Gold anti-fade mountant containing DAPI (Fisher Scientific) before analysis with a Leica LSM710

confocal microscope at the Institute of Medical Sciences, Microscopy and Histology Core Facility. Images were analysed using 'ZEN BLUE' software.

Flow Cytometry Analysis

For surface staining of markers using flow cytometry, following the appropriate stimulation protocol, cells were first washed twice with staining media (PBS + 0.5% BSA) and stained with fixable viability dye eFluor 780 as per the manufacturer's guidance. The cells were washed with staining media and suspended in blocking buffer (PBS + 5% BSA) at 4°C for 30 minutes. The cells were stained with the appropriate fluorochrome conjugated antibodies at 4°C for 30 minutes and fixed with BD Cytofix fixation solution (BD Biosciences) for 10 minutes at RT.

To measure phosphorylation of intracellular molecules, following T cell stimulation with plate bound anti-CD3 mAb (1 μg/mL), the cells were washed and treated with BD Cytofix/Cytoperm fixation/permeabilization solution (BD Biosciences) to arrest intracellular phosphorylation and subsequent activation of cells. The optimum time point for measurement was identified as 10 minutes following stimulation following a "sighting" experiment to determine the optimum time for assessment (**Figure 2A**). The cells were washed, blocked and stained for surface and intracellular molecules according to the protocol outlined above together with appropriate isotype controls. All fluorochrome conjugated antibodies specific to surface and intracellular molecules were acquired from BD biosciences. For all flow cytometry readouts, a minimum of 100,000 events were acquired using a BD LSR II flow cytometer (BD Biosciences) and analysed using FlowJo analysis software.

Statistics

Power calculations: For the main patient study and based on healthy donor PBMC sample responses to stimulation in the presence or absence of sCTLA-4 antibody blockade in which analysis of 20 samples provided statistically significant deviation from the null hypothesis, a comparative analysis of responses from 20 melanoma patient blood samples provided approximately 80% power to detect a 65% difference in responses at the 5% significance level. Differences between treatments were analyzed with an Anova one-way test with Tukey *post-hoc* analysis, Mann-Whitney U, two-tailed, unpaired t test or Pearson's r tests as outlined in figure legends. Where used, Bar and whisker plot error bars show the minimum and maximum value for each cohort.

Study Approval

Written informed consent was obtained from all donors. Ethical approvals were obtained from East of Scotland Research Ethics Committee (ref:13/NS/0126 & 10/S1401/20) and the study was performed and archived according to the protocols provided by the East of Scotland Research Ethics Committee. Exclusion criteria: Potential volunteer donors were included in the study if over the age of 18, independent of melanoma disease status from the dermatology, plastic surgery and oncology clinics at the University of Aberdeen. All potential volunteer candidates were provided with a patient information sheet prior to providing a blood donation. Patient weight was not recorded.

RESULTS

Immunosuppressive Properties of sCTLA-4

Most studies involving sCTLA-4 have used pan-specific anti-CTLA-4 antibodies, but here, we have used two selective anti-sCTLA-4 monoclonal antibodies, JMW-3B3 (IgG1λ) (36) and 73-B1 (IgG1κ), together with recombinant (rec.) sCTLA-4 to examine the immunoregulatory properties of sCTLA-4 primarily in melanoma patient donor samples. Both antibodies were raised against the unique C terminal sequence of sCTLA-4 and neither cross-react with the CTLA-4 receptor.

Previous reports have indicated sCTLA-4 to be immunosuppressive because selective removal from cell cultures can boost antigen-specific effector T cell responses (36). To determine whether or not sCTLA-4 is directly immunosuppressive, we added rec. human sCTLA-4 (sCTLA-4) or CTLA4-Ig to Jurkat T cells stimulated with PHA-L and either B7.1Ig or B7.2Ig costimulatory ligands before measuring IL-2 cell culture supernatant levels by ELISA after 48 hours (**Figure 1A**). Both reagents were comparatively immunosuppressive and demonstrated the suppressive capacity of natural sCTLA-4. We also assessed the immunosuppressive effects of sCTLA-4 in normal healthy donor PBMC cell cultures stimulated with plate-bound anti-CD3 mAb for 5 days and examined its effects on cell division by flow cytometry and T cell cytokine production by ELISA (**Figures 1B, C**). Addition of rec. human sCTLA-4 at 10 μg/ml was immunosuppressive, significantly decreasing both $CD8^+$ and $CD4^+$ T cell proliferation and PBMC culture supernatant levels of IFN-γ, and IL-17A and IL-10. The immunosuppressive effects of sCTLA-4 were significantly reversed by the addition of anti-sCTLA-4 mAb, 73-B1, demonstrating a sCTLA-4 specific effect. CTLA4-Ig also suppressed these PBMC responses, but its immunosuppressive effects were not reversible by anti-sCTLA-4 mAb, 73-B1, as CTLA4-Ig lacks the C terminal epitope recognised by the antibody, which is present on natural sCTLA-4.

Analysis of human healthy donor $CD11c^+$ antigen presenting cells stimulated with LPS, within the PBMC population, revealed that selective anti-sCTLA-4 antibody blockade also significantly increased detectable cell surface levels of CD80/CD86 (**Figure 1D**), while anti-panCTLA-4 antibody was slightly less effective. Thus, sCTLA-4 has direct immunosuppressive effects on activated PBMC by blocking or inhibiting costimulatory interactions and its removal enhances effector T cell activity.

Selective Antibody Blockade of sCTLA-4 Does Not Suppress Phosphorylation of T Cell Signalling Proteins Slp76 and ZAP-70

Previously, we demonstrated that selective blockade of sCTLA-4 significantly enhanced antigen-specific CD4 T helper T cell responses compared with pan anti-CTLA-4 antibody blockade (36). To further examine the effects of anti-CTLA-4 mAbs on T

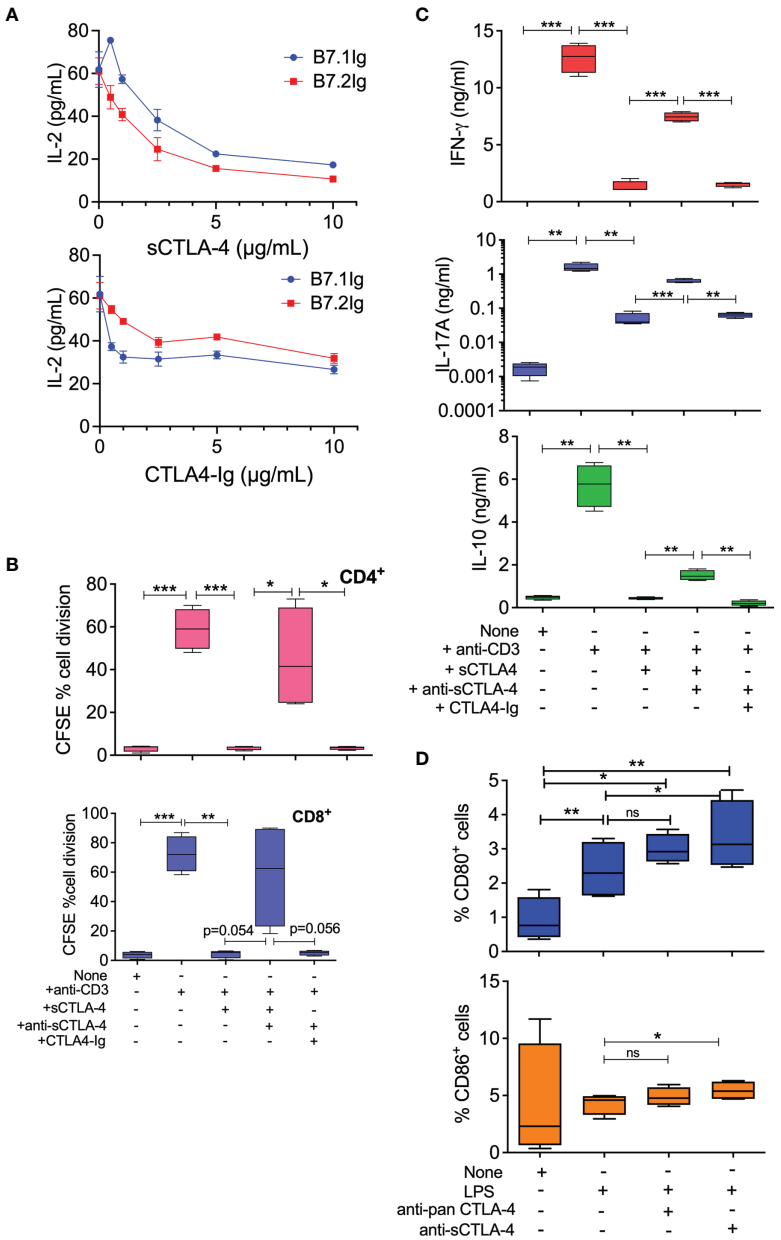

FIGURE 1 | Immunoregulatory properties of recombinant human sCTLA-4. **(A)** Comparison of immunosuppressive activity of rec. sCTLA-4 with CTLA4-Ig in Jurkat T cell cultures stimulated with PHA-L and either B7.1Ig or B7.2Ig costimulatory ligands for 48 hours at 37°C, 5% CO2 (Mean (SD); representative of n = 4). **(B, C)** Donor PBMC were stimulated with anti-CD3 mAb for 5 days at 37°C, 5% CO2 in the presence of human rec. sCTLA-4, CTLA4-Ig ± anti-sCTLA-4 antibody, 73-B1, before analysis of cell division by flow cytometry **(B)** and of cell culture supernatant cytokine levels by ELISA (**C**, n=5). **(D)** Effect of anti-CTLA-4 or sCTLA-4 antibody blockade on PBMC derived CD11c$^+$ APC CD80/CD86 T cell levels by flow cytometry. Cells were stimulated with 1 mg/mL LPS for 18 hrs in the presence or anti-sCTLA-4 (73-B1) or pan anti-CTLA-4 mAb (BNI3; both 10 µg/mL; n=4). (One-way Anova with Tukey *post-hoc* multiple comparison analysis, *P < 0.05 **P < 0.01, ***P < 0.001, ns, not significant).

cell activation, we stimulated PBMC with anti-CD3 mAb and assessed the effects by phosphoflow cytometry of anti-sCTLA-4 vs. pan anti-CTLA-4 antibody blockade on phosphorylation levels of both T cell receptor signalling cascade molecules Slp76 and ZAP-70 and compared to an isotype control antibody (**Figure 2**, white bars isotype, green/blue bars specific antibody). Stimulation of donor-derived T cells isolated from PBMC with anti-CD3 mAb, as expected, induced an increase in phosphorylated Slp76 and ZAP-70 in both CD4$^+$ and CD8$^+$ T cells compared with resting cells, and addition of anti-sCTLA-4 mAb clone 73-B1 did not affect phosphorylation levels. This is not surprising because anti-sCTLA-4 mAbs do not interact

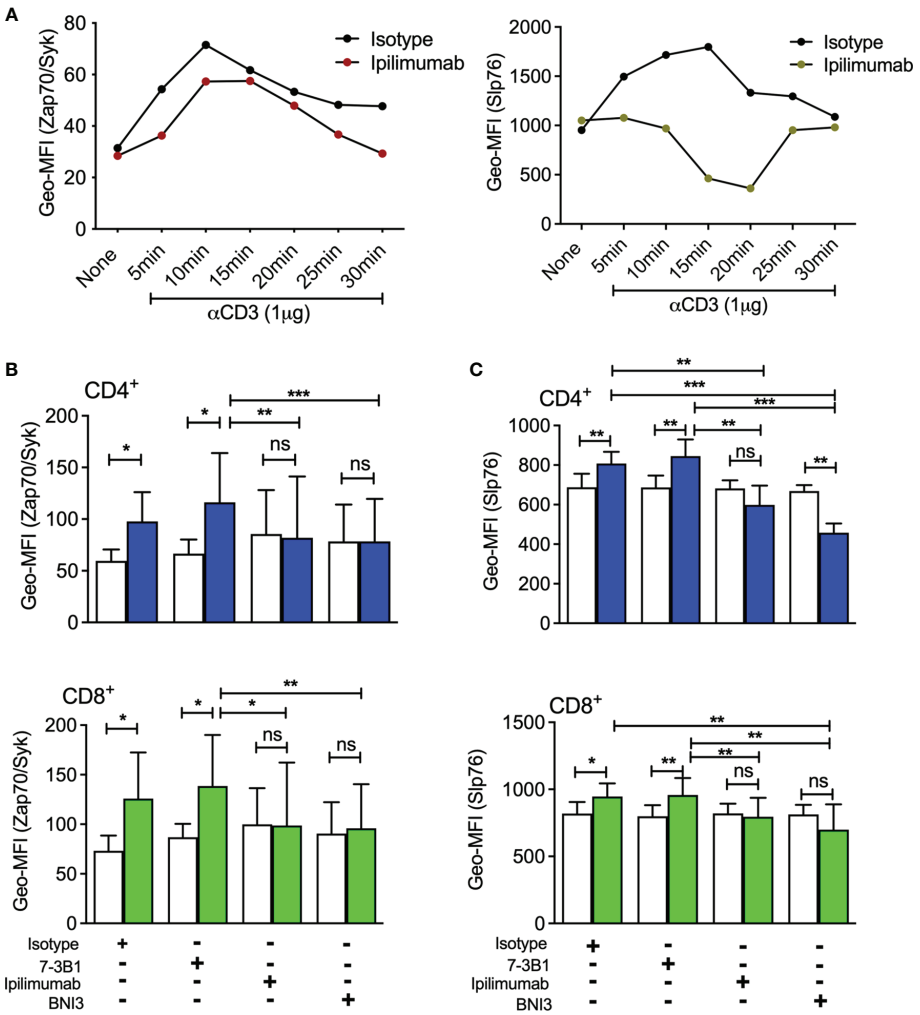

FIGURE 2 | Analysis of sCTLA-4 blockade on T cell receptor phosphorylation levels **(A)** Sighting experiment to evaluate the optimum period for measuring phosphorylation levels of Slp76 and ZAP-70 following stimulation of T cells with anti-CD3 Mab (n = 2). Effect of anti-CTLA-4 or anti-sCTLA-4 mAb blockade on phosphorylation levels of T cell signalling components **(B)** ZAP-70 and **(C)** Slp76 following activation. Healthy donor PBMC were stimulated with anti-CD3 mAb for 15 minutes, fixed and $CD4^+$ and $CD8^+$ T cells were analysed for ZAP-70 and Slp76 levels of phosphorylation by flow cytometry. White bars represent an IgG1 (Zap-70) or an IgG2a (Slp76) isotype control, while green/blue bars represent specific antibody. n=5; *P < 0.05 **P < 0.01, ***P < 0.001, ns, not significant, P values determined by Student t test; Mean (SD) values are shown).

directly with cells. Addition of anti-CTLA-4 mAbs BNI3 or ipilimumab, however, completely abolished any increase in phosphorylation in both anti-CD3 mAb stimulated $CD4^+$ and $CD8^+$ T cells compared with resting cells.

In summary, we find that selective targeting of sCTLA-4 has no inhibitory effect on phosphorylation levels of ZAP-70 and Slp76 after activation of $CD4^+$ and $CD8^+$ T cells.

A Mechanistic Relationship Between sCTLA-4 and TGF β2

Previous reports indicated that serum levels of sCTLA-4 are raised in some cancers including malignant melanoma, but those studies did not use sCTLA-4 selective antibodies and were, therefore, unable to distinguish fragments of CTLA-4 receptor cleaved from cell surfaces from native alternatively spliced sCTLA-4. We analysed and compared melanoma patient serum sCTLA-4 levels with those from healthy or lupus donor volunteers (**Figure 3**). Serum levels of sCTLA-4 were significantly higher in the melanoma patient cohort compared with both healthy and lupus patient volunteer donor serum cohorts.

The immunosuppressive TGFβ cytokine isoforms contribute to immune evasion strategies promoted by tumors and in a number of cancers, TGFβ2 has been closely implicated in epithelial to mesenchymal transition (EMT), a precursor to the metastatic process (39). We examined and compared serum levels of both TGFβ1 and 2 in the same healthy, melanoma and lupus cohorts (**Figure 3**). The lupus patient serum cohort

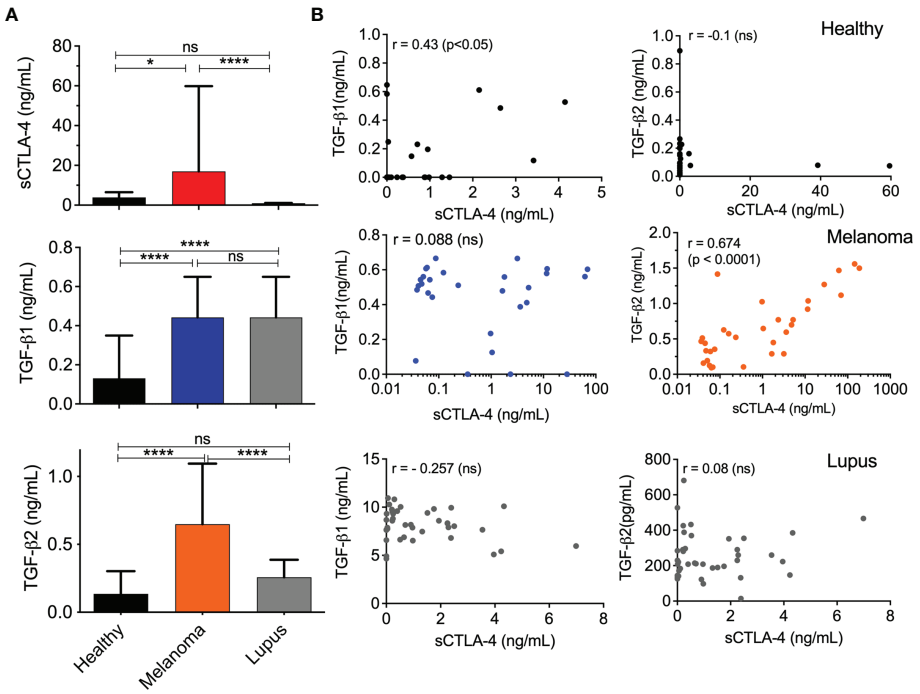

FIGURE 3 | Correlation analysis of TGFβ and sCTLA-4 serum levels in melanoma, lupus, and healthy volunteer donor cohorts. **(A)** Serum levels of TGFβ1, TGFβ2 and sCTLA-4 in healthy (n=27), melanoma patient (n = 32) and lupus patient donor cohorts (n = 40). (Mean (SD) *P < 0.05, ****P < 0.0001, ns, not significant, P values determined by one-way ANOVA test). **(B)** Regression analysis of TGFβ1 or TGFβ2 with sCTLA-4 levels in sera from healthy, melanoma patient and lupus patient donor cohorts (Pearson's r test, p values shown in figure).

was examined to determine whether any correlation between sCTLA-4 and TGFβ serum levels would be replicated in the context of an autoimmune disease. Serum levels of TGFβ1 were significantly raised in both the melanoma and lupus patient serum cohorts compared with healthy donors (**Figure 3A**), but TGFβ2 serum levels were significantly higher solely in the melanoma patient cohort. Correlative analysis of TGFβ1, TGFβ2 and sCTLA-4 levels in the three donor serum cohorts (**Figure 3B**) revealed a significant positive correlation between TGFβ2 and sCTLA-4 serum levels restricted solely to the melanoma serum cohort, and a weaker but significant correlation between TGFβ1 and sCTLA-4 in the healthy donor serum cohort. No other correlation was observed.

The analysis of the melanoma patient serum cohort revealed for the first time a potential relationship between TGFβ2 and sCTLA-4 in melanoma patients and also raised the prospect that TGFβ2 has a role in stimulating the production of sCTLA-4. TGFβ is important for the induction of peripheral Treg (pTreg) and has also been used in protocols to generate inducible Treg (iTreg) *in vitro* for clinical use (40). We fractionated $CD3^+$ T cells from healthy donor PBMC and stimulated them with anti-CD3 mAb in the presence of IL-2, while comparing the ability of all three TGFβ isoforms, TGFβ1, TGFβ2 and TGFβ3 to induce sCTLA-4 production (**Figure 4A**). Following an extended incubation of 8 days, sCTLA-4 supernatant levels were significantly increased after incubation with TGFβ2, with levels significantly higher than any other treatment. In some individual donors TGFβ1 was also able to increase sCTLA-4 levels, but collectively this population did not achieve significance. Finally, we repeated this sCTLA-4 induction experiment using BALB/c mouse splenocytes with very similar results (**Figure 4B**). Together, the data demonstrate that sCTLA-4 production can be consistently induced in activated T cells by the immunoregulatory cytokine TGFβ2, supporting the notion that TGFβ2, which is primarily produced by tumor cells in malignant melanoma, has the potential to induce immunosuppressive sCTLA-4 within the tumor environment.

Melanoma Derived Cell Lines Produce sCTLA-4

Contrary to current perceptions, studies of CTLA-4 in tumor cell biopsies and cell lines have revealed that CTLA-4 and sCTLA-4 may also be actively produced by cancer cells (28–30) perhaps as a means of tumor cell mediated immune evasion. The availability of anti-sCTLA-4 antibodies allowed us to selectively stain and differentiate both full-length CTLA-4 receptor and sCTLA-4 in human melanoma cancer cell lines, as well as those associated with other tumour types. Co-staining with pan anti-CTLA-4 (AF488, **Figure 5**) and anti-sCTLA-4 (AF555) revealed and differentiated the presence of both CTLA-4 receptor and sCTLA-4 in melanoma cell lines. Staining of the G-361 epithelial malignant melanoma cell line revealed sCTLA-4

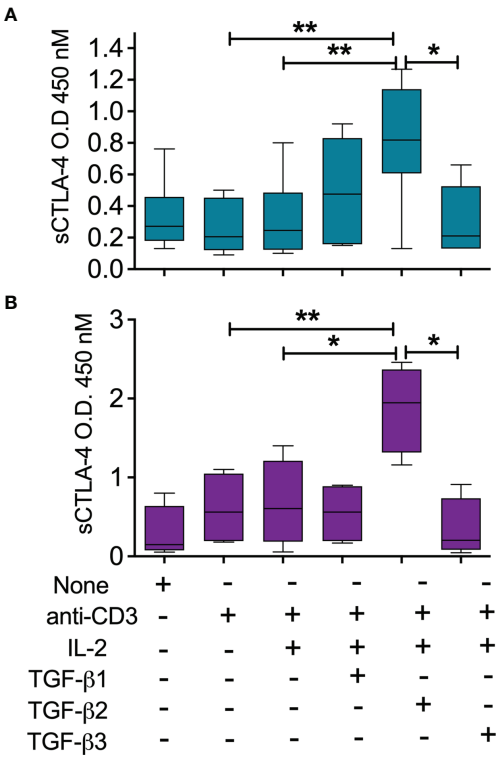

FIGURE 4 | Induction of sCTLA-4 by TGFβ2. **(A)** PBMC were isolated from healthy donors (n = 5, top panel) or **(B)** Balb/c mice (n = 4, lower panels) were incubated for 8 days after stimulation with anti-CD3 mAb (0.1 μg/mL), IL-2 (20 ng/mL) in the presence of TGFβ isoforms 1,2 and 3 each at 20 ng/mL before analysis of cell culture supernatant sCTLA-4. n=5; *P < 0.05 **P < 0.01, P values determined from a one-way test with Tukey *post-hoc* analysis; median values are shown).

detectable throughout individual cells, punctuated by CTLA-4 receptor within individual vesicles, by confocal microscopy (**Figure 5A**). Further, while all cancer cell lines tested to date stained positive for the CTLA-4 receptor, not all expressed sCTLA-4, including the A-375 epithelial malignant melanoma cell line (**Figure 5B**; see also **Supplementary Figure 1**).

Selective Blockade of sCTLA-4 Enhances Effector Cytokine Production by Melanoma Patient PBMC

In a previous study, we demonstrated that selective blockade of sCTLA-4 was as effective as pan-CTLA-4 blockade at reducing lung tumor frequency in the B16F10 model of melanoma lung metastasis (36). Thus, we examined the selective effects of anti-sCTLA-4 antibody blockade compared directly with anti-CTLA-4 mAb ipilimumab on supernatant effector cytokine levels from PBMC cell cultures provided by melanoma patient donors and stimulated with doses of plate-bound anti-CD3 mAb ranging from 0.02 to 1 μg/mL (**Table 1** and **Figure 6**). We also compared selective sCTLA-4 vs. pan-CTLA-4 blockade in cell cultures from healthy volunteer donors (**Figure 7**).

Analysis of IFN-γ, IL-17 and IL-10 supernatant levels from 5-day melanoma patient derived PBMC cell cultures (**Figure 6** and **Supplementary Figure 2**), revealed relatively modest differences in IFN-γ supernatant increases between treatments, with significant increases in levels observed only with both anti-sCTLA-4 mAbs JMW-3B3 and 73-B1 anti-sCTLA-4 clones stimulated significant increases in IFN-γ compared with ipilimumab at low levels of anti-CD3 mAb (0.02 μg/mL) stimulation. No other significant differences were detected.

Differences in supernatant levels of IL-17 were more apparent, with selective blockade of sCTLA-4 by antibody clone 73-B1 significantly increasing levels of IL-17 at every anti-CD3 mAb dose. An interesting observation here, was that significant differences between 73-B1 and ipilimumab arose because of increases in IL-17 in 73-B1 treated cultures but also decreases in ipilimumab treated cultures. No differences in IL-10 levels were detected in any of these treatments (**Supplementary Figure 2**).

Analysis of healthy donor PBMC responses also identified anti-sCTLA-4 clone 73-B1 to generate significant increases in IFN-γ and IL-17 supernatant levels in anti-CD3 mAb stimulated cultures compared with ipilimumab or isotype antibody (**Figure 7**). At higher anti-CD3 mAb doses, anti-sCTLA-4 clone JMW-3B3 also enhanced IL-17 but not IFN-γ levels compared with ipilimumab (**Figure 7**). Once again there were no effects on IL-10 levels by either pan-CTLA-4 or sCTLA-4 selective antibody blockade (**Supplementary Figure 2**). The increases in cytokine production from the healthy donor volunteer cohort were very similar to those identified previously (37).

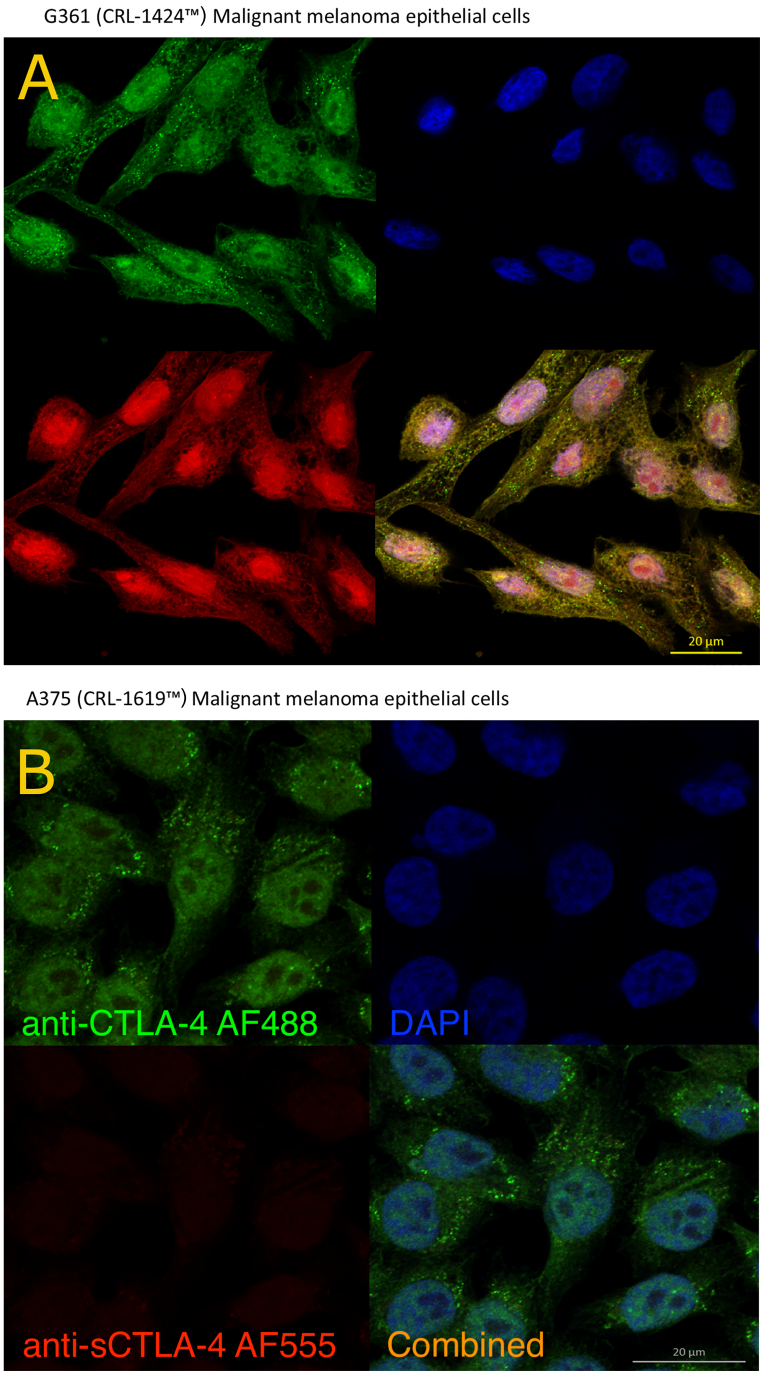

FIGURE 5 | Analysis of CTLA-4 receptor and sCTLA-4 in melanoma cell lines by confocal microscopy. G-361 **(A)** and A-375 **(B)** human melanoma cell lines were incubated on sterile poly-L-lysine coated coverslips until confluence was reached and stained with specific anti-sCTLA-4 mAb (JMW-3B3, AF555 - red) and rabbit polyclonal anti-panCTLA-4 (AF488 – green) together with DAPI (blue). Both antibodies bind sCTLA-4 yielding a yellow/orange color. Representative of n>3 experiments.

DISCUSSION

Having previously identified the soluble isoform of CTLA-4 as a candidate regulatory molecule capable of suppressing antigen specific effector T cell responses (36), we demonstrate here for the first time a novel mechanism based specifically on the TGFβ2 isoform that drives sCTLA-4 to be produced at high levels in human patients with melanoma and which we propose is used as a tumor immune evasion strategy. We provide further evidence that sCTLA-4 contributes to immune regulation through T cell

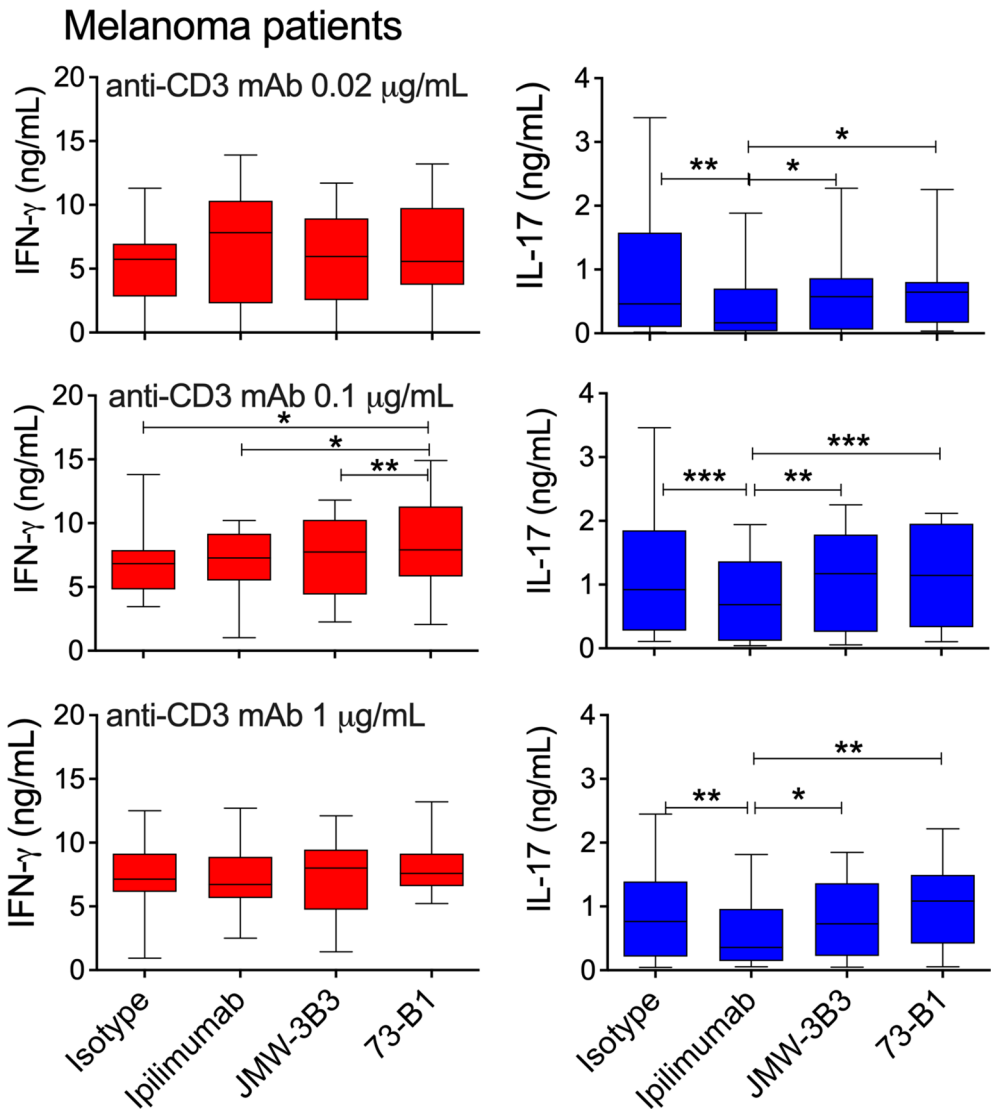

FIGURE 6 | Comparative effect of anti-sCTLA-4 or ipilimumab anti-CTLA-4 mAb blockade on melanoma patient donor PBMC responses to stimulation with 0, 0.1 or 1 μg/mL anti-CD3 mAb. PBMC were stimulated for five days at 37°C 5%CO2 in the presence of plate-bound anti-CD3 and 10 μg/mL IgG1 isotype control, anti-sCTLA-4 mAbs JMW-3B3 and 73-B1, and anti-CTLA-4 mAb, ipilimumab. Cell culture supernatants were measured by ELISA for levels of IFN-γ and IL-17A. n=12; *P < 0.05 **P < 0.01, ***P < 0.001, P values determined from a one-way test with Tukey post-hoc analysis).

suppression with efficacy similar to that of the artificial soluble form of CTLA-4, CTLA4-Ig and selective blockade of sCTLA-4 also enhanced culture supernatant levels of both IFN-γ and IL-17A effector cytokines in anti-CD3 activated PBMC from volunteer melanoma patients.

Although it is clear that CTLA-4 blockade can induce anti-tumor immunity with increased tumor infiltration of cytotoxic effector T cells, most studies do not account for any potential immunoregulatory contribution from the soluble isoform of CTLA-4. Is this second secretory isoform really of no consequence at all to immune regulation or indeed immunotherapy? Generally, sCTLA-4 is produced by resting immune cells including $CD8^+$ T cells, regulatory T cells, monocytes and B cells, but is also secreted by some non-immune cells, notably pituitary gland cells (41) and cancer cells (42). Recent work identified sCTLA-4 to be differentially expressed by thirty different cancer types building upon seminal observations of sCTLA-4 expression in melanoma and mesothelioma (28–30). Previous studies have indicated that sCTLA-4 is immunosuppressive, so to examine this more carefully, we produced recombinant human sCTLA-4 and incubated it with healthy donor PBMC stimulated with anti-CD3 mAb or Jurkat T cells stimulated with PHA-L and B7.1-Ig or B7.2-Ig. We demonstrated that sCTLA-4 was immunosuppressive and capable of inhibiting $CD4^+$ and $CD8^+$ T cell proliferation and cytokine secretion *in vitro*, partially reversible by selective antibody blockade. Further, sCTLA-4 blockade also increased cell surface

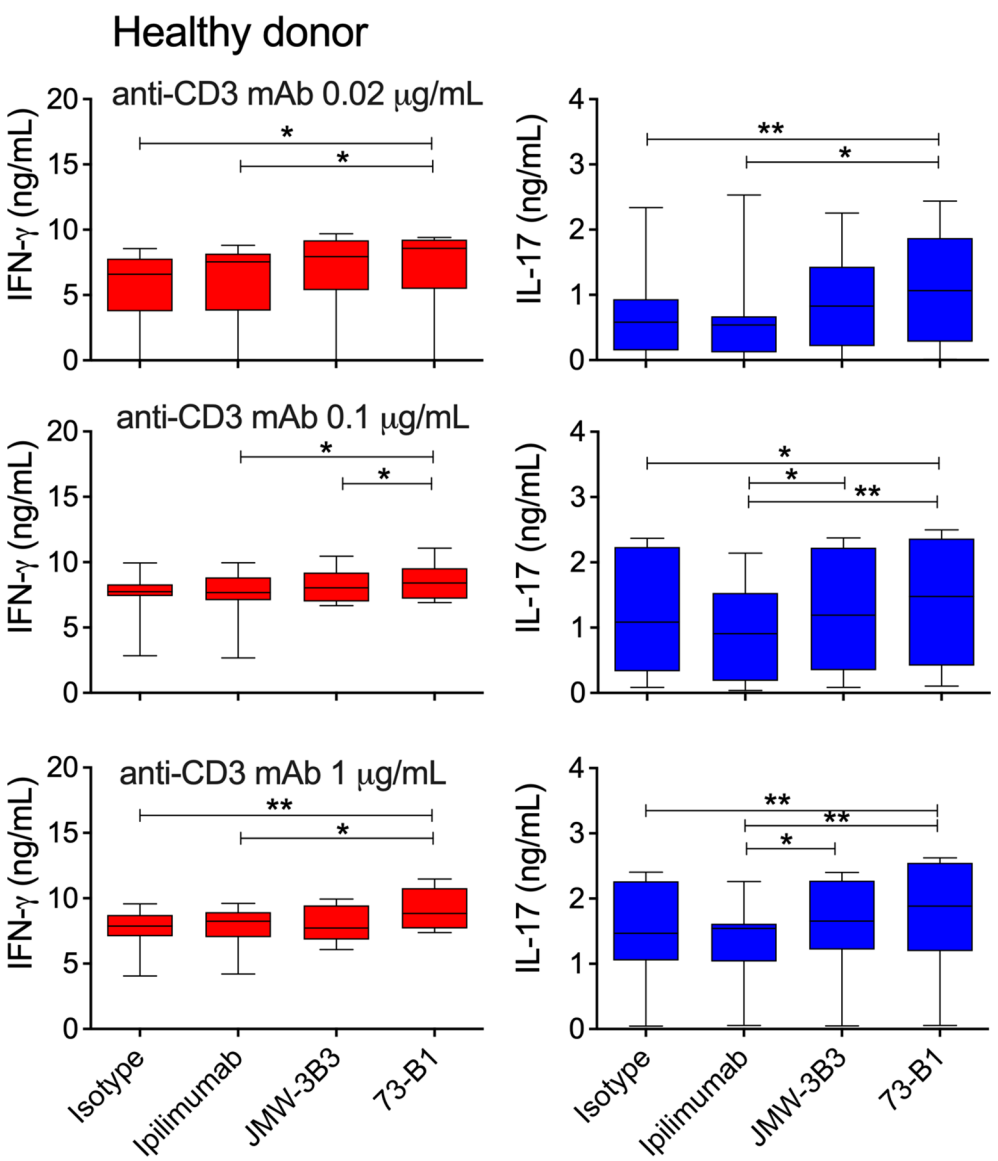

FIGURE 7 | Comparative effect of anti-sCTLA-4 or ipilimumab anti-CTLA-4 mAb blockade on healthy donor PBMC responses to stimulation with 0, 0.1 or 1 μg/mL anti-CD3 mAb. PBMC were stimulated for five days at 37°C 5%CO_2 in the presence of plate-bound anti-CD3 and 10 μg/mL IgG1 isotype control, anti-sCTLA-4 mAbs JMW-3B3 and 73-B1, and anti-CTLA-4 mAb, ipilimumab. Cell culture supernatants were measured by ELISA for levels of IFN-γ and IL-17A. n=8; *P < 0.05 **P < 0.01, P values determined a one-way test with Tukey *post-hoc* analysis).

levels of CD80/86 on $CD11c^+$ APC stimulated with LPS although it remains uncertain whether that arises from an increase in expression levels or is simply an effect of removing sCTLA-4 to allow free display of more CD80/CD86 receptors. In these studies, the immunosuppressive potential of sCTLA-4 approached that of CTLA4-Ig, the dimeric fusion protein, which has proven clinically useful for the treatment of rheumatoid arthritis. The CTLA-4 receptor has relatively high affinity for its B7 ligands largely because it forms a functional homo-dimeric complex resulting from a disulphide bridge between cysteine residues at position 127. This residue is lost in sCTLA-4, which has led to the notion that sCTLA-4 is monomeric and therefore deprived of the potent immunosuppressive qualities of its dimeric receptor counterpart and indeed recombinant CTLA4-Ig, which is also dimeric. However, recent data suggest a novel mechanism, in which sCTLA-4 binds to the RGMB receptor enhancing both sCTLA-4 engagement with B7 ligands and its immunosuppressive action thus potentially explaining its capacity for immunosuppression (30).

An interesting corollary to this work was the observation that antibody blockade of the CTLA-4 receptor in both $CD4^+$ and $CD8^+$ T cells stimulated with anti-CD3 mAb, suppressed phosphorylation levels of the key T cell signalling intermediates ZAP-70 and Slp76 implying that crosslinking of CTLA-4 by antibody can to some extent at least reduce the activational

capacity of individual effector T cells. This probably explains explain some of the inhibitory effects of anti-CTLA-4 antibody blockade originally observed during characterisation of CTLA-4 function (43). Antibodies that selectively target sCTLA-4 are in effect, functionally exempted from having this inhibitory capacity, because they do not bind to CTLA-4 on cells.

Previously, we identified regulatory T cells to express relatively high amounts of sCTLA-4 and set about investigating whether or not TGFβ plays a role in increasing levels of sCTLA-4. Initially our data were mixed with no clear evidence that TGFβ1 consistently contributed to induction in human T cells. Serum levels, however, of a second isoform, TGFβ2, have previously been demonstrated to correlate with disease progression from primary lesions to malignant distal metastasis (44). TGFβ2 was originally isolated from a glioblastoma cell line as Glioblastoma-derived T-cell suppressor factor (G-TSF) and since then several cancers including melanoma, have been found to secrete increased amounts of this immunosuppressive cytokine (32). Our study confirmed significantly higher serum levels of both TGFβ1 and TGFβ2 within the melanoma patient cohort, while analysis of lupus patient sera from a previous study identified high levels of TGFβ1 but little TGFβ2 compared with healthy donor sera. The correlation of sCTLA-4 with TGFβ2 serum levels exclusively in melanoma patient sera was surprising and immediately raised the notion of a potential new mechanism of immune cell evasion. This idea was supported by our data showing that a TGFβ2 based induction protocol did indeed drive both $CD4^+$ and $CD8^+$ T cells to produce significantly high culture supernatant levels of sCTLA-4 in both humans and mice. Collectively, these observations raise the notion that in melanoma, tumor cells can modulate sCTLA-4 production by T cells, perhaps as a mechanism of immune evasion. Our analysis of sCTLA-4 in the lupus patient cohort indicates that increased sCTLA-4 production is not, however, exclusively dependent on TGFβ2 but may be associated particularly with cancers known to secrete high levels of the TGFβ2 isoform. It is now essential that levels of both TGFβ2 and sCTLA-4 are assessed in other cancers associated with increased TGFβ2 levels, especially those that have previously been identified as "cold" tumor types (45).

We also examined whether or not selective anti-sCTLA-4 antibody blockade of sCTLA-4 influenced immune response intensity following stimulation of PBMC with increasing levels of stimulatory anti-CD3 mAb in patients suffering melanoma. Previously, we identified selective blockade of sCTLA-4 to enhance IL-17 and IFN-γ effector cytokine supernatant levels of healthy donor PBMC stimulated with recall antigens or anti-CD3 mAb (36). In a large cohort of lupus patients, however, we could not detect this cytokine enhancement (37). In this study, once again PBMC from the healthy donor cohort responded to sCTLA-4 antibody blockade as before by producing significantly increased levels of both IFN-γ and IL-17, whereas PBMC from the melanoma patient cohort was slightly less responsive to sCTLA-4 blockade with regard to IFN-γ but not IL-17. The increased effects of sCTLA-4 blockade by anti-sCTLA-4 clone 73-B1, particularly of IL-17, and to a lesser extent, IFN-γ, compared with ipilimumab arises partially because blockade with ipilimumab decreases cytokine levels slightly. This may point to a cross-linking effect in which anti-CTLA-4 antibodies rather than blocking CTLA-4 mediated inhibition, deliver an agonist inhibitory signal to the cytokine secreting effector T cell (see **Figure 2**), but this has not been confirmed. This does, however, need to be evaluated in more detail. It is likely that there are other immunosuppressive elements, e.g., IL-10 or TGFβ or PD-1 mediated inhibitory activity, influencing immune response intensity in melanoma patients. Another aspect that is being actively pursued is whether sCTLA-4 selectively inhibits the secretion of individual cytokines, or if it has general immunosuppressive effects like recombinant soluble CTLA4-Ig. Serum from patients with high levels of sCTLA-4 was immunosuppressive to healthy donor PBMC responses and was partially but not completely reversed by anti-sCTLA-4 blockade, supporting the notion that other soluble immunosuppressive factors are at play (data not shown).

Together, the data here support a role for sCTLA-4 in cancer immunoregulation, which is almost certain to influence current therapeutic approaches based on anti-CTLA-4 antibodies. Indeed, sCTLA-4 may itself form a therapeutic target.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by East of Scotland Research Ethics Committee (ref:13/NS/0126 & 10/S1401/20). The patients/participants provided their written informed consent to participate in this study. Ethical review and approval was not required for the animal study because tissues were obtained from mice already euthanised under the UK schedule 1 protocol outlined in the the Animals (Scientific Procedures) Act 1986.

AUTHOR CONTRIBUTIONS

RK: designed and performed research studies including acquiring and analysing data and contributed to writing the manuscript. CZ and FA-F: designed and performed research studies including acquiring and analysing data. LL: managed the clinical study including recruiting patients, maintaining patient logs and co-ordinating between the science and clinical teams. ID: contributed to study design, recruiting patient volunteers and providing clinical advice. FMe, FMu, and MN: contributed to study design, recruiting patient volunteers and providing clinical advice. LD and RA: contributed to study design and writing of the manuscript. SR: contributed to study design, recruiting patient volunteers and providing clinical advice. RB: contributed to study design and writing of the manuscript.

AO: contributed to study design, co-ordinated the clinical study, ethics applications and writing of the manuscript. FW: designed and co-ordinated the study, performed research studies, analysed data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.763877/full#supplementary-material

REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* (2021) 71:209–49. doi: 10.3322/caac.21660
2. Sharma P, Allison JP. The Future of Immune Checkpoint Therapy. *Science* (2015) 348:56–61. doi: 10.1126/science.aaa8172
3. Brunet JF, Denizot F, Luciani MF, Roux-Dosseto M, Suzan M, Mattei MG, et al. A New Member of the Immunoglobulin Superfamily–CTLA-4. *Nature* (2022) 328:267–70. doi: 10.1038/328267a0
4. Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, et al. CTLA-4 Can Function as a Negative Regulator of T Cell Activation. *Immunity* (1994) 1:405–13. doi: 10.1016/1074-7613(94)90071-X
5. Leach DR, Krummel MF, Allison JP. Enhancement of Antitumor Immunity by CTLA-4 Blockade. *Science* (1996) 271:1734–6. doi: 10.1126/science.271.5256.1734
6. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 Family Revisited. *Annu Rev Immunol* (2005) 23:515–48. doi: 10.1146/annurev.immunol.23.021704.115611
7. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved Survival With Ipilimumab in Patients With Metastatic Melanoma. *N Engl J Med* (2010) 363:711–23. doi: 10.1056/NEJMoa1003466
8. Postow MA, Chesney J, Pavlick AC, Robert C, Grossmann K, McDermott D, et al. Nivolumab and Ipilimumab Versus Ipilimumab in Untreated Melanoma. *N Engl J Med* (2015) 372:2006–17. doi: 10.1056/NEJMoa1414428
9. Motzer RJ, Tannir NM, McDermott DF, Aren Frontera O, Melichar B, Choueiri TK, et al. Nivolumab Plus Ipilimumab Versus Sunitinib in Advanced Renal-Cell Carcinoma. *N Engl J Med* (2018) 378:1277–90. doi: 10.1056/NEJMoa1712126
10. Overman MJ, Lonardi S, Wong KYM, Lenz HJ, Gelsomino F, Aglietta M, et al. Durable Clinical Benefit With Nivolumab Plus Ipilimumab in DNA Mismatch Repair-Deficient/Microsatellite Instability-High Metastatic Colorectal Cancer. *J Clin Oncol* (2018) 36:773–9. doi: 10.1200/JCO.2017.76.9901
11. Schadendorf D, Hodi FS, Robert C, Weber JS, Margolin K, Hamid O, et al. Pooled Analysis of Long-Term Survival Data From Phase II and Phase III Trials of Ipilimumab in Unresectable or Metastatic Melanoma. *J Clin Oncol* (2015) 33:1889–94. doi: 10.1200/JCO.2014.56.2736
12. Robert C, Ribas A, Wolchok JD, Hodi FS, Hamid O, Kefford R, et al. Anti-Programmed-Death-Receptor-1 Treatment With Pembrolizumab in Ipilimumab-Refractory Advanced Melanoma: A Randomised Dose-Comparison Cohort of a Phase 1 Trial. *Lancet* (2014) 384:1109–17. doi: 10.1016/S0140-6736(14)60958-2
13. Rizvi NA, Mazieres J, Planchard D, Stinchcombe TE, Dy GK, Antonia SJ, et al. Activity and Safety of Nivolumab, an Anti-PD-1 Immune Checkpoint Inhibitor, for Patients With Advanced, Refractory Squamous Non-Small-Cell Lung Cancer (CheckMate 063): A Phase 2, Single-Arm Trial. *Lancet Oncol* (2015) 16:257–65. doi: 10.1016/S1470-2045(15)70054-9
14. Kaufman HL, Russell J, Hamid O, Bhatia S, Terheyden P, D'Angelo SP, et al. Avelumab in Patients With Chemotherapy-Refractory Metastatic Merkel Cell Carcinoma: A Multicentre, Single-Group, Open-Label, Phase 2 Trial. *Lancet Oncol* (2016) 17:1374–85. doi: 10.1016/S1470-2045(16)30364-3
15. Massard C, Gordon MS, Sharma S, Rafii S, Wainberg ZA, Luke J, et al. Safety and Efficacy of Durvalumab (MEDI4736), an Anti-Programmed Cell Death Ligand-1 Immune Checkpoint Inhibitor, in Patients With Advanced Urothelial Bladder Cancer. *J Clin Oncol* (2016) 34:3119–25. doi: 10.1200/JCO.2016.67.9761
16. Rosenberg JE, Hoffman-Censits J, Powles T, van der Heijden MS, Balar AV, Necchi A, et al. Atezolizumab in Patients With Locally Advanced and Metastatic Urothelial Carcinoma Who Have Progressed Following Treatment With Platinum-Based Chemotherapy: A Single-Arm, Multicentre, Phase 2 Trial. *Lancet* (2016) 387:1909–20. doi: 10.1016/S0140-6736(16)00561-4
17. Michot JM, Bigenwald C, Champiat S, Collins M, Carbonnel F, Postel-Vinay S, et al. Immune-Related Adverse Events With Immune Checkpoint Blockade: A Comprehensive Review. *Eur J Cancer* (2016) 54:139–48. doi: 10.1016/j.ejca.2015.11.016
18. Daud AI, Wolchok JD, Robert C, Hwu WJ, Weber JS, Ribas A, et al. Programmed Death-Ligand 1 Expression and Response to the Anti-Programmed Death 1 Antibody Pembrolizumab in Melanoma. *J Clin Oncol* (2016) 34:4102–9. doi: 10.1200/JCO.2016.67.2477
19. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, et al. Signatures of Mutational Processes in Human Cancer. *Nature* (2013) 500:415–21. doi: 10.1038/nature12477
20. Alexandrov LB, Ju YS, Haase K, Van Loo P, Martincorena I, Nik-Zainal S, et al. Mutational Signatures Associated With Tobacco Smoking in Human Cancer. *Science* (2016) 354:618–22. doi: 10.1126/science.aag0299
21. Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, et al. Fc-Dependent Depletion of Tumor-Infiltrating Regulatory T Cells Co-Defines the Efficacy of Anti-CTLA-4 Therapy Against Melanoma. *J Exp Med* (2013) 210:1695–710. doi: 10.1084/jem.20130579
22. Bauche D, Mauze S, Kochel C, Grein J, Sawant A, Zybina Y, et al. Antitumor Efficacy of Combined CTLA4/PD-1 Blockade Without Intestinal Inflammation Is Achieved by Elimination of FcgammaR Interactions. *J Immunother Cancer* (2020) 8:10.1136/jitc–2020. doi: 10.1136/jitc-2020-001584
23. Magistrelli G, Jeannin P, Herbault N, Benoit De Coignac A, Gauchat JF, Bonnefoy JY, et al. A Soluble Form of CTLA-4 Generated by Alternative Splicing Is Expressed by Nonstimulated Human T Cells. *Eur J Immunol* (1999) 29:3596–602. doi: 10.1002/(SICI)1521-4141(199911)29:113.0.CO;2-Y
24. Oaks MK, Hallett KM, Penwell RT, Stauber EC, Warren SJ, Tector AJ. A Native Soluble Form of CTLA-4. *Cell Immunol* (2000) 201:144–53. doi: 10.1006/cimm.2000.1649
25. Leung AM, Lee AF, Ozao-Choy J, Ramos RI, Hamid O, O'Day SJ, et al. Clinical Benefit From Ipilimumab Therapy in Melanoma Patients may be

Associated With Serum CTLA4 Levels. *Front Oncol* (2014) 4:110. doi: 10.3389/fonc.2014.00110
26. Pistillo MP, Fontana V, Morabito A, Dozin B, Laurent S, Carosio R, et al. Soluble CTLA-4 as a Favorable Predictive Biomarker in Metastatic Melanoma Patients Treated With Ipilimumab: An Italian Melanoma Intergroup Study. *Cancer Immunol Immunother* (2019) 68:97–107. doi: 10.1007/s00262-018-2258-1
27. Simone R, Tenca C, Fais F, Luciani M, De Rossi G, Pesce G, et al. A Soluble Form of CTLA-4 Is Present in Paediatric Patients With Acute Lymphoblastic Leukaemia and Correlates With CD1d+ Expression. *PloS One* (2012) 7: e44654. doi: 10.1371/journal.pone.0044654
28. Laurent S, Queirolo P, Boero S, Salvi S, Piccioli P, Boccardo S, et al. The Engagement of CTLA-4 on Primary Melanoma Cell Lines Induces Antibody-Dependent Cellular Cytotoxicity and TNF-Alpha Production. *J Transl Med* (2013) 11:108–5876. doi: 10.1186/1479-5876-11-108
29. Roncella S, Laurent S, Fontana V, Ferro P, Franceschini MC, Salvi S, et al. CTLA-4 in Mesothelioma Patients: Tissue Expression, Body Fluid Levels and Possible Relevance as a Prognostic Factor. *Cancer Immunol Immunother* (2016) 65:909–17. doi: 10.1007/s00262-016-1844-3
30. Sekiya T, Takaki S. RGMB Enhances the Suppressive Activity of the Monomeric Secreted Form of CTLA-4. *Sci Rep* (2019) 9:6984. doi: 10.1038/s41598-019-43068-y
31. Massague J. TGFbeta in Cancer. *Cell* (2008) 134:215–30. doi: 10.1016/j.cell.2008.07.001
32. Batlle E, Massague J. Transforming Growth Factor-Beta Signaling in Immunity and Cancer. *Immunity* (2019) 50:924–40. doi: 10.1016/j.immuni.2019.03.024
33. Heldin CH, Vanlandewijck M, Moustakas A. Regulation of EMT by TGFbeta in Cancer. *FEBS Lett* (2012) 586:1959–70. doi: 10.1016/j.febslet.2012.02.037
34. David CJ, Huang YH, Chen M, Su J, Zou Y, Bardeesy N, et al. TGF-Beta Tumor Suppression Through a Lethal EMT. *Cell* (2016) 164:1015–30. doi: 10.1016/j.cell.2016.01.009
35. David CJ, Massague J. Contextual Determinants of TGFbeta Action in Development, Immunity and Cancer. *Nat Rev Mol Cell Biol* (2018) 19:419–35. doi: 10.1038/s41580-018-0007-0
36. Ward FJ, Dahal LN, Wijesekera SK, Abdul-Jawad SK, Kaewarpai T, Xu H, et al. The Soluble Isoform of CTLA-4 as a Regulator of T-Cell Responses. *Eur J Immunol* (2013) 43:1274–85. doi: 10.1002/eji.201242529
37. Dahal LN, Basu N, Youssef H, Khanolkar RC, Barker RN, Erwig LP, et al. Immunoregulatory Soluble CTLA-4 Modifies Effector T-Cell Responses in Systemic Lupus Erythematosus. *Arthritis Res Ther* (2016) 18:180–016. doi: 10.1186/s13075-016-1075-1
38. Hall AM, Ward FJ, Vickers MA, Stott LM, Urbaniak SJ, Barker RN. Interleukin-10-Mediated Regulatory T-Cell Responses to Epitopes on a Human Red Blood Cell Autoantigen. *Blood* (2002) 100:4529–36. doi: 10.1182/blood-2002-05-1383
39. Lamouille S, Xu J, Derynck R. Molecular Mechanisms of Epithelial-Mesenchymal Transition. *Nat Rev Mol Cell Biol* (2014) 15:178–96. doi: 10.1038/nrm3758
40. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, et al. Conversion of Peripheral CD4+CD25- Naive T Cells to CD4+CD25+ Regulatory T Cells by TGF-Beta Induction of Transcription Factor Foxp3. *J Exp Med* (2003) 198:1875–86. doi: 10.1084/jem.20030152
41. Iwama S, De Remigis A, Callahan MK, Slovin SF, Wolchok JD, Caturegli P. Pituitary Expression of CTLA-4 Mediates Hypophysitis Secondary to Administration of CTLA-4 Blocking Antibody. *Sci Transl Med* (2014) 6 (230):230ra45. doi: 10.1126/scitranslmed.3008002
42. Abdulkhaleq F, Larossi N, Ogbonda O, Abu-Eid R, Ward F. CTLA-4 Expression by Human Tumor Cells and Its Impact on Immunotherapeutic Strategies: A Systematic Review. *Immuno Oncol Insights* (2021) 2(3):151–69. doi: 10.18609/ioi.2021.024
43. Krummel MF, Allison JP. CD28 and CTLA-4 Have Opposing Effects on the Response of T Cells to Stimulation. *J Exp Med* (1995) 182:459–65. doi: 10.1084/jem.182.2.459
44. Reed JA, McNutt NS, Prieto VG, Albino AP. Expression of Transforming Growth Factor-Beta 2 in Malignant Melanoma Correlates With the Depth of Tumor Invasion. Implications for Tumor Progression. *Am J Pathol* (1994) 145:97–104.
45. Schlingensiepen KH, Schlingensiepen R, Steinbrecher A, Hau P, Bogdahn U, Fischer-Blass B, et al. Targeted Tumor Therapy With the TGF-Beta 2 Antisense Compound AP 12009. *Cytokine Growth Factor Rev* (2006) 17:129–39. doi: 10.1016/j.cytogfr.2005.09.002

Conflict of Interest: FW, RB, and LD are named inventors on patent entitled "*Antibodies specifically directed to a soluble form of CTLA-4*" (Patent No. US8697845 B2). FW is a director on the board of Aperio Pharma Ltd., which is developing anti-sCTLA-4 based antibody immunotherapy.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Predictive Value of *MAP2K1/2* Mutations on Efficiency of Immunotherapy in Melanoma

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Background: *MAP2K1/2* genes are mutated in approximately 8% of melanoma patients; however, the impact of *MAP2K1/2* gene alterations on the efficiency of immunotherapy has not been clarified. This study focused on the correlation between *MAP2K1/2* gene mutations and the treatment response.

Methods: Six metastatic melanoma clinical cohorts treated with immune checkpoint inhibitors [anti-cytotoxic T lymphocyte antigen-4 (CTLA-4) or anti-programmed cell death-1 (PD-1)] were recruited in this study. RNA expression profiling results from each of these six cohorts and the Cancer Genome Atlas (TCGA) melanoma cohort were analysed to explore the mechanism related to immune activation.

Results: Compared to patients with wild-type *MAP2K1/2*, those with *MAP2K1/2* mutations in an independent anti-CTLA-4-treated cohort had higher objective response rates, longer progression-free survival, and longer overall survival (OS). These findings were further validated in a pooled anti-CTLA-4-treated cohort in terms of the OS. However, there was no correlation between *MAP2K1/2* mutations and OS in the anti-PD-1-treated cohort. Subgroup Cox regression analysis suggested that patients with *MAP2K1/2* mutations received fewer benefits from anti-PD-1 monotherapy than from anti-CTLA-4 treatment. Furthermore, transcriptome profiling analysis revealed that melanoma tumours with *MAP2K* mutation was enriched in $CD8^+$ T cells, B cells, and neutrophil cells, also expressed high levels of CD33 and IL10, implying a potential mechanism underlying the benefit of melanoma patients with *MAP2K1/2* mutations from anti-CTLA-4 treatment.

Conclusions: *MAP2K1/2* mutations were identified as an independent predictive factor for anti-CTLA-4 therapy in melanoma patients. Anti-CTLA-4 treatment might be more effective than anti-PD-1 therapy for patients with *MAP2K1/2*-mutated melanoma.

Keywords: melanoma, CTLA-4 blockade, PD-1 blockade, MAPK pathway, immunotherapy

BACKGROUND

Treatment with immune checkpoint inhibitors (ICIs), including antibodies targeting cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death-1/programmed cell death ligand 1 (PD-1/PD-L1), is becoming a novel therapeutic paradigm for melanomas (1). Ipilimumab, an anti-CTLA-4 monoclonal antibody, has been found to significantly improve overall survival (OS) and progression-free survival (PFS) and increase the long-term survival rate of patients with advanced melanoma (2, 3). Compared to ipilimumab, second-generation ICIs targeting PD-1, namely nivolumab and pembrolizumab, have also been reported to induce an increased response rate, OS, and PFS, with superior toxicity profiles (4, 5). Moreover, ICIs are the standard of care in the systemic treatment of metastatic or unresectable melanomas (6).

Although ICIs significantly increase the survival of melanoma patients, only a subset of patients can benefit from the therapy, and the related mechanisms are still not fully understood. Therefore, the identification of biomarkers to select patients who will be more responsive to ICIs is of utmost importance. Several genomic features, such as high mutational load, high neoantigen load, and tumour clonality have been found to be predictive of a favourable response to anti-CTLA-4 therapy in melanoma patients (7, 8). In addition, it has been reported that aberrations of individual genes, such as *SERPINB3/SERPINB4*, *NRAS*, and *TP53*, are associated with the response to anti-CTLA-4 therapy (9–11). Either high PD-L1 expression or high tumour mutational burden (TMB) has also been recognized as predictors of the effectiveness of anti-PD-1 blockade in melanomas and other solid tumours (12, 13).

The status of driver mutations may also influence the response to ICIs. For example, *NRAS*-mutated melanoma is a distinct subtype in approximately 5%–20% of patients with melanomas and appears to have a poor prognosis (12, 13). In a retrospective analysis, compared to melanoma patients with wild-type *NRAS*, those with *NRAS* mutations were found to have higher objective response rates and prolonged stable disease in response to ICIs (11). *NRAS* encodes N-Ras, which is a component of the Ras/Raf/MEK/ERK signalling cascade (14). This cascade, also known as the Ras/MAPK signalling cascade, plays an important role in the pathogenesis of melanoma (15). MEK is one of the kinases involved in the Ras/MAPK signalling cascade. Moreover, the encoding genes *MAP2K1* and *MAP2K2* (*MAP2K1/2*) are frequently mutated in melanoma, with a frequency of approximately 8% of cases (16, 17). The occurrence of *MAP2K1/2* mutations was identified as a mechanism related to BRAF inhibitor resistance in melanoma (18). To date, there are no reports on effective small molecule inhibitors targeting *MAP2K1/2* in melanoma. It is also unclear whether mutations in these genes influence the efficacy of immunotherapy.

Preclinical studies have revealed that treatment with MEK inhibitors might improve the sensitivity of immunotherapy in melanoma. MEK inhibition in a melanoma cell line was found to increase the antigen levels, which might potentiate anti-tumour T-cell immunity (19). Moreover, MEK inhibition may reduce the number of Bregs while sparing anti-tumour B-cell function, thereby enhancing anti-tumour immunity (20). In mouse models, MEK inhibitors were found to inhibit tumour growth *via* increasing the number of intertumoral effector-phenotype $CD8^+$ T cells, and combination therapy with both MEK inhibitors and anti-PD-L1 agents exhibited a synergistic effect on antitumor growth (21).

In this study, through analysis of the sequencing and survival data for several public cohorts with metastatic melanoma, we investigated the association between *MAP2K1/2* mutations and the response to anti-CLTA-4 and anti-PD-1 immunotherapies. The influence of *MAP2K1/2* mutations on the expression of immunity-related genes was also evaluated by analysing the RNA expression profile data collected from these cohorts and from the Cancer Genome Atlas (TCGA) melanoma cohort. Our aim is to clarify the impact of *MAP2K1/2* gene alterations on the efficiency of immunotherapies and to provide guidance for treatment decision-making in *MAP2K1/2*-mutated melanomas.

Abbreviations: MAP2K, mitogen-activated protein kinase kinase; MAPK, mitogen-activated protein kinase; CTLA-4, cytotoxic T lymphocyte antigen-4; PD-1/PD-L1, programmed cell death-1/ligand-1; ICB, immune checkpoint blockade; ICIs, immune checkpoint inhibitors; CNAs, copy number alterations; WES, whole-exome sequencing; TMB, tumour mutational burden; MSI, microsatellite instability; SKCM, skin cutaneous melanoma; TCGA, The Cancer Genome Atlas; MSKCC, memorial Sloan Kettering cancer center; OS, overall survival; PFS, progression-free survival; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

METHODS

Eligible Literature Search

We performed a systematic computerized search of the MEDLINE (PubMed) database and the Embases database up to November 1, 2020. The search terms were as follows: (Melanomas [MeSH] OR "metastatic melanomas" [Title/Abstract]) AND ("PD-1 blockade"[Title/Abstract] OR "PD-L1 blockade" [Title/Abstract] OR "CTLA-4 blockade" [Title/Abstract] OR "immune checkpoint inhibitor" [Title/Abstract] OR "immune checkpoint inhibitors" [Title/Abstract] OR "ICI" [Title/Abstract] OR "ICIs" [Title/Abstract] OR "immune checkpoint blockade" [Title/Abstract] OR "immune checkpoint blockades" [Title/Abstract] OR "ICB" [Title/Abstract] OR "ICBs" [Title/Abstract]). Studies with eligible next-generation sequencing data were identified by hand and included if they met the criteria: (a) Clinical trials or study cohorts treated with ICIs; (b) Clinical outcomes of patients were available; (c) The number of evaluable patients was more than 30. We found six cohort studies of metastatic melanoma, specifically the Allen (8), Snyder (7), Hugo (22), and Liu (23) cohorts, and two metastatic pan-cancer cohorts comprising patients with melanoma, namely the Miao (24) and Samstein (25) cohorts.

Study Design and Data Acquisition

In this study, missense, nonsense, and frame-shift mutations in both *MAP2K1* and *MAP2K2* genes were defined as *MAP2K1/2* mutations.

In total, data for 753 melanoma patients were included in our study. Notably, melanoma patients treated with sequential CTLA-4 and PD-1 blockades were excluded from this study. First, we determined the predictive value for CTLA-4 monotherapy of *MAP2K1/2* mutations in the Allen cohort and validated this predictive value in a CTLA-4-monotherapy-pooled cohort comprising 239 melanoma samples from the Snyder, Miao, and Samstein cohorts. Thereafter, we explored the impact of *MAP2K1/2* mutations on OS in a PD-1-monotherapy-pooled cohort consisting of 285 melanoma samples from the Hugo, Liu, Miao, and Samstein cohorts. The TCGA-skin cutaneous melanoma (SKCM) cohort without ICI treatment (n = 455) and with chemotherapy (n = 73) was analysed to assess the prognostic value of *MAP2K1/2* (**Figure S1**).

Data accessibility: data for whole-exome sequencing (WES), copy number alterations (CNAs), gene expression, and clinicopathologic information were collected from the Allen, Snyder, Hugo, and TCGA-SKCM cohorts. Data for targeted-sequencing and OS were obtained from the Samstein cohort using the cBioPortal database. Data for the Liu and Miao cohorts were taken from previous publications.

Evaluation of Clinical Response

In evaluating the clinical response, responders were defined as melanoma patients with different degrees of clinical responses to immunotherapy, characterized as complete response (CR), partial response (PR), or stable disease (SD). Non-responders were defined as those patients with disease progression (PD) following immunotherapy.

Transcriptome Data Normalization and Processing

The transcriptome data were interpreted as fragments per kilobase million mapped reads (FPKM). To compensate for RNA-seq counts within and between samples, the FPKM for every gene was transformed into transcripts per kilobase million (TPM) values by dividing by the sum of FPKM in each sample. The immune filtration cells scores were estimated with the TIMER2.0 website tool using TPM data.

Statistical Analysis

In this study, all data analysis and graphic plotting were performed using the R software (v.3.6.3). Kaplan–Meier curves (log-rank test) of OS were plotted to compare survival outcomes between different subgroups. The proportion of death events of the patients in *MAP2K1/2*-mutated group and *MAP2K1/2*-wild-type groups was defined as risk ratio (RR). Pooled analysis was performed using the DerSimonian–Laird random-effects model to compare the RR with 95% of confidence intervals (CI) The degree of heterogeneity between cohorts was assessed *via* the I^2 index. No significant heterogeneity was defined as $I^2 < 50\%$ and P >0.1. Univariate and multivariate Cox proportional hazard regression analyses were performed to quantify the hazard ratio (HR) of various characteristics. Fisher's exact tests were used to determine if there were non-random associations between categorical variables. The Wilcoxon–Mann–Whitney test was used to compare the differences in discrete ordinal data between two independent groups.

RESULTS

MAP2K1/2 Mutations as a Biomarker to Predict Favourable Response to Anti-CTLA-4 Therapy and Survival in Metastatic Melanoma

Data analysis was performed using a cohort of 110 patients (Van Allen cohort) with metastatic melanoma treated with ipilimumab. The characteristics of the patients are summarized in **Table S1**. Seven patients (6.36%) in this cohort harboured *MAP2K1* or *MAP2K2* mutations and had longer OS (49.2 months vs 8.3 months; HR = 0.2; 95% confidence interval (CI), 0.05–0.83; p = 0.0262; **Figure 1A**) and PFS (19.4 months vs 2.8 months; HR = 0.37; 95% CI, 0.15–0.91; p = 0.0307; **Figure 1B**) than those with wild-type *MAP2K1/2*. Moreover, *MAP2K1/2* mutations were more frequent in responders (17.6% vs 1.3%; p = 0.0185; **Figure 1C**). In univariate analyses, three factors, namely *MAP2K1/2* mutations, tumour stage (stage 4 vs stage 3), and the presence of lactate dehydrogenase (LDH; 1 vs 0), were found to be associated with immunotherapeutic OS and PFS (**Table 1**). Furthermore, in the multivariable Cox proportional hazards regression model adjusted by tumour stage, LDH, and *MAP2K1/2* mutations, these three factors were still significantly associated with OS (HR = 0.24; 95% CI, 0.059–0.99; p = 0.048; **Table 1**) and PFS (HR = 0.39; 95% CI, 0.16–0.99; p = 0.048; **Table 1**). The results suggested that *MAP2K1/2* mutations might be an independent predictor for a favourable clinical response to anti-CTLA4 therapy in patients with melanoma.

To validate the predictive value of *MAP2K1/2* mutations for the efficacy of anti-CTLA4 therapy in melanoma, data analysis was also performed using a pooled cohort, which was a combination of three metastatic melanoma cohorts treated with ipilimumab (Miao, Samstein, and Snyder) (**Table S1**). This pooled cohort contained 239 patients, among which 24 patients (10%) harboured *MAP2K1* or *MAP2K2* mutations. According to the data analysis results, *MAP2K1/2* mutations were significantly correlated with longer OS in this pooled cohort (49.3 months vs 22.0 months; HR = 0.44; 95% CI, 0.22–0.91; p = 0.0255; **Figure 1D**) compared to the group with wild-type *MAP2K1/2*. The meta-analysis also demonstrated that the group with *MAP2K1/2* mutations exhibited a significantly reduced risk of death, as compared to the group with wild-type *MAP2K1/2* (fixed effects model; relative risk (RR) = 0.43; 95% CI, 0.24–0.77; **Figure 1E**). No substantial heterogeneity was observed across studies (p = 0.55, **Figure 1E**), indicating the conclusion was consistent, from data analysis between different cohorts to the association between *MAP2K1/2* mutations and the favourable clinical response to anti-CTLA-4 therapy. Further data analysis using a pooled cohort consisting of four cohorts also confirmed that *MAP2K1/2* mutations are a predictive factor for superior OS in most subgroups with diverse clinical and molecular characteristics (**Figure 1F**).

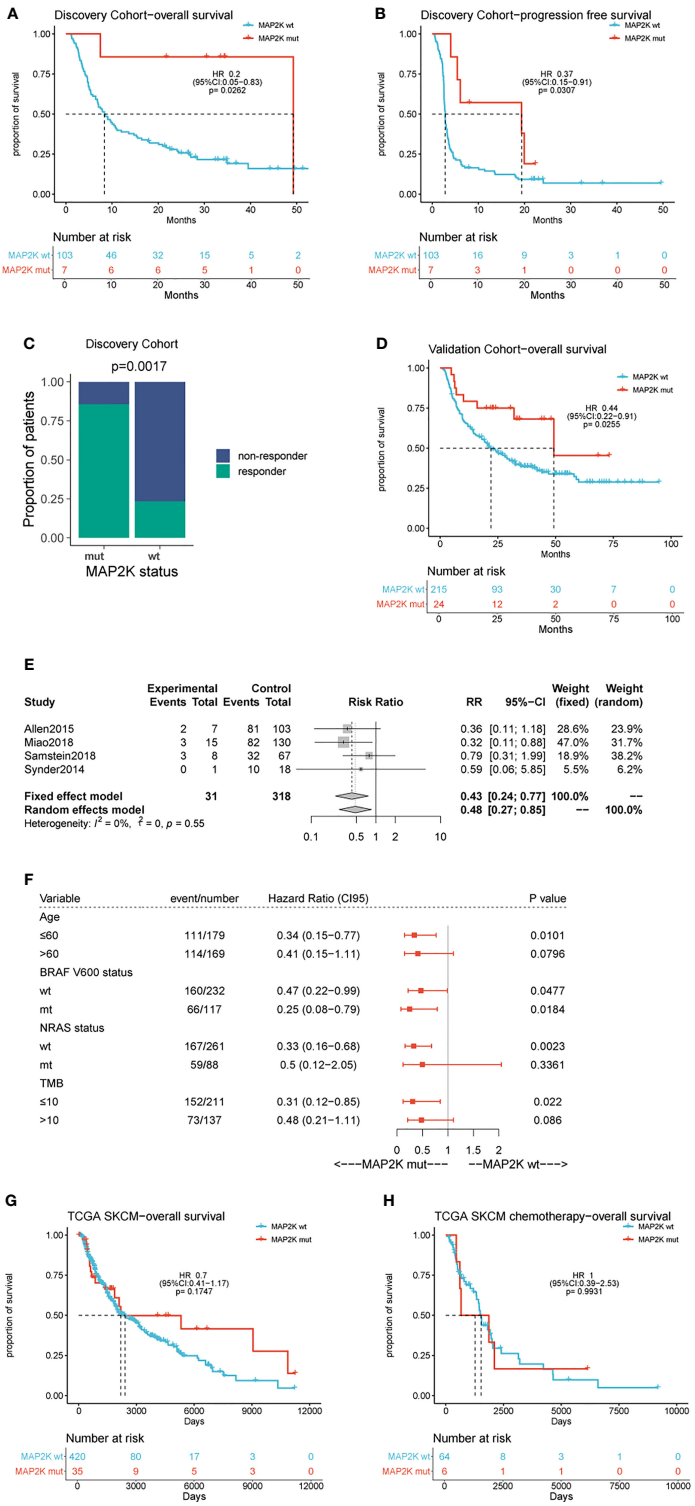

FIGURE 1 | Effect of *MAP2K1/2* gene mutations on treatment response in anti-CTLA-4 and non-immunotherapy-treated melanoma. Kaplan-Meier analyses of overall survival (OS) **(A)**, progression-free survival (PFS) **(B)**, and disease-control rate **(C)** in the anti-CTLA-4-treated discovery cohort. Kaplan-Meier analyses of overall survival (OS) **(D)** in the anti-CTLA-4-treated validation cohort. Pooled estimates of OS in four anti-CTLA-4-treated cohorts **(E)**. Subgroup Cox analysis of OS in pooled anti-CTLA-4-treated cohorts among patients with and without MAP2K1/2 gene mutations **(F)**. Kaplan-Meier analyses of OS in TCGA melanoma cohort **(G)** and TCGA chemotherapy-treated cohort **(H)**.

TABLE 1 | (a) Hazard ratio (HR) for OS *via* univariate and multivariate analyses in discovery cohort.

	Univariate		Multivariate	
Variable	**HR (95% CI)**	**P value (log-rank)**	**HR (95% CI)**	**P value (log-rank)**
Age (≤60 vs >60)	0.87 (0.57-1.35)	0.537		
Gender (male vs female)	0.78 (0.49-1.24)	0.301		
Stage (Stage 4 vs Stage 3)	4.58 (1.44-14.56)	0.001	3.76 (1.18-12)	0.025
LDH (1 vs 0)	2.07 (1.33-3.22)	0.001	2.04 (1.31-3.18)	0.002
BRAF V600 status (mut vs wt)	0.7 (0.43-1.16)	0.153		
NRAS status (mut vs wt)	1.14 (0.69-1.9)	0.610		
TMB (>median vs ≤median)	0.72 (0.47-1.11)	0.143		
TMB (top 20% vs bottom 80%)	0.73 (0.42-1.27)	0.252		
MAP2K status (mut vs wt)	0.2 (0.05-0.83)	0.004	0.24 (0.059-0.99)	0.048

TABLE 1 | (b) Hazard ratio (HR) for PFS *via* univariate and multivariate analyses in discovery cohort.

	Univariate		Multivariate	
Variable	**HR (95% CI)**	**P value (log-rank)**	**HR (95% CI)**	**P value (log-rank)**
Age (≤60 vs >60)	0.97 (0.65-1.44)	0.889		
Gender (male vs female)	0.93 (0.6-1.43)	0.735		
Stage (Stage 4 vs Stage 3)	2.41 (1.11-5.21)	0.012	1.83 (0.83-4.01)	0.025
LDH (1 vs 0)	2.04 (1.36-3.07)	0.001	2.05 (1.36-3.09)	<0.001
BRAF V600 status (mut vs wt)	0.87 (0.56-1.35)	0.53		
NRAS status (mut vs wt)	0.97 (0.61-1.55)	0.9		
TMB (>median vs ≤median)	0.86 (0.58-1.27)	0.442		
TMB (top 20% vs bottom 80%)	0.98 (0.6-1.59)	0.935		
MAP2K status (mut vs wt)	0.37 (0.15-0.91)	0.012	0.39 (0.16-0.99)	0.048

To clarify whether *MAP2K1/2* mutations are a predictive or prognostic biomarker, analysis of the data obtained from the TCGA database was performed, based on the *MAP2K1/2* mutational status of melanoma patients. No significant difference in OS was observed between the groups with mutated and wild-type *MAP2K1/2* in the total population (HR = 0.7; 95% CI, 0.41–1.17; p = 0.1747; **Figure 1G**) as well as in the chemotherapy population (HR = 1, 95% CI, 0.39–2.53; p = 0.99; **Figure 1H**). Taken together, these results suggested that *MAP2K1/2* gene mutations are a predictor for a favourable clinical response to anti-CTLA-4 therapy in metastatic melanoma rather than a prognostic factor for melanoma.

MAP2K1/2 Mutations Were Not Associated With the Clinical Benefits of Anti-PD-1/L1 Therapy for Metastatic Melanoma

Anti-PD-1 therapy with second-generation ICIs can prolong both PFS and OS in metastatic melanoma patients, with less high-grade toxicity than ipilimumab. To investigate whether *MAP2K1/2* mutations are also associated with a favourable clinical response to anti-PD-1/L1 therapy, we performed data analysis using a pooled cohort consisting of three public cohorts of 253 metastatic melanoma patients treated with anti-PD-1 agents (**Table S1**). Overall, 22 patients (8.7%) harboured *MAP2K1/2* mutations in this pooled cohort. The results showed that there was no significant difference in OS between mutated and wild-type *MAP2K1/2* groups (27.0 months vs 32.0 months; HR = 1.31; 95% CI, 0.68–2.53; p = 0.4151; **Figure 2A**). The meta-analysis showed that the risk of death was not reduced in the *MAP2K1/2*-mutated group as compared to the wild-type *MAP2K1/2* group (fixed effects model; RR = 1.13; 95% CI, 0.7–1.84; **Figure 2B**). Moreover, subgroup analysis indicated that *MAP2K1/2* mutations were not a predictor of OS in any subgroup (**Figure 2C**). These results revealed that *MAP2K1/2* gene mutations are not associated with the clinical benefits of anti-PD-1/L1 therapy for metastatic melanoma. Therefore, the predictive value of *MAP2K1/2* mutation in metastatic melanoma might be specific to anti-CTLA-4 therapy, rather than anti-PD-1/L1 therapy.

Mutation Status of *MAP2K1/2* Gene May Influence Systemic Treatment Options in Metastatic Melanoma

In this study, the clinical benefits (OS) of anti-CTLA-4 and anti-PD-1 therapies in patients with *MAP2K1/2*-mutated melanoma were compared in a pooled cohort comprising a combination of six cohorts. Analysis results showed that in the total population, the association of OS with anti-PD-1 therapy was superior to the association with anti-CLTA-4 therapy (median OS, 32.0 months vs 19.2 months; HR = 0.67; 95% CI, 0.53–0.85; p = 0.0009; **Figure 3A**), consistent with the results of a previous study (5). However, patients harbouring *MAP2K1/2* mutations treated with anti-PD-1 monotherapy had significantly poorer OS than those treated with anti-CTLA-4 monotherapy (median OS, 27.0 months vs 49.3 months; HR = 3.26; 95% CI, 1.18–9.02; p = 0.0225; **Figure 3B**). The difference in OS between the two immunotherapies could be attributed to the remarkably

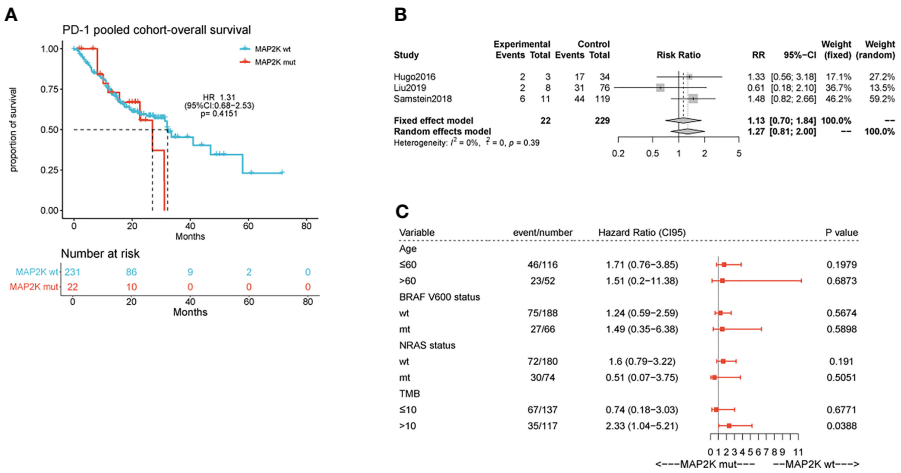

FIGURE 2 | Effect of *MAP2K1/2* gene mutations on treatment response in anti-PD-1-treated melanoma. Kaplan-Meier analyses of overall survival (OS) in the anti-PD-1-treated cohort **(A)**. Pooled estimates of OS in three anti-PD-1-treated cohorts **(B)**. Subgroup Cox analysis of OS in pooled anti-PD-1 treated cohorts among patients with and without *MAP2K1/2* gene mutations **(C)**.

improved survival rate in the *MAP2K1/2*-mutated group treated with anti-CTLA-4 therapy. Subgroup analysis also revealed that the *MAP2K1/2*-mutated group was more likely to obtain clinical benefits from anti-CTLA-4 monotherapy, as compared to anti-PD-1 monotherapy (HR = 0.31; 95% CI, 0.11−0.85; **Figure 3C**), suggesting that for *MAP2K1/2*-mutated melanomas, the efficacy of anti-CTLA-4 therapy might be superior to that of anti-PD-1 therapy.

Immunological Microenvironment of *MAP2K1/2*-Mutated Melanoma

To evaluate the impact of *MAP2K1/2* mutations on the transcription of immunity-related genes in melanoma, we integrated and analysed the gene expression data for patients from four clinical cohorts and the TCGA-SKCM cohort. To investigate the status of immune cell infiltration in melanoma patients treated with immunotherapies, TIMER2.0, a comprehensive resource for systematic analysis of immune infiltrates across diverse cancer types, was used to analyse the gene expression data of *MAP2K1/2*-mutated melanoma. Analysis results revealed that *MAP2K1/2*-mutated melanoma exhibited significantly increased densities of B cells (p = 0.015), $CD8^+$ T cells (p = 0.024), and neutrophils (p = 0.03) and a numerically higher level of myeloid dendritic cells (p = 0.089) compared to those in their wild-type counterparts, implying that melanoma patients with *MAP2K1/2* mutations have a favourable microenvironment for tumoral development (**Figure 4A**). However, there is no difference in macrophages or $CD4^+$ T cells between *MAP2K1/2*-mutated and wild-type melanomas (**Figure 4A**). Interestingly, the gene expression levels of CD33, a marker of myeloid-derived suppressor cells (MDSCs), and IL-10, which is mainly secreted by regulatory T cells (Tregs), were higher in *MAP2K1/2*-mutated melanoma, compared to those in wild-type melanoma (**Figure 4B**). Moreover, MDSCs and Tregs were reported to be associated with resistance to the PD-1 blockade (26, 27), consistent with the poor prognosis of patients with *MAP2K1/2*-mutated melanoma who received anti-PD-1 therapy. TMB analysis also revealed that melanoma patients harbouring *MAP2K1/2* mutations have a higher level of TMB (**Figure 4C**). Moreover, except for immunity-related genes, a total of 55 significantly differentially expressed genes were found between *MAP2K1/2*-mutated and wild-type melanoma in clinical cohort (**Table S2**), which might be associated with reshape of immunological microenvironment caused by MAPK mutation.

DISCUSSION

In recent years, immunotherapy has greatly improved the survival and quality of life for patients with melanoma, becoming one of the standard treatment regimens for metastatic or advanced melanoma. Although various molecules/antigens have been proposed as possible immunotherapy targets, only anti-CTLA4 antibody and anti-PD-1/L1 antibody are available for immunotherapy against melanoma in clinical practice. A large-scale phase III clinical trial (CheckMate 066) confirmed the efficacy of the anti-PD-1 antibody nivolumab in melanoma treatment. The results showed that nivolumab treatment was superior to standard chemotherapy as the first-line treatment with respect to the OS, PFS, and overall response rate. Later, another trial (Keynote-006) reported the superiority of pembrolizumab to ipilimumab. These studies laid the foundation for the approval of immunotherapy as the first-line treatment for melanoma by the Food and Drug Administration (FDA). Nevertheless, immunotherapy for melanoma treatment is still limited by the fact that only a portion of patients receive the clinical benefits of immunotherapy. For patients who do not respond to immunotherapy, this issue may lead to unnecessary

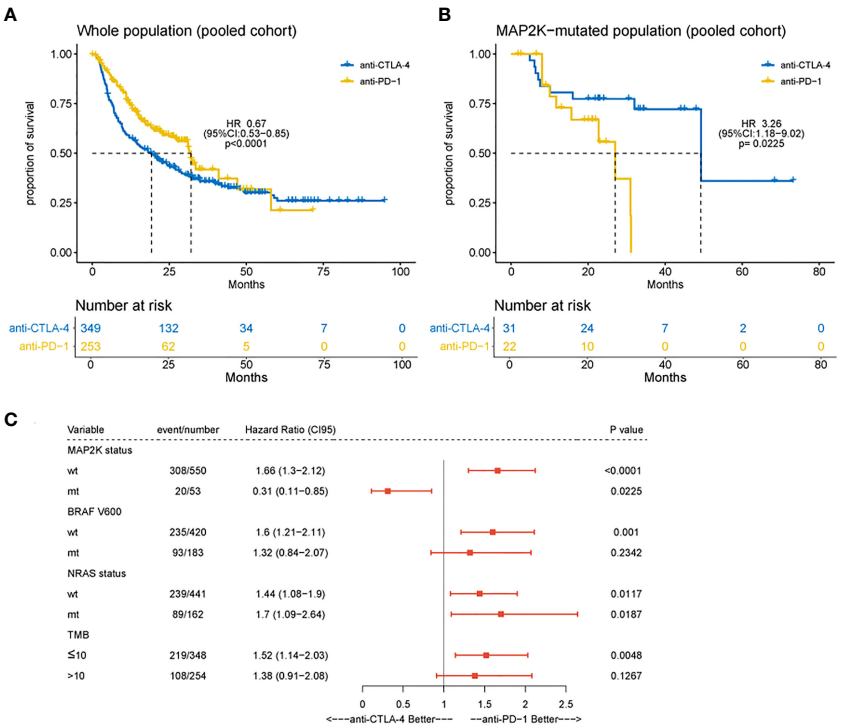

FIGURE 3 | Difference in overall survival between melanoma patients receiving anti-CTLA-4 or anti-PD-1 monotherapy. Kaplan-Meier analyses of overall survival (OS) in overall population **(A)** and *MAP2K1/2*-mutated subgroup **(B)** in the combined cohort of anti-CTLA-4 and anti-PD-1-treated patients. Subgroup Cox analysis of OS among patients receiving anti-CTLA-4 or anti-PD-1 monotherapy **(C)**.

costs (a heavy financial burden for the family) and loss of a valuable period for tumour treatment. Therefore, the identification of biomarkers for predicting the efficacy of immunotherapy would be of great importance.

Among potential biomarkers, CTLA-4 and PD-1 are the leading targets of ICIs in cancer immunotherapy. Previous studies have shown that CTLA-4 and PD-1 have distinct signalling pathways related to the different action mechanisms of immunotherapy (28). Anti-CTLA-4 therapy primarily interferes with the feedback mechanism to improve the proliferation and activation of more T cells, while anti-PD-1 treatment is assumed to attenuate the tumour-induced immunosuppression (29). Retrospective studies have identified different biomarkers that can predict the efficacy of anti-CTLA-4 or anti-PD-1 therapy. For example, loss of major histocompatibility complex (MHC) class I expression is a predictor of resistance to anti-CTLA-4 but not to anti-PD-1 therapy. In contrast, the expression of MHC class II, which is associated with interferon-γ-related signatures, can predict the treatment response to anti-PD-1 therapy, rather than anti-CTLA-4 therapy (23, 28, 30). To further identify more novel predictive biomarkers, we performed data analysis using several public cohorts and found that *MAP2K1/2* mutations might be a potential predictor for the clinical benefits of anti-CTLA-4 therapy in advanced melanomas. Our study revealed that patients with *MAP2K1/2* mutations had longer PFS and OS than their counterparts without mutations when both groups of patients received ipilimumab treatment. However, the predictive value of *MAP2K1/2* mutations is specific to anti-CTLA-4 therapy, rather than anti-PD-1 treatment, because no difference in the survival rate was observed between patients with *MAP2K1/2* mutations and their wild-type counterparts after anti-PD-1 treatment. Based on these observations, we investigated the possibility that the difference in clinical benefits between anti-PD-1/L1 and anti-CTLA-4 therapies in patients with *MAP2K1/2*-mutated melanoma can be used as a biomarker for the selection of the appropriate immunotherapy drug. According to this proposed hypothesis, further data analysis was conducted to compare the efficacy of anti-PD-1/L1 and anti-CTLA-4 therapies in patients with *MAP2K1/2*-mutated melanoma. Our results indicated that patients with *MAP2K1/2*-mutated melanoma who received anti-CTLA-4 therapy had better OS. In other words, anti-CTLA-4 therapy was superior to anti-PD-1/L1 therapy for patients with *MAP2K1/2*-mutated melanoma.

Compared with other proposed efficacy indicators of immunotherapy, such as TMB, microsatellite instability (MSI), and PD-L1 expression, the use of *MAP2K1/2* gene mutations as a qualitative biomarker could avoid the dilemma of setting cut-off values. In addition, *MAP2K1/2* mutations can be detected in peripheral blood ctDNA, providing a non-invasive approach to identify patients who may receive the benefits of ICI treatment.

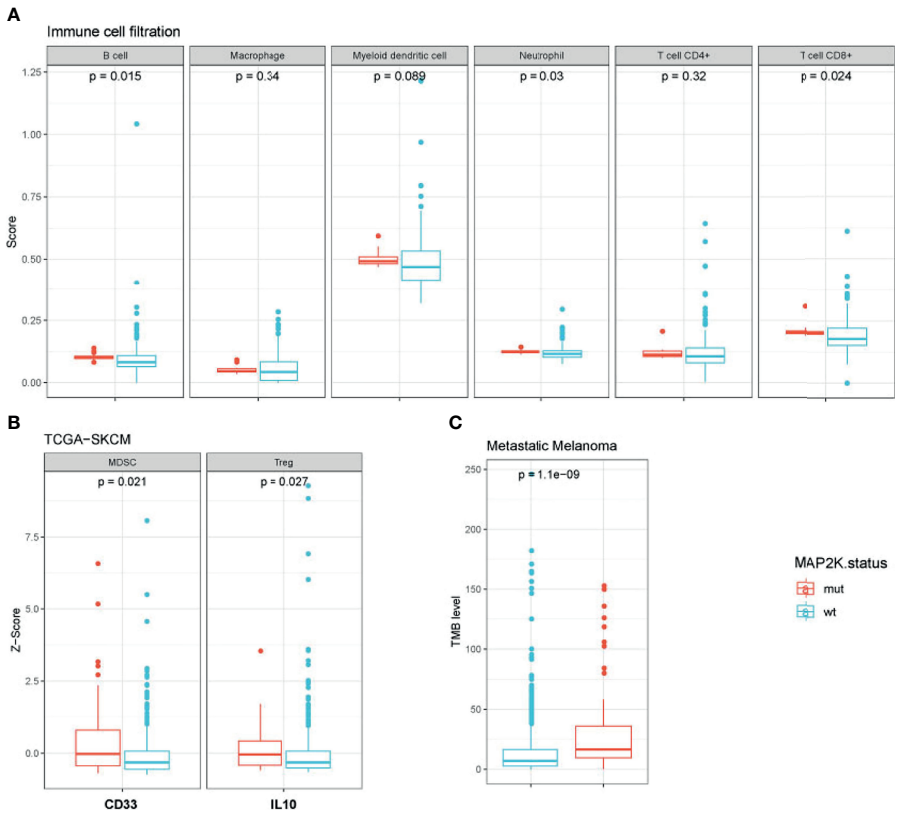

FIGURE 4 | Immunological microenvironment of *MAP2K1/2*-mutated melanoma. Boxplot comparing immune cell filtration between mutated and wild-type *MAP2K1/2* subgroup in metastatic melanoma cohort **(A)**, the expression of immune-related genes between the mutated and wild-type *MAP2K1/2* subgroups in TCGA-SKCM cohort **(B)**, and tumour mutational level between mutated and wild-type *MAP2K1/2* subgroup in metastatic melanoma cohort **(C)**.

In addition, we explored the mechanism underlying the predictive role of *MAP2K1/2* in the clinical response to ICIs. TMB, defined as the number of somatic mutations per megabase of interrogated genomic sequences, is considered to be related to the outcome of ICI treatment across multiple tumour types (31–33), though the exact mechanism remaining controversial (34). We found that *MAP2K1/2*-mutated melanomas exhibited higher TMB levels than *MAP2K1/2*-wild-type melanomas. This phenomenon could be associated with the predictive effect of TMB on anti-CTLA-4 monotherapy, which was previously reported (7). In addition, we observed an increase in B cells, $CD8^+$ T cells, and neutrophils in *MAP2K1/2*-mutated melanomas, as compared to *MAP2K1/2*-wild-type melanomas. Like dendritic cells, B cells can internalize antigens and deliver antigenic peptides to T-cell receptors (35). A previous study showed that high expression of immune cell-derived gene expression signatures in B cells is associated with better response to the anti-CTLA-4 antibody (36). Additionally, the number of MDSCs and Tregs is increased in *MAP2K1/2*-mutated melanomas. MDSCs are involved in immunosuppression *via* suppressing the functions of T-cells and natural killer-cells (27). It has been reported that MDSCs can induce the expansion of Tregs and reduce the anti-tumour activity of effector T cells (37), while Tregs can regulate immunosuppression by secreting cytokines, such as IL10, IL35, and TGF-β, thereby suppressing the effector T-cell response. These processes might account for the superiority of anti-CTLA-4 therapy over anti-PD-1 therapy in *MAP2K1/2*-mutated melanomas. Contrary to our expectations, there was no difference in the expression of MHC class I and II molecules between the *MAP2K1/2*-mutated and *MAP2K1/2*-wild-type groups (**Figures S2, S3**). For 55 significantly differentially expressed genes between MAP2K1/2-mutated and wild-type melanoma in clinical cohort, 14 genes (SMAD9, LRP6, PCDH18, TP53BP2, KDM1A, PKLR, GALNT5, RASGRF2, CTSK, ZNF845, ZNF384, TEK, MTHFD1, TAX1BP1) were associated with the immunological microenvironment as previous reports. Interestingly, TP53BP2, one of the 14 genes, has been proved to activate CD4+ and CD8+ immune and negatively regulate the MAPK signaling pathway in triple-negative breast cancers (38). The impact of *MAP2K1/2* gene mutations on the immunological microenvironment should be further assessed through *in vitro* or *in vivo* models. *MAP2K1/2*-mutated cases constitute approximately 8% of all melanoma patients, and the clinical studies of this population has been rarely reported. In fact, the inhibitory drugs of *MAP2K1/2*, more commonly called MEK inhibitors were identified to be effective

in the treatment of melanomas. The combination of MEK inhibitor with BRAF inhibitor has become the standard of care for patients with *BRAF*-mutated melanoma (39–41). Moreover, in *NRAS*-mutated melanoma patients, binimetinib has shown its treatment efficacy and represents a treatment option after failure of immunotherapy (42). It has been reported that previous treatment with BRAFi with or without MEKi result in shorter survival in *BRAF*-mutated melanoma patients when treated with anti-PD-1 antibody (43). Nevertheless, the influence of previous targeted therapies on the therapeutic effect of immunotherapy in *MAP2K1/2*-mutated melanoma is unknown. Since no effective targeted therapeutic drugs have been reported against *MAP2K1/2*-mutated melanomas, ICIs are still considered as the preferred systemic treatment for these patients. To the best of our knowledge, the present manuscript is the first report on the investigation of the association between MAP2K1/2 mutations and the clinical response to ICIs. This study can also provide a guideline for making treatment decisions for patients with *MAP2K1/2*-mutated melanoma. With an innovation-based view, we suggest that for patients with *MAP2K1/2*-mutated melanoma, anti-CTLA-4 therapy might be more effective than anti-PD-1 monotherapy. Due to the lack of available data, we could not compare the differences in the efficacy of anti-CTLA-4 monotherapy and combination therapy involving both anti-CTLA-4 anti-PD-1 in patients with *MAP2K1/2*-mutated melanoma in this study. Because of the possibility that combination therapy may increase the incidence of grade 3–4 immune-related adverse events, it would be especially meaningful for patients with poor physical conditions if a comparison study is performed for benefit-risk assessment in ICIs to determine the optimal benefit/risk potential for patients.

There are still some limitations in this study. Because the ICI-treated cohorts included in this study came from several research centres, analysis of the data from the pooled-cohort might introduce biases due to differences in the ICI regimen, dose usage, and treatment cycle between institutions. Additionally, there was no specific limitation on the number of previous therapies used in the observed cohorts, which may cause heterogeneity in the survival time and might account for the OS benefit in the whole population not being as obvious as that reported in the phase III clinical trials, although the trend was consistent (44, 45). Because of the limited sample size and lack of molecular information, we could not match the baseline characteristics such as PD-L1 expression and microsatellite stability. Therefore, prospective studies are additionally required to confirm the findings of this study. Further studies are also needed to elucidate the mechanisms underlying the clinical benefits of anti-CTLA-4 therapy in *MAP2K1/2*-mutated melanoma.

DATA AVAILABILITY STATEMENT

The data of the Allen cohort, Snyder cohort, Hugo cohort, Liu cohort, Miao cohort and Samstein cohort included in this analysis were provided online. The specific accession numbers and repository names are included in **Supplementary Table 1**.

AUTHOR CONTRIBUTIONS

Conceptualization: C-ZY and JC. Methodology: M-LH. Validation: X-YL, W-ZX, and Y-XC. Investigation: M-MY. Data curation: Y-HZ and S-YY. Writing—original draft preparation: TY and J-YZ. Writing—review and editing: CG. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

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REFERENCES

1. Robert C, Lanoy E, Besse B. One or Two Immune Checkpoint Inhibitors? *Cancer Cell* (2019) 36(6):579–81. doi: 10.1016/j.ccell.2019.11.005
2. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved Survival With Ipilimumab in Patients With Metastatic Melanoma. *N Engl J Med* (2010) 363(8):711–23. doi: 10.1056/NEJMoa1003466
3. Robert C, Thomas L, Bondarenko I, O'Day S, Weber J, Garbe C, et al. Ipilimumab Plus Dacarbazine for Previously Untreated Metastatic Melanoma. *N Engl J Med* (2011) 364(26):2517–26. doi: 10.1056/NEJMoa1104621
4. Schachter J, Ribas A, Long GV, Arance A, Grob JJ, Mortier L, et al. Pembrolizumab Versus Ipilimumab for Advanced Melanoma: Final Overall Survival Results of a Multicentre, Randomised, Open-Label Phase 3 Study (KEYNOTE-006). *Lancet* (2017) 390(10105):1853–62. doi: 10.1016/S0140-6736(17)31601-X
5. Wolchok JD, Chiarion-Sileni V, Gonzalez R, Rutkowski P, Grob JJ, Cowey CL, et al. Overall Survival With Combined Nivolumab and Ipilimumab in Advanced Melanoma. *N Engl J Med* (2017) 377(14):1345–56. doi: 10.1056/NEJMoa1709684
6. Herrscher H, Robert C. Immune Checkpoint Inhibitors in Melanoma in the Metastatic, Neoadjuvant, and Adjuvant Setting. *Curr Opin Oncol* (2020) 32 (2):106–13. doi: 10.1097/CCO.0000000000000610
7. Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, et al. Genetic Basis for Clinical Response to CTLA-4 Blockade in Melanoma. *N Engl J Med* (2014) 371(23):2189–99. doi: 10.1056/NEJMoa1406498
8. Van Allen EM, Miao D, Schilling B, Shukla SA, Blank C, Zimmer L, et al. Genomic Correlates of Response to CTLA-4 Blockade in Metastatic Melanoma. *Science* (2015) 350(6257):207–11. doi: 10.1126/science.aad0095
9. Riaz N, Havel JJ, Kendall SM, Makarov V, Walsh LA, Desrichard A, et al. Recurrent SERPINB3 and SERPINB4 Mutations in Patients Who Respond to

Anti-CTLA4 Immunotherapy. *Nat Genet* (2016) 48(11):1327–9. doi: 10.1038/ng.3677
10. Xiao W, Du N, Huang T, Guo J, Mo X, Yuan T, et al. TP53 Mutation as Potential Negative Predictor for Response of Anti-CTLA-4 Therapy in Metastatic Melanoma. *EBioMedicine* (2018) 32:119–24. doi: 10.1016/j.ebiom.2018.05.019
11. Johnson DB, Lovly CM, Flavin M, Panageas KS, Ayers GD, Zhao Z, et al. Impact of NRAS Mutations for Patients With Advanced Melanoma Treated With Immune Therapies. *Cancer Immunol Res* (2015) 3(3):288–95. doi: 10.1158/2326-6066.CIR-14-0207
12. Cristescu R, Mogg R, Ayers M, Albright A, Murphy E, Yearley J, et al. Pan-Tumor Genomic Biomarkers for PD-1 Checkpoint Blockade-Based Immunotherapy. *Science* (2018) 362(6411):eaar3593. doi: 10.1126/science.aar3593
13. Hellmann MD, Ciuleanu TE, Pluzanski A, Lee JS, Otterson GA, Audigier-Valette C, et al. Nivolumab Plus Ipilimumab in Lung Cancer With a High Tumor Mutational Burden. *N Engl J Med* (2018) 378(22):2093–104. doi: 10.1056/NEJMoa1801946
14. Vu HL, Aplin AE. Targeting Mutant NRAS Signaling Pathways in Melanoma. *Pharmacol Res* (2016) 107:111–6. doi: 10.1016/j.phrs.2016.03.007
15. Murugan AK, Dong J, Xie J, Xing M. MEK1 Mutations, But Not ERK2 Mutations, Occur in Melanomas and Colon Carcinomas, But None in Thyroid Carcinomas. *Cell Cycle* (2009) 8(13):2122–4. doi: 10.4161/cc.8.13.8710
16. Nikolaev SI, Rimoldi D, Iseli C, Valsesia A, Robyr D, Gehrig C, et al. Exome Sequencing Identifies Recurrent Somatic MAP2K1 and MAP2K2 Mutations in Melanoma. *Nat Genet* (2011) 44(2):133–9. doi: 10.1038/ng.1026
17. Hayward NK, Wilmott JS, Waddell N, Johansson PA, Field MA, Nones K, et al. Whole-Genome Landscapes of Major Melanoma Subtypes. *Nature* (2017) 545(7653):175–80. doi: 10.1038/nature22071
18. Van Allen EM, Wagle N, Sucker A, Treacy DJ, Johannessen CM, Goetz EM, et al. The Genetic Landscape of Clinical Resistance to RAF Inhibition in Metastatic Melanoma. *Cancer Discovery* (2014) 4(1):94–109. doi: 10.1158/2159-8290.CD-13-0617
19. Kono M, Dunn IS, Durda PJ, Butera D, Rose LB, Haggerty TJ, et al. Role of the Mitogen-Activated Protein Kinase Signaling Pathway in the Regulation of Human Melanocytic Antigen Expression. *Mol Cancer Res* (2006) 4(10):779–92. doi: 10.1158/1541-7786.MCR-06-0077
20. Yarchoan M, Mohan AA, Dennison L, Vithayathil T, Ruggieri A, Lesinski GB, et al. MEK Inhibition Suppresses B Regulatory Cells and Augments Anti-Tumor Immunity. *PloS One* (2019) 14(10):e0224600. doi: 10.1371/journal.pone.0224600
21. Ebert PJR, Cheung J, Yang Y, McNamara E, Hong R, Moskalenko M, et al. MAP Kinase Inhibition Promotes T Cell and Anti-Tumor Activity in Combination With PD-L1 Checkpoint Blockade. *Immunity* (2016) 44(3):609–21. doi: 10.1016/j.immuni.2016.01.024
22. Hugo W, Zaretsky JM, Sun L, Song C, Moreno BH, Hu-Lieskovan S, et al. Genomic and Transcriptomic Features of Response to Anti-PD-1 Therapy in Metastatic Melanoma. *Cell* (2016) 165(1):35–44. doi: 10.1016/j.cell.2016.02.065
23. Liu D, Schilling B, Liu D, Sucker A, Livingstone E, Jerby-Arnon L, et al. Integrative Molecular and Clinical Modeling of Clinical Outcomes to PD1 Blockade In Patients With Metastatic Melanoma. *Nat Med* (2019) 25(12):1916–27. doi: 10.1038/s41591-019-0654-5
24. Miao D, Margolis CA, Gao W, Voss MH, Li W, Martini DJ, et al. Genomic Correlates of Response to Immune Checkpoint Therapies in Clear Cell Renal Cell Carcinoma. *Science* (2018) 359(6377):801–6. doi: 10.1126/science.aan5951
25. Samstein RM, Lee CH, Shoushtari AN, Hellmann MD, Shen R, Janjigian YY, et al. Tumor Mutational Load Predicts Survival After Immunotherapy Across Multiple Cancer Types. *Nat Genet* (2019) 51(2):202–6. doi: 10.1038/s41588-018-0312-8
26. Dyck L, Wilk MM, Raverdeau M, Misiak A, Boon L, Mills KH. Anti-PD-1 Inhibits Foxp3(+) Treg Cell Conversion and Unleashes Intratumoural Effector T Cells Thereby Enhancing the Efficacy of a Cancer Vaccine in a Mouse Model. *Cancer Immunol Immunother* (2016) 65(12):1491–8. doi: 10.1007/s00262-016-1906-6
27. Najjar YG, Finke JH. Clinical Perspectives on Targeting of Myeloid Derived Suppressor Cells in the Treatment of Cancer. *Front Oncol* (2013) 3:49. doi: 10.3389/fonc.2013.00049
28. Rodig SJ, Gusenleitner D, Jackson DG, Gjini E, Giobbie-Hurder A, Jin C, et al. MHC Proteins Confer Differential Sensitivity to CTLA-4 and PD-1 Blockade in Untreated Metastatic Melanoma. *Sci Transl Med* (2018) 10(450):eaar3342. doi: 10.1126/scitranslmed.aar3342
29. Topalian SL, Drake CG, Pardoll DM. Immune Checkpoint Blockade: A Common Denominator Approach to Cancer Therapy. *Cancer Cell* (2015) 27(4):450–61. doi: 10.1016/j.ccell.2015.03.001
30. Sade-Feldman M, Jiao YJ, Chen JH, Rooney MS, Barzily-Rokni M, Eliane JP, et al. Resistance to Checkpoint Blockade Therapy Through Inactivation of Antigen Presentation. *Nat Commun* (2017) 8(1):1136. doi: 10.1038/s41467-017-01062-w
31. Ott PA, Bang YJ, Piha-Paul SA, Razak ARA, Bennouna J, Soria JC, et al. T-Cell-Inflamed Gene-Expression Profile, Programmed Death Ligand 1 Expression, and Tumor Mutational Burden Predict Efficacy in Patients Treated With Pembrolizumab Across 20 Cancers: KEYNOTE-028. *J Clin Oncol* (2019) 37(4):318–27. doi: 10.1200/JCO.2018.78.2276
32. Riaz N, Havel JJ, Makarov V, Desrichard A, Urba WJ, Sims JS, et al. Tumor and Microenvironment Evolution During Immunotherapy With Nivolumab. *Cell* (2017) 171(4):934–949.e16. doi: 10.1016/j.cell.2017.09.028
33. Carbone DP, Reck M, Paz-Ares L, Creelan B, Horn L, Steins M, et al. First-Line Nivolumab in Stage IV or Recurrent Non-Small-Cell Lung Cancer. *N Engl J Med* (2017) 376(25):2415–26. doi: 10.1056/NEJMoa1613493
34. McGrail DJ, Pilie PG, Rashid NU, Voorwerk L, Slagter M, Kok M, et al. High Tumor Mutation Burden Fails to Predict Immune Checkpoint Blockade Response Across All Cancer Types. *Ann Oncol* (2021) 32(5):661–72. doi: 10.1016/j.annonc.2021.02.006
35. Schroeder HW. B-Cell Development and Differentiation. In: *Clinical Immunology, Book, 5th Edition* Elsevier, (2019) pp. 107–18.e1. doi: 10.1016/B978-0-7020-6896-6.00007-7
36. Varn FS, Wang Y, Cheng C. A B Cell-Derived Gene Expression Signature Associates With an Immunologically Active Tumor Microenvironment and Response to Immune Checkpoint Blockade Therapy. *Oncoimmunology* (2019) 8(1):e1513440. doi: 10.1080/2162402X.2018.1513440
37. Pistoia V, Morandi F, Bianchi G, Pezzolo A, Prigione I, Raffaghello L. Immunosuppressive Microenvironment in Neuroblastoma. *Front Oncol* (2013) 3:167. doi: 10.3389/fonc.2013.00167
38. Quist J, Mirza H, Cheang MCU, Telli ML, O'Shaughnessy JA, Lord CJ, et al. A Four-Gene Decision Tree Signature Classification of Triple-Negative Breast Cancer: Implications for Targeted Therapeutics. *Mol Cancer Ther* (2019) 18(1):204–12. doi: 10.1158/1535-7163.MCT-18-0243
39. Long GV, Stroyakovskiy D, Gogas H, Levchenko E, de Braud F, Larkin J, et al. Dabrafenib and Trametinib Versus Dabrafenib and Placebo for Val600 BRAF-Mutant Melanoma: A Multicentre, Double-Blind, Phase 3 Randomised Controlled Trial. *Lancet* (2015) 386(9992):444–51. doi: 10.1016/S0140-6736(15)60898-4
40. Larkin J, Ascierto PA, Dreno B, Atkinson V, Liszkay G, Maio M, et al. Combined Vemurafenib and Cobimetinib in BRAF-Mutated Melanoma. *N Engl J Med* (2014) 371(20):1867–76. doi: 10.1056/NEJMoa1408868
41. Dummer R, Ascierto PA, Gogas HJ, Arance A, Mandala M, Liszkay G, et al. Encorafenib Plus Binimetinib Versus Vemurafenib or Encorafenib in Patients With BRAF-Mutant Melanoma (COLUMBUS): A Multicentre, Open-Label, Randomised Phase 3 Trial. *Lancet Oncol* (2018) 19(5):603–15. doi: 10.1016/S1470-2045(18)30142-6
42. Dummer R, Schadendorf D, Ascierto PA, Arance A, Dutriaux C, Di Giacomo AM, et al. Binimetinib Versus Dacarbazine in Patients With Advanced NRAS-Mutant Melanoma (NEMO): A Multicentre, Open-Label, Randomised, Phase 3 Trial. *Lancet Oncol* (2017) 18(4):435–45. doi: 10.1016/S1470-2045(17)30180-8
43. Puzanov I, Ribas A, Robert C, Schachter J, Nyakas M, Daud A, et al. Association of BRAF V600E/K Mutation Status and Prior BRAF/MEK Inhibition With Pembrolizumab Outcomes in Advanced Melanoma: Pooled Analysis of 3 Clinical Trials. *JAMA Oncol* (2020) 6(8):1256–64. doi: 10.1001/jamaoncol.2020.2288
44. Hodi FS, Chesney J, Pavlick AC, Robert C, Grossmann KF, McDermott DF, et al. Combined Nivolumab and Ipilimumab Versus Ipilimumab Alone in Patients With Advanced Melanoma: 2-Year Overall Survival Outcomes in a Multicentre, Randomised, Controlled, Phase 2 Trial. *Lancet Oncol* (2016) 17(11):1558–68. doi: 10.1016/S1470-2045(16)30366-7
45. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Rutkowski P, Moshyk A, et al. Five-Year Survival With Combined Nivolumab and Ipilimumab in

Advanced Melanoma. *N Engl J Med* (2019) 381(16):1535–46. doi: 10.1056/NEJMoa1910836

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Novel Molecular Determinants of Response or Resistance to Immune Checkpoint Inhibitor Therapies in Melanoma

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Background: Immune checkpoint inhibitor (ICI) therapy dramatically prolongs melanoma survival. Currently, the identified ICI markers are sometimes ineffective. The objective of this study was to identify novel determinants of ICI efficacy.

Methods: We comprehensively curated pretreatment somatic mutational profiles and clinical information from 631 melanoma patients who received blockade therapy of immune checkpoints (i.e., CTLA-4, PD-1/PD-L1, or a combination). Significantly mutated genes (SMGs), mutational signatures, and potential molecular subtypes were determined. Their association with ICI responses was assessed simultaneously.

Results: We identified 27 SMGs, including four novel SMGs (*COL3A1*, *NRAS*, *NARS2*, and *DCC*) that are associated with ICI efficacy and well-known driver genes. *COL3A1* mutations were associated with improved ICI overall survival (hazard ratio (HR): 0.64, 95% CI: 0.45–0.91, $p = 0.012$), whereas immune resistance was observed in patients with *NRAS* mutations (HR: 1.42, 95% CI: 1.10–1.82, $p = 0.006$). The presence of the tobacco smoking-related signature was significantly correlated with inferior prognoses (HR: 1.42, 95% CI: 1.11–1.82, $p = 0.005$). In addition, the signature resembling that of alkylating agents and a newly discovered signature both exhibited extended prognoses (both HR < 1, $p < 0.05$). Based on the activities of the extracted 6 mutational signatures, we identified one immune subtype that was significantly associated with better ICI outcomes (HR: 0.44, 95% CI: 0.23–0.87, $p = 0.017$).

Conclusion: We uncovered several novel SMGs and re-annotated mutational signatures that are linked to immunotherapy response or resistance. In addition, an immune subtype was found to exhibit favorable prognoses. Further studies are required to validate these findings.

Keywords: melanoma, immunotherapy, SMGs, mutational signatures, molecular subtypes, predictive biomarkers

INTRODUCTION

The blockade of cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed cell death-1 (PD-1), or its ligand PD-L1 with monoclonal antibodies (e.g., ipilimumab, pembrolizumab, or nivolumab) considerably prolongs the survival of patients with advanced or metastatic melanoma (1). The insight that inhibition of immune checkpoints can result in the reversion of inactivated T cells has dramatically changed cancer therapy patterns (2). Despite impressive durable clinical benefits, immune checkpoint inhibitors (ICIs) offer a long-term response only to a subset of patients with melanoma (3). Therefore, selecting among patients the subpopulation that will respond to ICI therapy remains a problem that needs to be urgently solved.

Initial clinical trials of anti-PD-1 showed that tumors expressing high PD-L1 levels were associated with benefits to treatment (4–6). However, further studies have reported that a greater proportion of responders were patients with negative PD-L1 expression (7–9). Neoantigens are computationally obtained based on somatic mutational profiles, and an elevated neoantigen burden (NB) has been shown to underlie the responses to ICI treatment. Tumor mutational burden (TMB) is consistently correlated with elevated benefits to ICI agents, initially in trials of melanoma and non-small cell lung cancer (NSCLC) (10–12). The association of high TMB with improved ICI response has also been observed in several other cancers (13–15). Nevertheless, TMB is an unstable indicator because it does not exhibit an association with the response in other cancers, such as renal cell cancer (16), Hodgkin's lymphoma (17), and virally mediated Merkel-cell carcinoma (18). The above observations drive us to explore novel determinants of the benefits of checkpoint inhibition treatment.

Several recent studies have reported that mutations in single genes, such as *POLE* (19), *POLD1* (19), *PBRM1* (20), *TTN* (21), and *MUC16* (22), were correlated with favorable ICI response or survival. Nevertheless, mutations in *B2M*, which stabilize intracellular peptides on the cell surface and play a vital role in antigen presentation, were demonstrated to be associated with acquired resistance to CTLA-4 and PD-1 inhibitors in melanoma (23). Similarly, *JAK1* or *JAK2* mutations have also been linked with primary or acquired resistance to anti-PD-1 therapy in advanced melanoma and colon carcinoma (24, 25).

Specific mutational signatures, which are characteristic patterns of mutation types produced by distinct mutation processes, have been shown to be associated with ICI response (2). Lung cancer patients harboring tobacco smoking-related mutational signatures exhibited a better clinical benefit than those without such signatures (12). Tumors with a durable anti-PD-1 response displayed an accumulation of a mutational signature correlated with apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) (26, 27). Moreover, melanoma patients who harbored ultraviolet light exposure-related mutational signatures were more likely to experience favorable responses when receiving immune checkpoint-based therapies (27). It will be of interest to explore whether other DNA-damaging mutational signatures are linked with immunotherapy responses and to uncover novel signatures that were not previously annotated in melanoma.

Immune molecular subtypes based on multi-omics data have recently been identified in melanoma (28–30). However, most of these identified subtypes are employed to predict tumor intrinsic prognoses and cannot be used to evaluate the therapeutic effect. Current immunotherapy studies of malignant melanoma are mostly focused on somatic mutation levels, and fewer studies included continuous data (e.g., gene expression profiles). Feasibly, potential molecular subtypes could be obtained by clustering the mutational signature activities extracted from mutational profiles (31), and a further selection of immune subtypes could be achieved by evaluating the association between distinct subgroups and immunotherapy efficacy.

We hypothesized that an expanded clinically annotated melanoma cohort could more effectively be used to detect significant correlations between pretreatment genomic features and ICI efficacy. Therefore, we curated pretreatment somatic data from melanoma samples treated with ICI agents. By integrating mutational profiles and clinicopathologic characteristics across 631 samples, we aimed to identify novel significantly mutated genes (SMGs) and potential immune subtypes that are associated with response or resistance to ICI treatment and to re-annotate the mutational signatures in the setting of immunotherapy.

METHODS

Genomic Data and Clinical Information

A total of 333,968 pretreatment whole-exome sequencing non-synonymous somatic alterations in 631 melanoma patients treated with ICIs (i.e., anti-CTLA-4, anti-PD-1/PD-L1, or combined therapy) from eight previously published studies were collected (11, 25, 27, 32–36). Mutation types in this study included missense mutations, nonsense mutations, frameshift del/ins, in frame del/ins, and splice site mutations. All somatic mutations were uniformly re-annotated using the Oncotator (37). Gene expression profiles were curated in three of eight studies (33, 34, 36). Clinicopathologic characteristics including age, sex, ICI response status, follow-up information on overall survival (OS) and progression-free survival (PFS), and ICI types of the above eight studies are shown in **Table S1**. Of the aggregated 631 melanoma patients, 627 had data regarding OS times and status, and 390 had information on PFS times and status. Other available data for all patients are shown in **Table S2**. Objective response rates (ORRs) indicate the proportion of patients with complete response (CR) or partial response (PR) status. Disease control rates (DCRs) reflect the proportion of patients who achieve a non-progressive disease status (i.e., CR, PR, and stable disease [SD]).

A total of 313 ICI-treated melanoma samples, which are subjected to the Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) assay of a targeted 468-gene panel at Memorial Sloan Kettering Cancer Center (MSKCC), were also collected for specific validation (38). Detailed clinical

characteristics are illustrated in **Table S3**. Clinical information and somatic mutational profiles of 457 melanoma samples from The Cancer Genome Atlas (TCGA) were downloaded from the Genome Data Commons (https://gdc.cancer.gov).

Identification of Significantly Mutated Genes

SMGs were identified using the MutSigCV algorithm against the hg19 genome (39). MutSigCV detects significantly enriched non-silent somatic alterations in one gene by considering the background mutation rate estimated through silent mutations. In addition to being statistically significant by this algorithm ($q < 0.1$), a putative SMG must meet the criterion of expressing in TCGA melanoma dataset (40). The mutational patterns of SMGs were visualized using the R package GenVisR (41).

Deciphering Mutational Signatures Operative in the Genome

The algorithm published by Kim et al. (42) was applied to detect mutational signatures in the integrated melanoma cohort. The core of this method is Bayesian variant non-negative matrix factorization (NMF), which can automatically calculate the optimal number of mutational signatures and eliminate manual inspection. Specifically, NMF was applied to decompose mutation portrait matrix *A*, which contained 96 base substitution classes with trinucleotide sequence patterns. Matrix A was factorized into two non-negative matrices *W* and *H* (i.e., $A \approx WH$), where *W* indicates the extracted mutational signatures and *H* represents the mutation activities of each corresponding signature. The column of matrix *A* is the count of detected signatures, and rows represent the 96 base substitution types, which are the permutation and combination of six main mutational categories (i.e., C > A, C > G, C > T, T > A, T > C, and T > G) and their surrounding adjacent bases. The rows and columns of matrix *H* indicate the individual signatures and their corresponding mutational activities, respectively. All extracted mutational signatures were then compared with the 30 annotated signatures stored in the Catalogue of Somatic Mutations in Cancer (COSMIC; version 2) based on cosine similarity. The detected mutational signatures were defined as binary variables (i.e., yes and no) in survival analyses and multivariate Cox regression models according to the principle proposed by a recent study: a signature was supposed to exist in a sample if it contributed to greater than 100 substitutions or 25% of the total mutations (43).

Detection of Potential Molecular Subtypes

We employed consensus clustering to determine the potential molecular subtypes of the integrated melanoma patients. After obtaining the activities of extracted mutational signatures of all patients, we then used the partition around medoids (PAM) algorithm with the Euclidean distance metric and performed 500 bootstraps, each comprising 80% of patients in the aggregated cohort. The clustering number was explored from 2 to 10, and the optimal number was determined by evaluating the cluster consensus coefficient and consensus matrix. Consensus clustering analysis was conducted using the R package ConsensusClusterPlus (44).

Estimation of Tumor Infiltration Lymphocytes

The CIBERSORT algorithm was used to calculate the proportion of infiltrating immune cell subsets in tumors, which is an analytical tool that imputes gene expression profiles and provides an estimation of the abundance of 22 human hematopoietic cell phenotypes with 547 genes from the leukocyte gene signature matrix, termed LM22 (45). The 22 cell subsets include 7 T-cell types, naive and memory B cells, plasma cells, NK cells, and myeloid subsets, which exert distinct functionalities in antitumor immune responses.

Differential Analysis and Gene Set Enrichment Analysis

Differential expression of each gene in distinct subgroups was calculated using the R package limma (46) and edgeR (47). Especially, read counts of gene expression profiles were normalized using the calcNormFactors function in the package edgeR, and then as input to lmFit and eBayes functions in the limma package. The differential expression *t* statistics obtained from eBayes function were subsequently used to conduct gene set enrichment analysis (GSEA) implemented by R fgsea package (http://bioconductor.org/packages/release/bioc/html/fgsea.html). Cell signaling pathways and biological processes in the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) were utilized as background datasets. The false discovery rate (FDR) and normalized enrichment score (NES) were calculated based on 1 million permutations.

Association of Gene Mutations With Tumor Mutational Burden and Neoantigen Burden

Genome instability is markedly influenced by mutations in the genomic maintenance genes (48). Therefore, in addition to univariate analysis of the association of specific gene mutations with TMB and NB, multivariate logistic regression models with mutations in DNA damage repair genes (i.e., *BRCA1/2*, *TP53*, and *POLE*) and mismatch repair (MMR) genes (i.e., *MLH1*, *MSH2*, *MSH6*, and *PMS2*) taken into account were also conducted to control false positives. In this study, TMB was defined as the log2 transformation of total non-synonymous mutations per megabase. The neoantigen data of 340 melanoma patients were downloaded from The Cancer Immunome Atlas (TCIA; https://www.tcia.at/home).

Statistical Analyses

Statistical analyses were performed employing R software (version 4.0.1). A genomic overview of the aggregated melanoma cohort was achieved using the maftools package (49). The Kaplan–Meier survival analyses and multivariate Cox regression models implemented by survival and forest model packages, respectively, were used to evaluate the associations of SMG mutations, the presence of mutational signatures, and

potential subtypes with survival outcomes. Furthermore, the log-rank test was applied to compare the significant differences between the survival curves. The correlation of continuous and categorical variables with specific binary factors was evaluated using Wilcoxon's rank-sum test and Fisher's exact test, respectively. A two-sided *p*-value of less than 0.05 was considered to be statistically significant.

RESULTS

Pretreatment Genomic Features and Significantly Mutated Genes Linked With Immune Checkpoint Inhibitor Response

The integrated somatic mutational profiles and clinically annotated information of 631 melanoma patients derived from eight previously published ICI studies were obtained (**Table S1**). A genomic mutation overview of the aggregated cohort is shown in **Figure S1**. Among 631 ICI-treated tumors, 193 (30.6%) showed CR/PR, 89 (14.1%) SD, 341 (54.1%) PD, and four (0.6%) mixed response, and four (0.6%) were not evaluated. Overall, 324 (51.4%) patients were treated with anti-CTLA-4, 163 (25.8%) were treated with anti-PD-1/PD-L1, and 144 (22.8%) received combined therapy (i.e., anti-CTLA-4 plus anti-PD-1/PD-L1). The median ICI OS and PFS were 19.2 and 3.5 months, respectively.

We calculated the TMB of this integrated cohort and compared it with that of 33 cancer types in TCGA. Consistent with previous observations (2), melanoma and NSCLC were two cancers with the highest TMB (**Figure S2**). We treated TMB as a continuous variable to evaluate its association with ICI efficacy. The results demonstrated that elevated TMB was significantly correlated with improved ICI OS and PFS in multivariate Cox regression models ($p < 0.001$ and $p = 0.065$, respectively; **Figures S3A, B**). In addition, we observed that high TMB was more enriched in patients with better ICI efficacy (i.e., objective response and disease control) in univariate analysis (Wilcoxon's rank-sum test, $p < 0.001$ and $p = 0.003$, respectively; **Figures S3C, D**) and multivariate logistic models (both $p < 0.001$; **Figures S3E, F**).

We employed the MutSigCV algorithm to detect SMGs. In total, 27 SMGs were identified, including well-known driver genes (e.g., *BRAF*, *NF1*, *TP53*, *ARID2*, *PTEN*, *PPP6C*, and *DDX3X*) and several novel genes (**Figure 1** and **Table S4**). We then explored the associations of all identified SMGs with ICI OS, PFS, ORR, and DCR. We observed that numerous gene mutations exhibited a significant association with ICI efficacy (e.g., *CFH*, *MKRN3*, *NF1*, and *THSD7B*); nevertheless, the associations were not found to be significant by multivariate-adjusted analysis (**Table S4**). Finally, we identified four novel SMGs (*COL3A1*, *NRAS*, *NARS2*, and *DCC*), whose alterations were linked with ICI response or resistance (**Table S4**). The detailed mutational patterns of these four genes are shown in **Figure S4**. *COL3A1* is a member of the fibrillar collagen family that functions in extensible connective tissues such as the skin, and alterations in *COL3A1* have been demonstrated to be

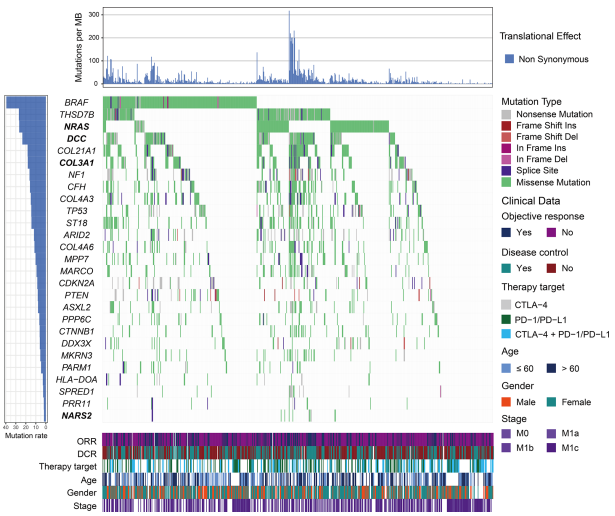

FIGURE 1 | Mutational patterns of significantly mutated genes (SMGs) in melanoma. The left panel depicts the mutation rate of each SMG, the top panel represents non-synonymous mutation burden across integrated patients, the middle panel indicates mutational patterns of identified SMGs with distinct mutation types colored distinctly, and the bottom panel shows clinical characteristics such as age, gender, stage, therapy target, objective response status, and disease control status. SMGs associated with immune checkpoint inhibitor (ICI) efficacy are highlighted in bold.

associated with melanoma metastasis. *NRAS* is an oncogene typically found in melanoma, and multiple targeted therapy agents have been developed for the treatment of *NRAS*-mutated melanoma. *NARS2*, mutated in 1.9% of the total patients, was found to be involved in the prognosis of neurodegenerative disorders (e.g., Alzheimer's disease). The transmembrane protein DCC is a member of the immunoglobulin superfamily of cell adhesion molecules and functions as a tumor suppressor in several cancers, including melanoma.

COL3A1 Mutations Predictive of Improved Immune Checkpoint Inhibitor Survival

The Kaplan–Meier analysis indicated that patients with *COL3A1* mutations showed a significantly improved ICI OS compared with patients without such mutations (median OS: 45.0 [95% CI, 34.5–NA] vs. 24.9 [95% CI, 21.5–28.2] months; log-rank test $p < 0.001$; **Figure 2A**). This association remained significant in the multivariate Cox regression model when age, sex, stage, therapy type, and TMB were taken into consideration (hazard ratio (HR): 0.64, 95% CI: 0.45–0.91, $p = 0.012$; **Figure 2B**). Consistently, an improved PFS was also observed in patients with *COL3A1* mutations in survival analysis (median PFS: 11.43 [95% CI, 5.43–NA] vs. 4.47 [95% CI, 3.57–6.03] months; log-rank test $p = 0.017$; **Figure 2C**) and multivariate analysis (HR: 0.66, 95% CI: 0.44–0.99, $p = 0.042$; **Figure 2D**). We further explored the association of *COL3A1* mutations with ICI ORR and DCR. The results suggested that *COL3A1*-mutated tumors exhibited an elevated ORR (42.9% vs. 28.4%; Fisher's exact test $p = 0.003$;

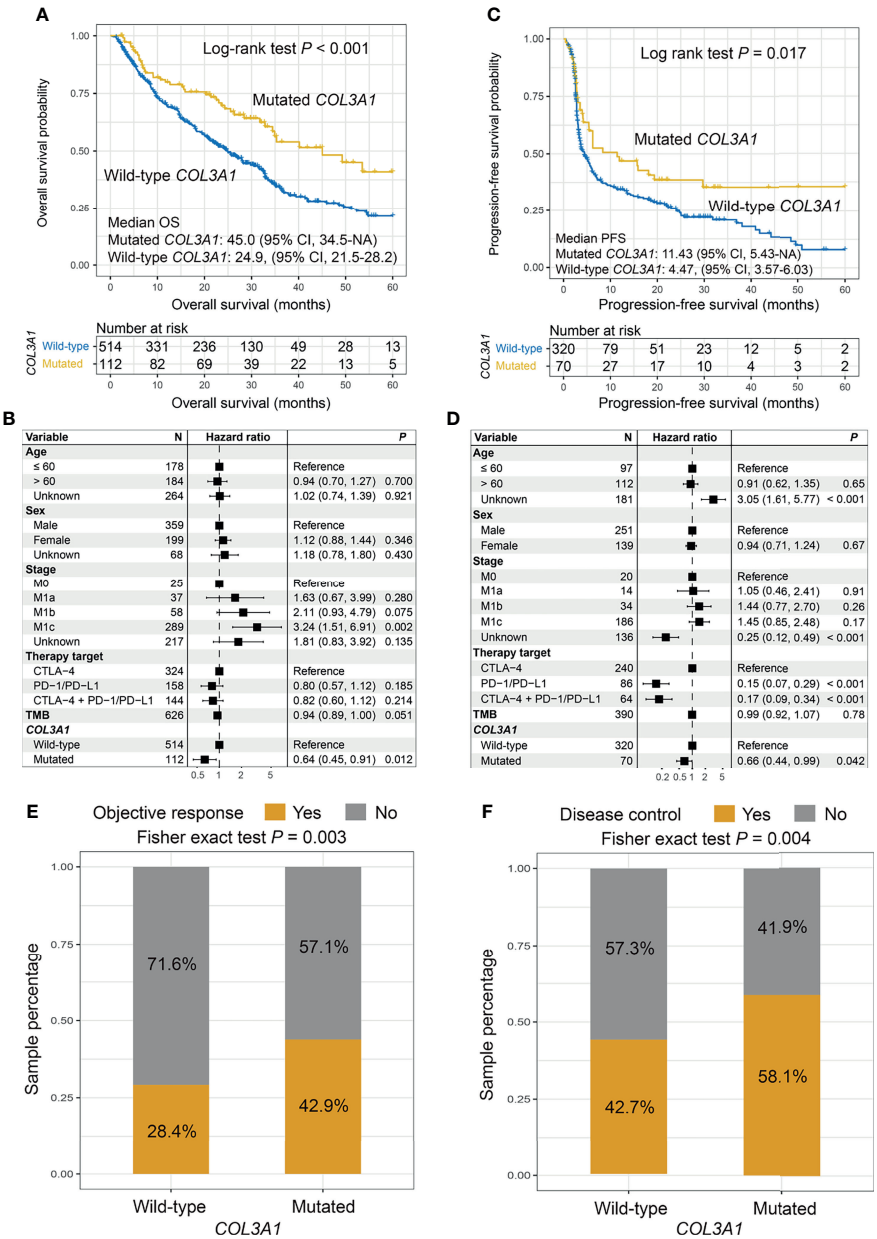

FIGURE 2 | Association of *COL3A1* mutations with immune checkpoint inhibitor (ICI) survival outcome and response. Kaplan–Meier survival analyses and multivariate Cox regression models with confounding factors taken into account were conducted to evaluate the links of *COL3A1* mutations with **(A, B)** overall survival (OS) and **(C, D)** progression-free survival (PFS). *COL3A1* mutations are associated with **(E)** objective response rate (ORR) and **(F)** disease control rate (DCR).

Figure 2E) and DCR (58.1% vs. 42.7%; Fisher's exact test p = 0.004; **Figure 2F**), and marginal statistical significance was observed in the multivariate logistic regression model (p = 0.091 and 0.076, respectively; **Figures S5A, B**). The association of *COL3A1* mutations with ICI survival in distinct ICI types was assessed. We found that *COL3A1* mutations were associated with improved OS in anti-CTLA-4 and combined therapies (log-rank test p = 0.045 and 0.007, respectively; **Figures S6A, C**). In the anti-PD-1/PD-L1 therapy, a trend of better prognosis was observed in *COL3A1*-mutated patients, although it did not reach statistical significance (log-rank test p = 0.094; **Figure S6B**). *COL3A1* mutation associations with ORR (**Figures S6D–F**) and DCR (**Figures S6G–I**) in the three ICI types were also evaluated and illustrated. Six of seven individual cohorts showed trends of improved OS of patients with *COL3A1* mutations (**Figure S7**); the Zaretsky et al. cohort was not evaluated because it harbors only four melanoma patients. We evaluated the prognostic power of *COL3A1* mutations in TCGA melanoma

cohort. No significant associations were observed between *COL3A1* mutations and OS (log-rank test $p = 0.946$, multivariate Cox $p = 0.602$; **Figures S8A, B**) and PFS (log-rank test $p = 0.813$, multivariate Cox $p = 0.618$; **Figures S8C, D**).

We further investigated the possible mechanisms underlying the *COL3A1* mutations. First, an enhanced TMB was observed in the *COL3A1*-mutated patients (Wilcoxon's rank-sum test $p < 0.001$; **Figure S9A**). This link remained significant even after adjusting for mutations in *BRCA1/2*, *TP53*, *POLE*, and MMR genes (OR: 16.11, 95% CI: 8.23–35.46, $p < 0.001$; **Figure S9B**). Consistent results were also observed for NB in univariate analysis (Wilcoxon's rank-sum test $p < 0.001$; **Figure S9C**) and multivariate logistic regression (OR: 4.94, 95% CI: 2.32–11.34, $p < 0.001$; **Figure S9D**). Second, immune cell infiltration analysis revealed that CD8 T cells, activated CD4 memory T cells, and resting NK cells infiltrated tumors of patients with *COL3A1* mutations (Wilcoxon's rank-sum test all $p < 0.05$; **Figure S9E**). Noticeably, *COL3A1* mutant tumors exhibited increased infiltration of pro-inflammatory M1 macrophages (Wilcoxon's rank-sum test $p < 0.001$; **Figure S9E**) and decreased infiltration of immune-suppressive M2 macrophages (Wilcoxon's rank-sum test $p = 0.011$; **Figure S9E**). Third, GSEA results suggested that antigen processing and presentation-related pathways in KEGG and GO databases were enriched in patients with *COL3A1* mutations (all FDR < 0.05; **Figures S9F–J**). Collectively, favorable genomic traits and the immune microenvironment may underlie the better ICI response of *COL3A1* mutations.

NRAS, *NARS2*, and *DCC* Mutations Associated With Immune Checkpoint Inhibitor Efficacy

Patients with *NRAS* mutations exhibited a trend of worse ICI OS than patients without *NRAS* mutations (median OS: 24.4 [95% CI, 19.1–32.9] vs. 28.1 [95% CI, 24.9–33.5] months; log-rank test $p = 0.089$; **Figure 3A**). This result was more significant in the multivariate Cox model (HR: 1.42, 95% CI: 1.10–1.82, $p = 0.006$; **Figure 3B**). No significant difference was observed between patients with and without *NRAS* mutations in relation to ICI PFS (HR: 1.00, 95% CI: 0.74–1.33, $p = 0.998$; **Figures S10A, B**). The tendencies of decreased ORR (OR: 1.34, 95% CI: 0.89–2.04, $p = 0.161$; **Figure S10C**) and DCR (OR: 1.27, 95% CI: 0.86–1.88, $p = 0.231$; **Figure S10D**) were observed in *NRAS*-mutated tumors. The associations between *NRAS* mutations and ICI OS in the three distinct treatments were also assessed. The results

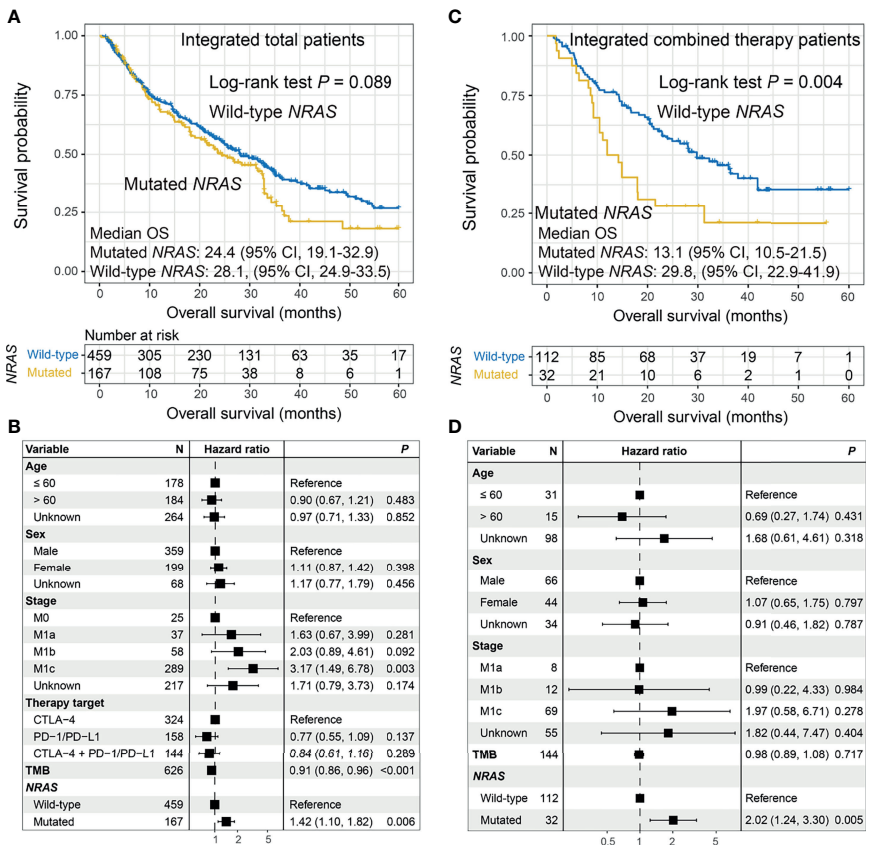

FIGURE 3 | Association between *NRAS* mutations and immune checkpoint inhibitor (ICI) survival. **(A)** Overall survival (OS) curves stratified by *NRAS* mutational status and **(B)** forest plot representation of the connection of *NRAS* mutations with OS outcome in the aggregated melanoma cohort. **(C)** OS curves stratified by *NRAS* mutational status and **(D)** forest plot representation of the association of *NRAS* mutations with ICI outcome in patients who received combined therapy.

demonstrated that *NRAS* mutations were consistently correlated with immune resistance in combined therapy using the Kaplan–Meier analysis (log-rank test p = 0.004; **Figure 3C**) and multivariate Cox model (HR: 2.02, 95% CI: 1.24–3.30, p = 0.005; **Figure 3D**), as well as anti-CTLA-4 therapy (log-rank test p = 0.132; multivariate Cox HR: 1.65, 95% CI: 1.16–2.36, p = 0.005; **Figures S11A, B**). No significant correlation of *NRAS* mutations with anti-PD-1/PD-L1 outcomes was observed (**Figures S11C, D**). Verifiably, *NRAS* mutations were marginally associated with ICI resistance in the combined therapy in the MSKCC cohort (log-rank test p = 0.189; multivariate Cox HR: 1.93, 95% CI: 0.90–4.12, p = 0.085; **Figures S12A, B**).

NARS2 mutations were associated with an elevated ORR (OR: 0.15, 95% CI: 0.03–0.57, p = 0.008; **Figure S13A**), and a similar tendency was also observed in DCR (OR: 0.31, 95% CI: 0.07–1.13, p = 0.096; **Figure S13B**). No differences were detected between the OS curves stratified by *NARS2* status (HR: 1.57, 95% CI: 0.69–3.61, p = 0.286; **Figure S13C**). However, a shortened PFS was observed in patients with *NARS2* mutations (HR: 2.52, 95% CI: 1.12–5.68, p = 0.033; **Figure S13D**).

DCC mutations were correlated with enhanced ORR (OR: 0.62, 95% CI: 0.39–0.98, p = 0.041; **Figure S14A**), and a similar tendency was also observed in DCR (OR: 0.69, 95% CI: 0.44–1.08, p = 0.102; **Figure S14B**). Survival and Cox regression analyses indicated that patients with *DCC* mutations exhibited the trends of improved OS (HR: 0.80, 95% CI: 0.59–1.10, p = 0.167; **Figure S14C**) and PFS (HR: 0.71, 95% CI: 0.49–1.05, p = 0.082; **Figure S14D**), although not statistically significant.

Mutational Signatures Associated With Immune Checkpoint Inhibitor Response or Resistance

The overall mutational pattern of pooled melanoma patients was dominated by C > T (or G > A) mutations with a mutational proportion of 86.7% (**Figure 4A**). We extracted six mutational signatures from melanoma and subsequently compared them with 30 validated signatures from COSMIC. Finally, signatures 1, 4, 7, 11, and 21 were determined according to the COSMIC nomenclature, and a novel signature (named as the unmatched signature) that did not match the previously annotated mutational signatures was also uncovered (**Figure 4B** and **Figure S15**). The distribution of six mutational signatures in each patient varied, as illustrated in **Table S5** and **Figure S16**. Clock-like signature 1, characterized by C > T mutations at CpG dinucleotides, was associated with age-related accumulation of spontaneous deamination of 5-methylcytosine. Signature 4 is featured by C > A mutations and has been reported to be connected with exposure to tobacco carcinogens (e.g., benzo[*a*]pyrene). Mutational profiles of signatures 7 and 11, which both exhibited mainly C > T substitutions and predominantly existed

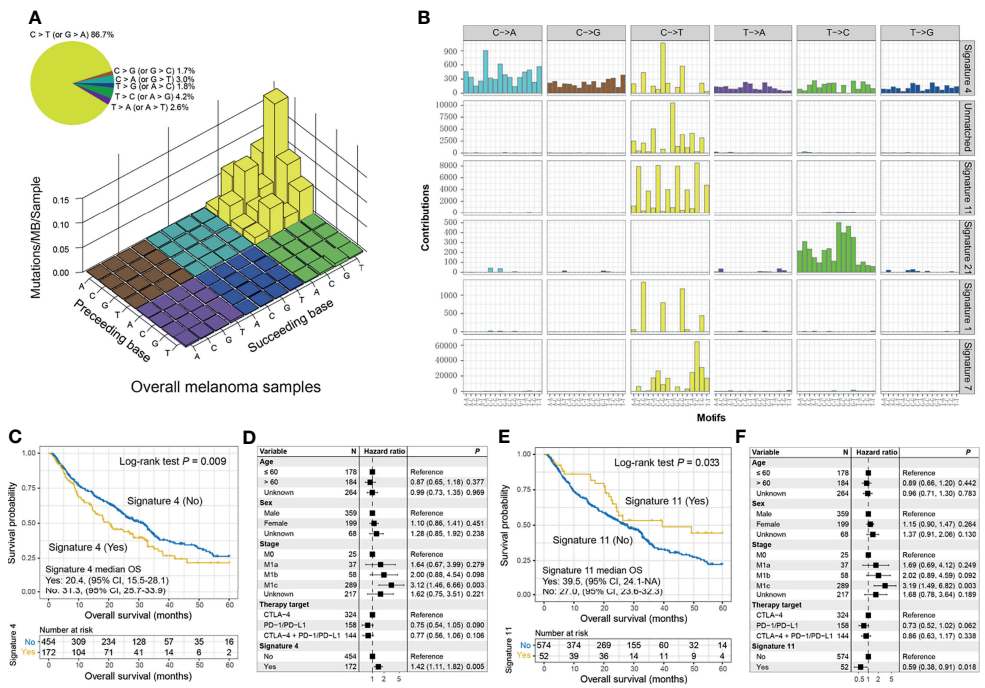

FIGURE 4 | Mutational signatures extracted from the integrated melanoma cohort and their association with immunotherapy prognosis. **(A)** Lego plot representation of mutation patterns in 631 melanoma cases. Single-nucleotide substitutions are divided into 6 categories with 16 surrounding flanking bases. The inset pie chart displays the proportion of 6 mutational patterns. **(B)** The activities of corresponding extracted mutational signatures (i.e., signatures 1, 4, 7, 11, and 21, and unmatched signature). The trinucleotide base substitution types are shown on the x-axes, whereas the y-axes illustrate the contribution percentage of distinct mutation types in each mutational signature. The Kaplan–Meier overall survival (OS) analysis of **(C)** signature 4 and **(E)** signature 11. Multivariate Cox regression models of **(D)** signature 4 and **(F)** signature 11 with age, sex, stage, and therapy type taken into account.

in melanoma, are likely due to exposure to ultraviolet light and treatment with alkylating agents, respectively. Signature 21, dominated by T > C mutations, is probably linked to microsatellite unstable tumors. The unmatched signature was characterized by C > T mutations.

We observed that the presence of signature 4 was significantly correlated with ICI resistance in OS analysis (median OS: 20.4 [95% CI, 15.5–28.1] vs. 31.3 [95% CI, 25.7–33.9] months; log-rank test $p = 0.009$; **Figure 4C**) and multivariate-adjusted model (HR: 1.42, 95% CI: 1.11–1.82, $p = 0.005$; **Figure 4D**). A tendency of worse PFS outcome was also observed in patients with signature 4 (log-rank test $p = 0.196$; multivariate Cox $p = 0.152$; **Figure S17A**). Consistently, decreased ORR (20.6% vs. 34.9%; Fisher's exact test $p < 0.001$; multivariate logistic $p = 0.001$; **Figure S17B**) and DCR (33.9% vs. 49.8%; Fisher's exact test $p < 0.001$; multivariate logistic $p < 0.001$; **Figure S17C**) were associated with the tumors with signature 4. We also compared the genomic and microenvironmental features of patients with and without signature 4. A decreased TMB was observed in patients with signature 4 (Wilcoxon's rank-sum text $p < 0.001$; multivariate logistic OR: 0.10, 95% CI: 0.06–0.16, $p < 0.001$; **Figures S18A, B**). In addition, the lower infiltration of M1 macrophages (Wilcoxon's rank-sum text $p = 0.007$; **Figure S18C**) and higher infiltration of M2 macrophages (Wilcoxon's rank-sum text $p = 0.045$; **Figure S18C**) may be another reason for the ICI resistance of patients with signature 4.

Conversely, the presence of signature 11 was linked to improved ICI OS in survival analysis (median OS: 39.5 [95% CI, 24.1–NA] vs. 27.0 [95% CI, 23.6–32.3]; log-rank test $p = 0.033$; **Figure 4E**) and multivariate Cox regression model (HR: 0.59, 95% CI: 0.38–0.91, $p = 0.018$; **Figure 4F**). Improved ICI OS was also observed in patients with the unmatched signature (HR: 0.59, 95% CI: 0.39–0.90, $p = 0.014$; **Figures S19A, B**). We also treated the above three signatures as continuous variables to conduct a multivariate Cox analysis. The associations of signature 4 (HR: 2.04, 95% CI: 1.26–3.29, $p = 0.003$; **Figure S20A**), signature 11 (HR: 0.47, 95% CI: 0.23–0.97, $p = 0.041$; **Figure S20B**), and unmatched signature (HR: 0.35, 95% CI: 0.12–1.01, $p = 0.052$; **Figure S20C**) with ICI OS were still present.

Potential Molecular Subtypes Contributed to Immune Checkpoint Inhibitor Overall Survival

We could detect latent molecular subtypes based on the activities of extracted mutational signatures. Consensus clustering analysis was performed with cluster numbers ranging from 2 to 10. We observed that the preferable clustering consensus was exhibited when clustering numbers were selected as three or five (**Figure S21A**). More subtle subtypes could be virtually microdissected with an increase in clustering numbers as shown in the cluster tracking plot (**Figure S21B**). Therefore, we selected the clusters as five (i.e., C1, C2, C3, C4, and C5) to explore their association with ICI OS. The plots of the cluster consensus and consensus matrix are separately illustrated in **Figures S21C, D**.

The Kaplan–Meier analysis suggested that patients from the C4 cluster (23 of 626 patients [3.7%]) could achieve the best ICI OS as compared with the other four clusters (log-rank test $p = 0.062$; **Figure 5A**). In the multivariate Cox model, we treated the C4 cluster as the reference subgroup and observed that the other four clusters exhibited worse ICI OS ($p = 0.004$, 0.033, 0.012, and 0.086; **Figure 5B**). In this study, we termed the C4 cluster as "Immune subtype" and the rest as "Non-immune subtype". The improved ICI OS of the immune subtype was still observed when compared with the non-immune subtype in univariate analysis (median OS: 49.3 [95% CI, 22.9–NA] vs. 27.0 [95% CI, 24.3–31.8] months; log-rank test $p = 0.039$; **Figure 5C**) and multivariate Cox model (HR: 0.44, 95% CI: 0.23–0.87, $p = 0.017$; **Figure 5D**).

The Combined Biomarker Predictive of Immune Checkpoint Inhibitor Overall Survival

Considering the predictive implications of *COL3A1* mutations and signature 4, we integrated *COL3A1* mutations and lack of mutational signature 4 as a combined biomarker to evaluate the improved ICI OS (**Table S6**). Patients with the combined marker harbored a significantly better ICI OS than patients without the combined marker (median OS: 45.0 [95% CI, 34.5–NA] vs. 24.9 [95% CI, 21.5–28.2] months; log-rank test $p < 0.001$; **Figure S22A**). The association remained still significant even after adjusting for the confounding factors in the multivariate Cox regression model (HR: 0.60, 95% CI: 0.42–0.87, $p = 0.007$; **Figure S22B**). The presence of the combined marker was also associated with improved ICI PFS according to the Kaplan–Meier analysis (median PFS: 11.43 [95% CI, 6.23–NA] vs. 4.47 [95% CI, 3.50–6.03] months; log-rank test $p = 0.013$; **Figure S22C**) and multivariate Cox model (HR: 0.62, 95% CI: 0.41–0.94, $p = 0.026$; **Figure S22D**). Consistently, an elevated ORR was observed in patients with the combined marker in the univariate analysis (45.2% vs. 28.1%; Fisher's exact test $p = 0.001$; **Figure S23A**) and multivariate logistic regression (OR: 0.67, 95% CI: 0.41–1.11, $p = 0.043$; **Figure S23B**). A similar association between the combined marker and DCR was also found by employing univariate (60.6% vs. 42.5%; Fisher's exact test $p < 0.001$; **Figure S23C**) and multivariate analysis (OR: 0.59, 95% CI: 0.36–0.97, $p = 0.035$; **Figure S23D**).

DISCUSSION

Immune checkpoint-based treatments have revolutionized therapeutic strategies for melanoma. In this study, we comprehensively explored the mutational profiles of 631 melanoma patients treated with ICI agents. We identified four novel SMGs that were previously not recognized to be associated with ICI response/resistance. We further annotated three mutational signatures with respect to ICI efficacy. In addition, a latent immune subtype was demonstrated to be linked to improved ICI outcomes.

Mutations in single genes, such as *MUC16* (22), *POLE* (19), and *PBRM1* (20), exhibited vital effects on the prediction of tumor prognoses or immunotherapeutic outcomes. Our results showed that mutations in the newly identified *COL3A1* SMG were linked with improved ICI response and survival. Subsequently, genomic and immunologic analyses explained that enhanced TMB and

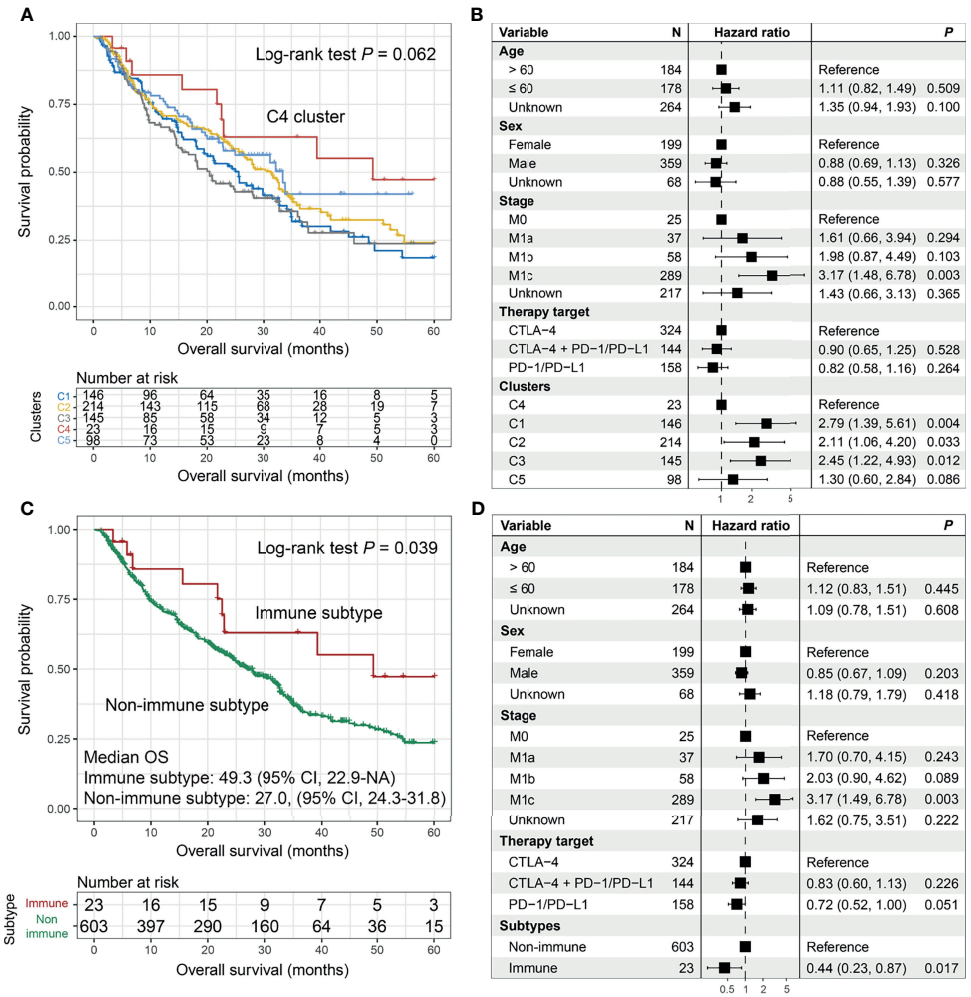

FIGURE 5 | The prediction roles of the identified melanoma immune subtype for immune checkpoint inhibitor (ICI) survival. **(A)** Kaplan–Meier survival analysis and **(B)** forest plot illustration of 5 clusters derived from the consensus clustering. Prognostic significances of the immune subtype vs. non-immune subtype under **(C)** Kaplan–Meier overall survival (OS) analysis and **(D)** multivariate Cox model with confounding variables taken into consideration.

NB, and a hot immune microenvironment characterized patients with *COL3A1* mutations. Indeed, *COL3A1* also participates in immune response regulation at the gene expression level (50, 51), and further studies are needed to explore the link between *COL3A1* mutations and protein expression in immunotherapy. In this study, we also observed that melanoma patients with and without *COL3A1* mutations exhibited a survival difference in the setting of anti-CTLA-4 therapy, but not in the anti-PD-1/PD-L1 therapy. This may be attributed to the following three reasons: 1) the distinct interactions of *COL3A1* mutations with CTLA-4 and PD-1/PD-L1, for example, synergistic and antagonistic roles; 2) the tumor microenvironment may be distinctly influenced by the two ICI treatments, which would generate differential immunogenicity in patients with *COL3A1* mutations; and 3) the sample size used for the two ICI types (324 vs. 158) may also be a potential reason for the distinct survival differences.

The association of *NRAS* mutations with ICI efficacy was only reported by Johnson et al. (52), and their observations indicated trends of improved ICI OS (19.5 vs. 15.2 months) and PFS (4.1 vs. 2.9 months) for patients with *NRAS* mutations, although the results were not statistically significant (log-rank test $p = 0.51$ and 0.08, respectively). Conversely, our study revealed that *NRAS* mutations were linked with inferior ICI OS in the aggregated cohort (multivariate Cox HR: 1.42, $p = 0.006$), and this result was also obtained in both combined therapy (multivariate Cox HR: 2.02, $p = 0.005$) and anti-CTLA-4 cohort (multivariate Cox HR: 1.65, $p = 0.005$). Furthermore, a similar tendency of poorer OS was also observed in patients with *NRAS* mutations who received combined therapy in the MSKCC cohort (multivariate Cox HR: 1.93, $p = 0.085$). The inconsistent results may be attributed to the following two reasons: 1) the sample sizes used, 631 samples of our study vs. 229 of Johnson et al. study; 2) in the multivariate-adjusted analysis, we performed multivariate Cox regression

models adjusting for confounding factors (e.g., age, sex, stage, therapy types, and TMB); however, no adjusted analyses were applied in the Johnson et al. study. On the other hand, *NRAS*-mutated patients had a higher TMB, although ICI resistance developed in these patients. This indicates that a high TMB may be a spurious participant in ICI response. Similar results were also reported by Marinelli et al. (53); that is, *KEAP1*-driven co-mutations were associated with unresponsiveness to immunotherapy, although an elevated TMB was observed in this subset. The cold microenvironment and other immunological factors present in these patients may significantly contribute to immunotherapy efficacy.

NARS2 mutations were linked to elevated ORR. However, worse PFS was observed in *NARS2*-mutated patients. These results indicate that *NARS2* mutations may be a favorable indicator for shorter treatment responses; however, they may play a negative role in disease prognosis.

Smoking-related mutational signature 4, which commonly occurs in lung, head, and neck, and esophageal cancers, was also detected in the pooled melanoma cohort. Lung cancer patients harboring this mutational signature have been demonstrated to show a higher response to ICI treatment (12). However, our study indicated that the smoking signature was associated with ICI resistance in melanoma patients, and distinct tumor types may generate inconsistent results. Findings from a recent study (54) revealed that melanoma patients with cigarette smoking behavior exhibited inferior melanoma-specific survival, which was due to smoking-associated decreased immune infiltration. In our study, the smoking signature was also correlated with a weaker immune microenvironment *via* the regulation of M1 and M2 macrophages. Overall, smoking and its relevant traits may influence immune responses and thus determine the prognosis and immunotherapeutic efficacy in melanoma.

In our study, melanoma patients with alkylating agent exposure-related mutational signature 11 showed prolonged survival as compared with those without such signature. Consistently, patients who received ICI agents were more likely to experience an enhanced ORR if their tumors had this alkylating agent signature in melanoma (27). We also identified a mutational signature that featured C > T substitutions, which was associated with improved survival following ICI treatment. The discovery of this novel mutational signature would further enrich COSMIC data and provide implications for immunotherapy.

The immune molecular subtypes were commonly identified based on immunologic and microenvironment characterizations derived from mixed gene expression profiles. However, currently, a majority of immunotherapy studies have mainly focused on the somatic mutation level, and fewer included gene expression data. In this integrated analysis, only three of eight cohorts had mRNA sequencing data; thus, it may be inappropriate to conduct molecular subtyping by employing mRNA expression data with the limited coverage of melanoma patients. The utilization of activities of mutational signatures extracted from tumor samples is a good choice to determine immanent subclasses in patients with only or mainly mutation data. We detected five clusters with distinct survival outcomes using the six mutational signatures. One cluster with the best ICI prognosis was termed the immune subtype in this study. Interestingly, we observed that patients of the immune subtype were a subset of patients with a lack of signature 4 and the presence of signature 11 (**Table S6**), further verifying the favorable prognostic outcome of this immune subtype.

Based on the findings of this study, prospective clinical trials should be performed to confirm the potential implications of *COL3A1* mutations, mutational signature 4, the identified immune subtype, and other immunotherapy determinants in melanoma and other cancer types, which will provide more clues for guiding clinical practice and individualized treatment. However, there are several limitations to this research. First, the integrated melanoma cohort was derived from multiple distinct cohorts, which may produce deviations in the data processing. Second, transcriptomic data were obtained from only three of the eight included studies, which may not fully elucidate the potential mechanisms of the determinants. Finally, the associations of the identified gene mutations with immunological features remained at a theoretical level and need to be experimentally validated.

Overall, our study integrated 631 ICI-treated melanoma patients and uncovered several clinically related ICI determinants, which provide helpful biomarkers for melanoma immunotherapy prediction.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://xenabrowser.net/hub/ https://www.tcia.at/home.

ETHICS STATEMENT

All studies have been reviewed and approved by the Institutional Research Board.

AUTHOR CONTRIBUTIONS

QW designed this study. QW, SW, WZ, and YK developed the methodology and acquired the related data. QW, WZ, YK, YL, FS, JL, and CS performed the data analysis and interpretation. WZ, YK, and QW drafted and revised the manuscript. QW supervised this study. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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REFERENCES

1. Wolchok JD, Chiarion-Sileni V, Gonzalez R, Rutkowski P, Grob JJ, Cowey CL, et al. Overall Survival With Combined Nivolumab and Ipilimumab in Advanced Melanoma. *N Engl J Med* (2017) 377(14):1345–56. doi: 10.1056/NEJMoa1709684
2. Keenan TE, Burke KP, Van Allen EM. Genomic Correlates of Response to Immune Checkpoint Blockade. *Nat Med* (2019) 25(3):389–402. doi: 10.1038/s41591-019-0382-x
3. Gide TN, Wilmott JS, Scolyer RA, Long GV. Primary and Acquired Resistance to Immune Checkpoint Inhibitors in Metastatic Melanoma. *Clin Cancer Res* (2018) 24(6):1260–70. doi: 10.1158/1078-0432.CCR-17-2267
4. Garon EB, Rizvi NA, Hui R, Leighl N, Balmanoukian AS, Eder JP, et al. Pembrolizumab for the Treatment of non-Small-Cell Lung Cancer. *N Engl J Med* (2015) 372(21):2018–28. doi: 10.1056/NEJMoa1501824
5. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, Activity, and Immune Correlates of Anti-PD-1 Antibody in Cancer. *N Engl J Med* (2012) 366(26):2443–54. doi: 10.1056/NEJMoa1200690
6. Tumeh PC, Harview CL, Yearley JH, Shintaku IP, Taylor EJ, Robert L, et al. PD-1 Blockade Induces Responses by Inhibiting Adaptive Immune Resistance. *Nature* (2014) 515(7528):568–71. doi: 10.1038/nature13954
7. Socinski MA, Jotte RM, Cappuzzo F, Orlandi F, Stroyakovskiy D, Nogami N, et al. Atezolizumab for First-Line Treatment of Metastatic Nonsquamous NSCLC. *N Engl J Med* (2018) 378(24):2288–301. doi: 10.1056/NEJMoa1716948
8. El-Khoueiry AB, Sangro B, Yau T, Crocenzi TS, Kudo M, Hsu C, et al. Nivolumab in Patients With Advanced Hepatocellular Carcinoma (CheckMate 040): An Open-Label, non-Comparative, Phase 1/2 Dose Escalation and Expansion Trial. *Lancet* (2017) 389(10088):2492–502. doi: 10.1016/S0140-6736(17)31046-2
9. Motzer RJ, Rini BI, McDermott DF, Redman BG, Kuzel TM, Harrison MR, et al. Nivolumab for Metastatic Renal Cell Carcinoma: Results of a Randomized Phase II Trial. *J Clin Oncol* (2015) 33(13):1430–7. doi: 10.1200/JCO.2014.59.0703
10. Anagnostou V, Smith KN, Forde PM, Niknafs N, Bhattacharya R, White J, et al. Evolution of Neoantigen Landscape During Immune Checkpoint Blockade in Non-Small Cell Lung Cancer. *Cancer Discov* (2017) 7(3):264–76. doi: 10.1158/2159-8290.CD-16-0828
11. Van Allen EM, Miao D, Schilling B, Shukla SA, Blank C, Zimmer L, et al. Genomic Correlates of Response to CTLA-4 Blockade in Metastatic Melanoma. *Science* (2015) 350(6257):207–11. doi: 10.1126/science.aad0095
12. Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, et al. Cancer Immunology. Mutational Landscape Determines Sensitivity to PD-1 Blockade in non-Small Cell Lung Cancer. *Science* (2015) 348(6230):124–8. doi: 10.1126/science.aaa1348
13. Hellmann MD, Nathanson T, Rizvi H, Creelan BC, Sanchez-Vega F, Ahuja A, et al. Genomic Features of Response to Combination Immunotherapy in Patients With Advanced Non-Small-Cell Lung Cancer. *Cancer Cell* (2018) 33(5):843–52.e844. doi: 10.1016/j.ccell.2018.03.018
14. Hellmann MD, Callahan MK, Awad MM, Calvo E, Ascierto PA, Atmaca A, et al. Tumor Mutational Burden and Efficacy of Nivolumab Monotherapy and in Combination With Ipilimumab in Small-Cell Lung Cancer. *Cancer Cell* (2018) 33(5):853–61.e854. doi: 10.1016/j.ccell.2018.04.001
15. Rosenberg JE, Hoffman-Censits J, Powles T, van der Heijden MS, Balar AV, Necchi A, et al. Atezolizumab in Patients With Locally Advanced and Metastatic Urothelial Carcinoma Who Have Progressed Following Treatment With Platinum-Based Chemotherapy: A Single-Arm, Multicentre, Phase 2 Trial. *Lancet* (2016) 387(10031):1909–20. doi: 10.1016/S0140-6736(16)00561-4
16. McDermott DF, Huseni MA, Atkins MB, Motzer RJ, Rini BI, Escudier B, et al. Clinical Activity and Molecular Correlates of Response to Atezolizumab Alone or in Combination With Bevacizumab Versus Sunitinib in Renal Cell Carcinoma. *Nat Med* (2018) 24(6):749–57. doi: 10.1038/s41591-018-0053-3
17. Armand P, Engert A, Younes A, Fanale M, Santoro A, Zinzani PL, et al. Nivolumab for Relapsed/Refractory Classic Hodgkin Lymphoma After Failure of Autologous Hematopoietic Cell Transplantation: Extended Follow-Up of the Multicohort Single-Arm Phase II CheckMate 205 Trial. *J Clin Oncol* (2018) 36(14):1428–39. doi: 10.1200/JCO.2017.76.0793
18. Nghiem PT, Bhatia S, Lipson EJ, Kudchadkar RR, Miller NJ, Annamalai L, et al. PD-1 Blockade With Pembrolizumab in Advanced Merkel-Cell Carcinoma. *N Engl J Med* (2016) 374(26):2542–52. doi: 10.1056/NEJMoa1603702
19. Wang F, Zhao Q, Wang YN, Jin Y, He MM, Liu ZX, et al. Evaluation of POLE and POLD1 Mutations as Biomarkers for Immunotherapy Outcomes Across Multiple Cancer Types. *JAMA Oncol* (2019) 5(10):1504–6. doi: 10.1001/jamaoncol.2019.2963
20. Braun DA, Ishii Y, Walsh AM, Van Allen EM, Wu CJ, Shukla SA, et al. Clinical Validation of PBRM1 Alterations as a Marker of Immune Checkpoint Inhibitor Response in Renal Cell Carcinoma. *JAMA Oncol* (2019) 5(11):1631–3. doi: 10.1001/jamaoncol.2019.3158
21. Jia Q, Wang J, He N, He J, Zhu B. Titin Mutation Associated With Responsiveness to Checkpoint Blockades in Solid Tumors. *JCI Insight* (2019) 4(10):e127901. doi: 10.1172/jci.insight.127901
22. Li X, Pasche B, Zhang W, Chen K. Association of MUC16 Mutation With Tumor Mutation Load and Outcomes in Patients With Gastric Cancer. *JAMA Oncol* (2018) 4(12):1691–8. doi: 10.1001/jamaoncol.2018.2805
23. Sade-Feldman M, Jiao YJ, Chen JH, Rooney MS, Barzily-Rokni M, Eliane JP, et al. Resistance to Checkpoint Blockade Therapy Through Inactivation of Antigen Presentation. *Nat Commun* (2017) 8(1):1136. doi: 10.1038/s41467-017-01062-w
24. Shin DS, Zaretsky JM, Escuin-Ordinas H, Garcia-Diaz A, Hu-Lieskovan S, Kalbasi A, et al. Primary Resistance to PD-1 Blockade Mediated by JAK1/2 Mutations. *Cancer Discov* (2017) 7(2):188–201. doi: 10.1158/2159-8290.CD-16-1223
25. Zaretsky JM, Garcia-Diaz A, Shin DS, Escuin-Ordinas H, Hugo W, Hu-Lieskovan S, et al. Mutations Associated With Acquired Resistance to PD-1 Blockade in Melanoma. *N Engl J Med* (2016) 375(9):819–29. doi: 10.1056/NEJMoa1604958
26. Wang S, Jia M, He Z, Liu XS. APOBEC3B and APOBEC Mutational Signature as Potential Predictive Markers for Immunotherapy Response in non-Small Cell Lung Cancer. *Oncogene* (2018) 37(29):3924–36. doi: 10.1038/s41388-018-0245-9
27. Miao D, Margolis CA, Vokes NI, Liu D, Taylor-Weiner A, Wankowicz SM, et al. Genomic Correlates of Response to Immune Checkpoint Blockade in Microsatellite-Stable Solid Tumors. *Nat Genet* (2018) 50(9):1271–81. doi: 10.1038/s41588-018-0200-2
28. Tsoi J, Robert L, Paraiso K, Galvan C, Sheu KM, Lay J, et al. Multi-Stage Differentiation Defines Melanoma Subtypes With Differential Vulnerability to Drug-Induced Iron-Dependent Oxidative Stress. *Cancer Cell* (2018) 33(5):890–904.e895. doi: 10.1016/j.ccell.2018.03.017
29. Cancer Genome Atlas N. Genomic Classification of Cutaneous Melanoma. *Cell* (2015) 161(7):1681–96. doi: 10.1016/j.cell.2015.05.044
30. Hu B, Wei Q, Zhou C, Ju M, Wang L, Chen L, et al. Analysis of Immune Subtypes Based on Immunogenomic Profiling Identifies Prognostic Signature for Cutaneous Melanoma. *Int Immunopharmacol* (2020) 89(Pt A):107162. doi: 10.1016/j.intimp.2020.107162
31. Li XC, Wang MY, Yang M, Dai HJ, Zhang BF, Wang W, et al. A Mutational Signature Associated With Alcohol Consumption and Prognostically

Significantly Mutated Driver Genes in Esophageal Squamous Cell Carcinoma. *Ann Oncol* (2018) 29(4):938–44. doi: 10.1093/annonc/mdy011
32. Roh W, Chen PL, Reuben A, Spencer CN, Prieto PA, Miller JP, et al. Integrated Molecular Analysis of Tumor Biopsies on Sequential CTLA-4 and PD-1 Blockade Reveals Markers of Response and Resistance. *Sci Transl Med* (2017) 9(379):eaah3560. doi: 10.1126/scitranslmed.aah3560
33. Hugo W, Zaretsky JM, Sun L, Song C, Moreno BH, Hu-Lieskovan S, et al. Genomic and Transcriptomic Features of Response to Anti-PD-1 Therapy in Metastatic Melanoma. *Cell* (2016) 165(1):35–44. doi: 10.1016/j.cell.2016.02.065
34. Riaz N, Havel JJ, Makarov V, Desrichard A, Urba WJ, Sims JS, et al. Tumor and Microenvironment Evolution During Immunotherapy With Nivolumab. *Cell* (2017) 171(4):934–49.e916. doi: 10.1016/j.cell.2017.09.028
35. Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, et al. Genetic Basis for Clinical Response to CTLA-4 Blockade in Melanoma. *N Engl J Med* (2014) 371(23):2189–99. doi: 10.1056/NEJMoa1406498
36. Liu D, Schilling B, Liu D, Sucker A, Livingstone E, Jerby-Arnon L, et al. Integrative Molecular and Clinical Modeling of Clinical Outcomes to PD1 Blockade in Patients With Metastatic Melanoma. *Nat Med* (2019) 25 (12):1916–27. doi: 10.1038/s41591-019-0654-5
37. Ramos AH, Lichtenstein L, Gupta M, Lawrence MS, Pugh TJ, Saksena G, et al. Oncotator: Cancer Variant Annotation Tool. *Hum Mutat* (2015) 36(4): E2423–9. doi: 10.1002/humu.22771
38. Samstein RM, Lee CH, Shoushtari AN, Hellmann MD, Shen R, Janjigian YY, et al. Tumor Mutational Load Predicts Survival After Immunotherapy Across Multiple Cancer Types. *Nat Genet* (2019) 51(2):202–6. doi: 10.1038/s41588-018-0312-8
39. Lawrence MS, Stojanov P, Polak P, Kryukov GV, Cibulskis K, Sivachenko A, et al. Mutational Heterogeneity in Cancer and the Search for New Cancer-Associated Genes. *Nature* (2013) 499(7457):214–8. doi: 10.1038/nature12213
40. Kandoth C, McLellan MD, Vandin F, Ye K, Niu B, Lu C, et al. Mutational Landscape and Significance Across 12 Major Cancer Types. *Nature* (2013) 502 (7471):333–9. doi: 10.1038/nature12634
41. Skidmore ZL, Wagner AH, Lesurf R, Campbell KM, Kunisaki J, Griffith OL, et al. GenVisR: Genomic Visualizations in R. *Bioinformatics* (2016) 32 (19):3012–4. doi: 10.1093/bioinformatics/btw325
42. Kim J, Mouw KW, Polak P, Braunstein LZ, Kamburov A, Kwiatkowski DJ, et al. Somatic ERCC2 Mutations are Associated With a Distinct Genomic Signature in Urothelial Tumors. *Nat Genet* (2016) 48(6):600–6. doi: 10.1038/ng.3557
43. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, et al. Signatures of Mutational Processes in Human Cancer. *Nature* (2013) 500 (7463):415–21. doi: 10.1038/nature12477
44. Wilkerson MD, Hayes DN. ConsensusClusterPlus: A Class Discovery Tool With Confidence Assessments and Item Tracking. *Bioinformatics* (2010) 26 (12):1572–3. doi: 10.1093/bioinformatics/btq170
45. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, et al. Robust Enumeration of Cell Subsets From Tissue Expression Profiles. *Nat Methods* (2015) 12(5):453–7. doi: 10.1038/nmeth.3337
46. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. Limma Powers Differential Expression Analyses for RNA-Sequencing and Microarray Studies. *Nucleic Acids Res* (2015) 43(7):e47. doi: 10.1093/nar/gkv007
47. Robinson MD, McCarthy DJ, Smyth GK. Edger: A Bioconductor Package for Differential Expression Analysis of Digital Gene Expression Data. *Bioinformatics* (2010) 26(1):139–40. doi: 10.1093/bioinformatics/btp616
48. Martin SA, Hewish M, Lord CJ, Ashworth A. Genomic Instability and the Selection of Treatments for Cancer. *J Pathol* (2010) 220(2):281–9. doi: 10.1002/path.2631
49. Mayakonda A, Lin DC, Assenov Y, Plass C, Koeffler HP. Maftools: Efficient and Comprehensive Analysis of Somatic Variants in Cancer. *Genome Res* (2018) 28(11):1747–56. doi: 10.1101/gr.239244.118
50. Zhang T, Wang BF, Wang XY, Xiang L, Zheng P, Li HY, et al. Key Genes Associated With Prognosis and Tumor Infiltrating Immune Cells in Gastric Cancer Patients Identified by Cross-Database Analysis. *Cancer Biother Radiopharm* (2020) 35(9):696–710. doi: 10.1089/cbr.2019.3423
51. Tian Y, Ke Y, Ma Y. High Expression of Stromal Signatures Correlated With Macrophage Infiltration, Angiogenesis and Poor Prognosis in Glioma Microenvironment. *PeerJ* (2020) 8:e9038. doi: 10.7717/peerj.9038
52. Johnson DB, Lovly CM, Flavin M, Panageas KS, Ayers GD, Zhao Z, et al. Impact of NRAS Mutations for Patients With Advanced Melanoma Treated With Immune Therapies. *Cancer Immunol Res* (2015) 3(3):288–95. doi: 10.1158/2326-6066.CIR-14-0207
53. Marinelli D, Mazzotta M, Scalera S, Terrenato I, Sperati F, D'Ambrosio L, et al. KEAP1-Driven Co-Mutations in Lung Adenocarcinoma Unresponsive to Immunotherapy Despite High Tumor Mutational Burden. *Ann Oncol* (2020) 31(12):1746–54. doi: 10.1016/j.annonc.2020.08.2105
54. Pozniak J, Nsengimana J, Laye JP, O'Shea SJ, Diaz JMS, Droop AP, et al. Genetic and Environmental Determinants of Immune Response to Cutaneous Melanoma. *Cancer Res* (2019) 79(10):2684–96. doi: 10.1158/0008-5472.CAN-18-2864

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Comprehensive Testing of Chemotherapy and Immune Checkpoint Blockade in Preclinical Cancer Models Identifies Additive Combinations

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Antibodies that target immune checkpoints such as cytotoxic T lymphocyte antigen 4 (CTLA-4) and the programmed cell death protein 1/ligand 1 (PD-1/PD-L1) are now a treatment option for multiple cancer types. However, as a monotherapy, objective responses only occur in a minority of patients. Chemotherapy is widely used in combination with immune checkpoint blockade (ICB). Although a variety of isolated immunostimulatory effects have been reported for several classes of chemotherapeutics, it is unclear which chemotherapeutics provide the most benefit when combined with ICB. We investigated 10 chemotherapies from the main canonical classes dosed at the clinically relevant maximum tolerated dose in combination with anti-CTLA-4/anti-PD-L1 ICB. We screened these chemo-immunotherapy combinations in two murine mesothelioma models from two different genetic backgrounds, and identified chemotherapies that produced additive, neutral or antagonistic effects when combined with ICB. Using flow cytometry and bulk RNAseq, we characterized the tumor immune milieu in additive chemo-immunotherapy combinations. 5-fluorouracil (5-FU) or cisplatin were additive when combined with ICB while vinorelbine and etoposide provided no additional benefit when combined with ICB. The combination of 5-FU with ICB augmented an inflammatory tumor microenvironment with markedly increased $CD8^+$ T cell activation and upregulation of IFNγ, TNFα and IL-1β signaling. The effective anti-tumor immune response of 5-FU chemo-immunotherapy was dependent on $CD8^+$ T cells but was unaffected when TNFα or IL-1β cytokine signaling pathways were blocked. Our study identified additive and non-additive chemotherapy/ICB combinations and suggests a possible role for increased inflammation in the tumor microenvironment as a basis for effective combination therapy.

Keywords: chemo-immunotherapy combinations, immune checkpoint blockade (ICB) therapy, T cells, proinflammatory cytokine, TNFa = tumor necrosis factor–a, IL-1b, fluorouracil (5-FU), cisplatin

INTRODUCTION

Drugs that block immune checkpoint receptors such as CTLA-4, PD-1 or PD-L1 have revolutionised cancer treatment, with durable anti-tumor responses observed in a subset of cancer patients (1, 2). However, the majority of patients treated with immune checkpoint blockade (ICB) demonstrate little or no benefit. Conventional chemotherapy remains standard treatment for many cancers. In addition to cytotoxic effects on cancer cells, many chemotherapeutics are immunostimulatory, capable of; inducing immunogenic cell death (3), increasing antigen cross-presentation (4), increasing immune cell infiltration (5), depleting immunosuppressive cells (6, 7), and altering expression of immune checkpoint ligands (8, 9). As these characteristics have been linked to ICB efficacy, some chemotherapeutics could potentially enhance anti-tumor immune responses when combined with ICB and therefore combination therapy warrants further investigation. Combination ICB and chemotherapy has shown efficacy in several cancer types. In fact, of the many different drug classes that have been combined with ICB, classical cancer chemotherapy remains one of the most successful (10). Particularly in thoracic cancers, chemotherapy/ICB combinations have shown efficacy, with FDA approval in non-small cell lung cancer (11) and small cell lung cancer (12), and with promising results in malignant pleural mesothelioma (13).

Although the effects of individual chemotherapeutics on discrete components of the immune system have been extensively described, a systematic analysis of how different chemotherapies combine with ICB *in vivo* is lacking, and the molecular mechanisms underlying additive chemo-immunotherapy combinations remains unknown. In this study, we systematically interrogated the therapeutic interaction between ICB and different canonical classes of cancer chemotherapeutics, given at maximum tolerated dose (MTD), in two preclinical cancer models, and mapped the molecular and cellular profiles of additive combinations, with the aim of prioritizing combinations to take forward into clinical trials.

MATERIALS AND METHODS

Mice

Female BALB/c and C57BL/6 mice (RRID: IMSR_ARC:BC, RRID: IMSR_ARC:B6) were bred and maintained at the Animal Resource Centre (Murdoch, WA, Australia) or Harry Perkins Institute of Medical Research (Murdoch, WA, Australia). All mice used were between 8-10 weeks of age and were maintained under standard specific pathogen free housing conditions at the Harry Perkins Bioresources North Facility (Nedlands, WA, Australia). All experiments were conducted in accordance with the code of conduct of the National Health and Medical Research Council (NHMRC) of Australia, and under the approval of the Harry Perkins Institute of Medical Research Animal Ethics Committee (protocols AE029, AE100, AE179).

Cell Lines

Murine mesothelioma cell lines AB1 (CBA, Cat# CBA-0144, RRID: CVCL_4403), AB1-HA (CBA, Cat# CBA-1374, RRID: CVCL_G361) and AE17 (CBA, Cat#CBA-0156, RRID: CVCL_4408) were derived as previously described (14, 15). Cell lines were maintained in RPMI 1640 (ThermoFisher Scientific, Scoresby VIC, Australia) supplemented with 20 mM HEPES, 0.05 mM 2-mercaptoethanol, 100 units/mL penicillin (CSL, Melbourne VIC, Australia), 50 μg/mL gentamicin (David Bull Labs, Kewdale VIC, Australia), 10% Newborn Calf Serum (NCS; ThermoFisher Scientific, Scoresby VIC, Australia) and 50 mg/mL of geneticin for AB1-HA only (G418; Life Technologies). Cells were cultured for a minimum of 4 passages after thawing before inoculation into mice. Cell lines were validated yearly by flow cytometry for MHC-I molecules H2-K^b (consistent with C57BL/6) and H2-K^d (consistent with BALB/c), and for fibroblast markers E-cadherin, epithelial cell adhesion molecule, and platelet-derived growth factor receptor α (negative). All cell lines were tested for Mycoplasma spp., every 3 months by PCR and found to be negative.

Tumor Cell Inoculation

Cells were harvested when they reached 80% confluence. The right-hand flanks of mice were inoculated subcutaneously with 5 x 10^5 tumor cells suspended in 100 μL of PBS. Mice were randomized prior to treatment, when tumors were palpable. Tumor dimensions (length and width) were measured with digital calipers by an investigator blinded for treatment allocation and tumor growth was represented as area (mm^2).

Chemotherapy, ICB and Antibody Treatments

Chemotherapy and ICB were administered on the same day, initiating treatment when tumors were approximately 20-25 mm^2 in size. Chemotherapies were provided by Sir Charles Gardiner Pharmacy (Nedlands, WA, Australia) and was administered at the predetermined MTD as previously reported (16), except 5-FU which was administered at 75 mg/kg because MTD 5-FU with ICB caused severe toxicity (**Table S1**). Anti-CTLA-4 (clone 9H10, JJP Biologics) was dosed once at 100 μg/mouse and anti-PD-L1 (clone MIH5, JJP Biologics) was dosed 3 times with 2-day intervals at 100 μg/mouse (17). For depletion experiments, anti-CD4 (clone GK1.5, BioXcell), anti-CD8 (clone YTS 169, BioXcell) or anti-IL1β (clone B122, BioXcell) antibodies were administered 3 times with 3-day intervals at 100 μg/mouse with the first dose commencing 3 days before chemo-immunotherapy. Anti-TNFα (clone XT3.11, BioXcell) was administered using the above schedule but at 2 mg/mouse. All treatments were diluted in sterile 0.9 % sodium chloride and administered intraperitoneally (i.p.) or intravenously (i.v.) as described in **Table S1**.

Preparation of Single Cell Suspensions

Spleen and draining lymph nodes (DLNs) were digested with 1 mg/mL type IV collagenase (Worthington Biochemical) and 1 μg/mL DNase (Sigma Aldrich) in RPMI-1640 supplemented with 2% NCS and 20 mM HEPES for 25 minutes at room temperature. Red blood cells were lysed with Pharm Lyse (BD Biosciences). All cell suspensions were resuspended in EDTA-BSS-NCS. Absolute numbers of leucocytes in DLNs were obtained using the Z2 Coulter Counter Analyzer (Beckman Coulter).

Tumors were processed using the Miltenyi Biotec mouse tumor dissociation kit, as per manufacturer's protocol. Briefly, tumors were cut into 2-4 mm pieces and added to GentleMACs C tubes with 2.35 mL RPMI media supplemented with 10% NCS. Prioprietary enzyme mix was added, and samples mechanically digested using the GentleMACS Octo Dissociator 37C_M_TDK_2 protocol.

Flow Cytometry

Three flow cytometry panels outlined in **Table S2** were used to characterize lymphoid and myeloid cell subsets. CD16/32 Fc block (eBioscience) and Zombie UV™ (Biolegend) viability dye were diluted in PBS and added to samples prior to surface antigen staining. All antibodies for surface staining were diluted in PBS + 2% NCS. Cells were permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Cells were washed with Permeabilization Buffer (eBioscience) and subjected to intracellular staining. Single stain and fluorescence minus-one (FMO) controls were also performed. To measure granzyme B (GzmB) and IFNγ, samples were incubated in Brefeldin A (Biolegend) for 4 hours at 37°C before antibody staining. Data was acquired using a BD LSRFortessa™ SORP with 50,000 live events collected per sample where possible. All flow cytometry analyses were completed using FlowJo™ Software version 10 (BD Biosciences). Summary of antibody concentrations and gating strategies are outlined (**Table S2**; **Figure S1**).

Flow Cytometry Data Analysis

FCS files were subjected to automatic quality control of signal acquisition and dynamic range by the flowAI (v1.8) package using default parameters. Bad events (defined by negative outliers) were excluded, and manual gating was performed as outlined in **Figure S1**. For clustering analysis on the lymphoid cells, each sample was downsampled to 5,000 $CD45^+$ cells using the DownSample (v3.1.0) package. All samples from all groups were then concatenated. The UMAP (v2.2) and Phenograph (v1.3) packages using default parameters (k = 30) were performed in FlowJo using the concatenated FCS file. Clusters were manually grouped into the final subsets described in **Figure 3**. Clusters were combined based on similar location on the UMAP plot and similar expression of key markers. Clusters that were $CD45^+$ but had no expression of other phenotypic markers in the panel were colored grey and excluded from the analysis.

Tumor Preparation for Bulk RNAseq

Whole tumors were harvested and stored in RNAlater (Life Technologies) at -80°C. RNA was extracted from frozen tumors using the RNeasy Plus Mini Kit and Tissue Ruptor (QIAGEN). RNA quality was confirmed on the Bioanalyzer (Agilent Technologies). Library preparation and sequencing (100-base pair single-end on an Illumina HiSeq platform) were performed by the Australian Genome Research Facility (Melbourne, VIC, Australia).

Bulk RNAseq Analysis

Raw FASTQ files were aligned to the GRCm38/mm10 reference genome using Kallisto (18). Transcripts with low counts were removed and two count matrices were compiled using Tximport (19). The DESeq2 package (20) was used to identify differentially expressed genes (DEGs) between the following comparisons: PBS vs ICB, 5-FU, cisplatin, 5-FU+ICB or cisplatin+ICB; ICB alone vs 5FU+ICB or cisplatin+ICB, 5-FU alone vs 5-FU+ICB and cisplatin alone vs cisplatin+ICB. P values were adjusted for multiple comparisons using the Benjamini-Hochberg (B-H) method. A p value < 0.05 and a Log2 fold change cut-off of 0.5 were used to select DEGs. A full list of DEGs between each comparison can be found in **Supplementary File 1**.

Pathway analysis on up-and down-regulated DEGs between each comparison were performed using Enrichr (21). Over-representation of pathways from KEGG Mouse 2019 and Reactome 2016 databases were mapped using DEGs as input. The enrichment of upregulated ligands from the LINCS L1000 connectivity map were also analyzed using DEGs in Enrichr. Upstream regulator analysis was performed with DEGs and associated log fold changes as input, using the Ingenuity Systems program (22). Default settings were used and activation *Z*-scores were used to determine the activation state of each upstream regulator. Those with activation *Z*-scores of ≥ 2 were considered 'activated' while activation *Z*-scores of ≤ -2 were considered 'inhibited'. Upstream regulators included cytokines, transcription regulators, complexes, enzymes and kinases. For these analyses, p values were adjusted for multiple comparisons using the Benjamini-Hochberg method and $p < 0.05$ was considered significant.

Count data was scaled up to library size using Tximport (19) resulting in scaled transcripts per million (TPM) normalized count matrices. Heatmaps with unsupervised hierarchical clustering of the top 200 variable DEGs, determined by standard deviation were performed using the pheatmap package in R (v3.6). Gene set enrichment analysis (GSEA) was completed on the normalized gene expression data using 50 MSigDB hallmark gene sets on the Broad Institute software (23). Gene sets enriched with a FDR > 0.25 were considered significant. A total of 1000 permutations were performed, and all other default parameters were used. CIBERSORTx was used to identify immune cell populations in normalised RNAseq data as previously described (17).

Statistical Analysis

Data are presented as mean ± SD. For flow cytometry experiments, statistical analyses were performed using Mann-Whitney U tests with multiple comparisons to compare between monotherapies and combination chemo/immunotherapy-treated samples using GraphPad Prism v8. Survival data were analyzed using Log-rank (Mantel-Cox) test in GraphPad Prism v8. To compare combination treatments to monotherapy controls, hazard ratios (HR) were calculated using logrank analysis of survival curves to determine agonistic or antagonistic effects. To further define additive interactions, as described before (24), HR was calculated for each treatment group compared to PBS or best monotherapy treated controls. Additive effects were defined as HR(combination) < [HR (combination) - HR(mono 1) - HR(mono 2) + 1]. Results of these analyses are displayed in **Table S3**.

RESULTS

5-FU and Cisplatin Generate Robust Anti-Tumor Responses When Combined With ICB

The addition of ICB with chemotherapy regimens are increasingly being trialed in the clinic to improve patient outcomes (25). However, the impact of individual chemotherapies on ICB efficacy remains unclear. To assess anti-tumor responses of chemotherapy when combined with ICB *in vivo*, we screened 10 chemotherapeutics from different canonical classes in combination with anti-CTLA-4/anti-PD-L1 antibodies in two murine mesothelioma models (**Figures 1A, B**). As there is a difference in the therapeutic response to the different chemotherapies (**Figures S2, 3**), we compared survival of the combination therapy with survival of the best monotherapy (either chemotherapy or ICB alone) and plotted each as a hazard ratio (HR). ICB alone induced complete tumor regression in 0-30% of AB1 tumor bearing animals

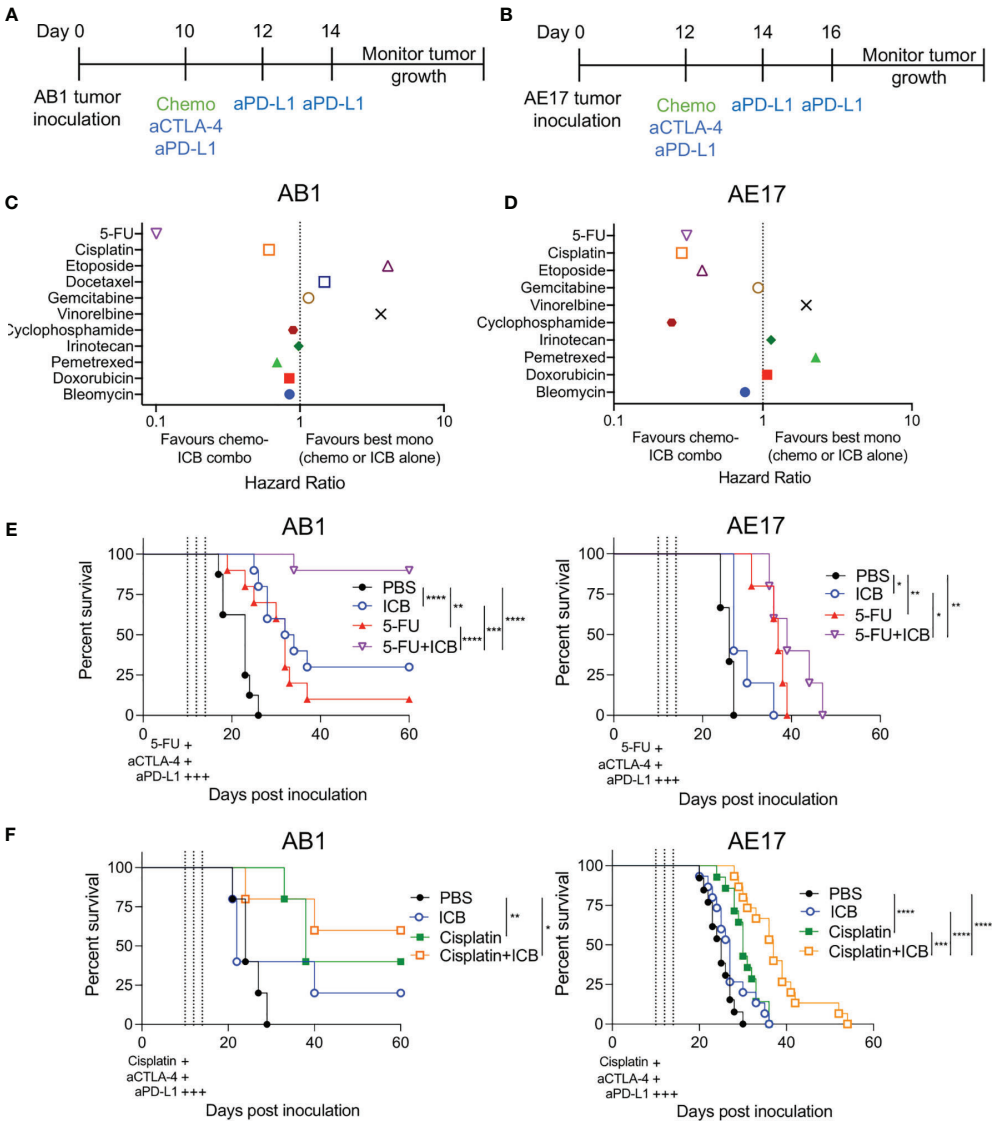

FIGURE 1 | Different combinations of chemotherapy and immune checkpoint blockade (ICB) demonstrate additive and antagonistic responses. **(A, B)** Treatment schedule for mice inoculated with AB1 **(A)** or AE17 **(B)** mesothelioma cell lines. **(C, D)** Hazard ratio (HR) analysis of survival plots comparing combination chemotherapy and ICB (anti-CTLA-4/anti-PD-L1) to the best performing monotherapy in AB1 **(C)** and AE17 **(D)**. HR is defined as the risk of a negative (death) outcome occurring in one group at the next instance of time, compared to another group at the same time. A lower ratio i.e., less than 1 indicates a higher rate of survival in the chemo-immunotherapy combination compared to monotherapy. **(E)** Survival curves of 5-FU chemo-immunotherapy combinations in AB1 (left; n = 8-10 per group, two pooled experiments) and AE17 (right; n = 5 per group, one experiment). **(F)** Survival curves of cisplatin chemo-immunotherapy combinations in AB1 (left; n = 5 per group, one experiment) and AE17 (right; n = 13-15 per group, two pooled experiments). Mantel-Cox survival test; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

but not in the AE17 model. When combined with ICB, all tested chemotherapies had varying effects on anti-tumor efficacy across the two models (**Figures 1C, D**, **S2, 3**, **Table S3**). Gemcitabine, irinotecan, doxorubicin and bleomycin provided no benefit when combined with ICB in either AB1 (HR = 1.14, 0.979, 0.845, 0.845, 0.692 respectively) or AE17 (HR = 0.929, 1.14, 1.07, 0.759 respectively). ICB provided no further benefit when added to cyclophosphamide in AB1 (HR = 0.895), but the combination significantly improved median survival in AE17 (HR = 0.244). The combination of vinorelbine or etoposide with ICB was antagonistic in AB1 (HR = 3.65, 4.08) but had no effect in AE17 (HR = 1.96, 0.391). The reverse was the case for pemetrexed (HR = 0.692 in AB1, 2.261 in AE17).

The combination of 5-FU with ICB (5-FU+ICB) resulted in robust anti-tumor responses in the AB1 model (HR = 0.101), with significant increase in median survival compared to both 5-FU ($p = 0.0001$) and ICB ($p = 0.005$) monotherapy. Complete tumor regression occurred in >80% of 5-FU+ICB treated animals, compared to 0-20% or 20-30% complete responders in 5-FU or ICB monotherapy, respectively (**Figure 1E**). 5-FU+ICB was also additive in AE17 (HR = 0.308), with an increase in median survival compared to the monotherapies (5-FU: $p = 0.199$, ICB: $p = 0.0142$). Cisplatin and ICB (cisplatin+ICB) were additive in both AB1 (HR = 0.610) and AE17 (HR = 0.286) (**Figure 1F**). In AE17, cisplatin+ICB significantly increased median survival compared to cisplatin ($p = 0.0025$) and ICB ($p<0.0001$) monotherapy. Taken together, these data demonstrate that different chemotherapies display additive, antagonistic or neutral interactions with ICB and that these interactions could be variable between models.

Combination ICB With 5-FU or Cisplatin Induces Profound Expansion of Tumor Draining Lymph Nodes

To understand how 5-FU and cisplatin enhance the anti-tumor immune response when combined with ICB, we first analyzed tumor draining lymph nodes (DLNs) and spleens from treated, tumor-bearing mice (**Figure S4A**). We focused on the AB1 model because 5-FU and cisplatin produced the most robust responses in this model. DLNs from both cisplatin+ICB and 5-FU+ICB groups were larger in size relative to monotherapy groups (**Figures 2A, B**). The absolute number of leucocytes in DLNs of 5-FU+ICB treated animals was significantly greater compared to DLNs from either 5-FU ($p < 0.0001$) or ICB ($p < 0.0001$) monotherapies. Cisplatin+ICB treated DLNs contained a significantly greater number of leucocytes compared to those treated with cisplatin alone ($p = 0.015$). The proportions of $CD8^+$, $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ T cells in DLNs and spleens were similar between all treatment groups (**Figures S4B, C**). We observed increased proportions of activated, proliferating $CD4^+Foxp3^+ICOS^+Ki67^+$ T cells in DLNs (**Figure 2C**) and spleens (**Figure S4D**) of combination treated animals compared to 5-FU or cisplatin chemotherapy alone. We found minor depletion of neutrophils ($CD11b^+Ly6C^+Ly6G^+$) and inflammatory monocytes ($CD11b^+Ly6C^{hi}Ly6G^-$) in DLNs (**Figure 2D**) and spleens (**Figures S5A, B**) in 5-FU treated groups as reported previously (26). These data suggest that additive chemo-immunotherapy combinations induce a profound expansion of leucocytes in tumor DLNs.

Additive Chemo-Immunotherapy Combinations Increase the Frequency of Intratumoral T Cells

To determine if there were specific intratumoral immune cells involved in additive chemo-immunotherapy combinations, we first characterized tumor infiltrating immune cell populations of 5-FU and cisplatin chemo/immunotherapy-treated animals by flow cytometry and CIBERSORT analysis of bulk RNAseq data. In terms of overall immune cell populations, we did not see consistent significant differences between combination therapy to chemotherapy or ICB alone (**Figures 3A, B**). Tumors from 5-FU+ICB treated mice displayed increased $CD8^+$ T cell infiltration as identified by both CIBERSORT and flow cytometry analyses ($p = 0.04$; **Figures 3A, B**, **S6A, B**). The number of intratumoral $CD4^+$ helper T cells were significantly greater in cisplatin+ICB treated mice compared to cisplatin only ($p = 0.01$; **Figures 3A**, **S6A**). The frequency of $CD4^+Foxp3^+$ regulatory T cells (T_{regs}) was reduced in tumors from 5-FU+ICB treated mice compared to PBS controls in both data sets. T_{regs} in cisplatin+ICB treated tumors were significantly reduced compared to PBS controls in flow cytometry data ($p = 0.02$; **Figures S6A, B**). The frequency of monocytes significantly decreased in 5-FU+ICB tumors compared to PBS in the CIBERSORT analysis ($p = 0.028$) but did not reach statistical significance in the flow cytometry data (**Figures S6C, D**).

We further characterized the activation status of tumor infiltrating lymphocytes (TILs). Dimensional reduction analyses on $CD45^+$ cells using UMAP and Phenograph produced 13 distinct phenotypic clusters (**Figure 3C**). The frequency of activated ($ICOS^+$) and proliferating ($Ki67^+$) $CD8^+$ T cells (cluster 7) was significantly greater in tumors from 5-FU+ICB treated mice (23.3 ± 8.69%) compared to 5-FU alone (3.62 ± 1.21%; $p = 0.03$, **Figure 3D**). We also characterized GzmB expression and IFNγ secretion from $CD8^+$ T cells and found a significant increase in the total number of $IFN\gamma^+$ $CD8^+$ TILs in chemo-immunotherapy treated tumors compared to 5-FU treated tumors (**Figure S7**). Tumors from both cisplatin+ICB and 5-FU+ICB treated mice were enriched with increased $CD4^+Foxp3^-ICOS^+Ki67^+$ T cells (cluster 10) compared to chemotherapy alone (5-FU+ICB vs 5-FU; 8.81 ± 5.94% vs 2.74 ± 1.22%; $p = 0.04$; cisplatin+ICB vs cisplatin; 6.09 ± 1.37% vs 1.69 ± 0.70%; $p = 0.005$). Activated tumor-infiltrating T_{regs} ($CD4^+Foxp3^+ICOS^+Ki67^-$; cluster 13) were also significantly reduced in both combination chemo-immunotherapy treated groups (5-FU+ICB: 0.25 ± 0.20%; cisplatin+ICB: 0.29 ± 0.31%) compared to PBS controls (2.73 ± 1.01%; $p = 0.015$; $p = 0.014$ respectively) (**Figure 3D**).

As both 5-FU and cisplatin chemo-immunotherapies increased activated and proliferating $CD8^+$ and $CD4^+Foxp3^-$ T cells, we depleted $CD8^+$ or $CD4^+$ T cells throughout the treatment

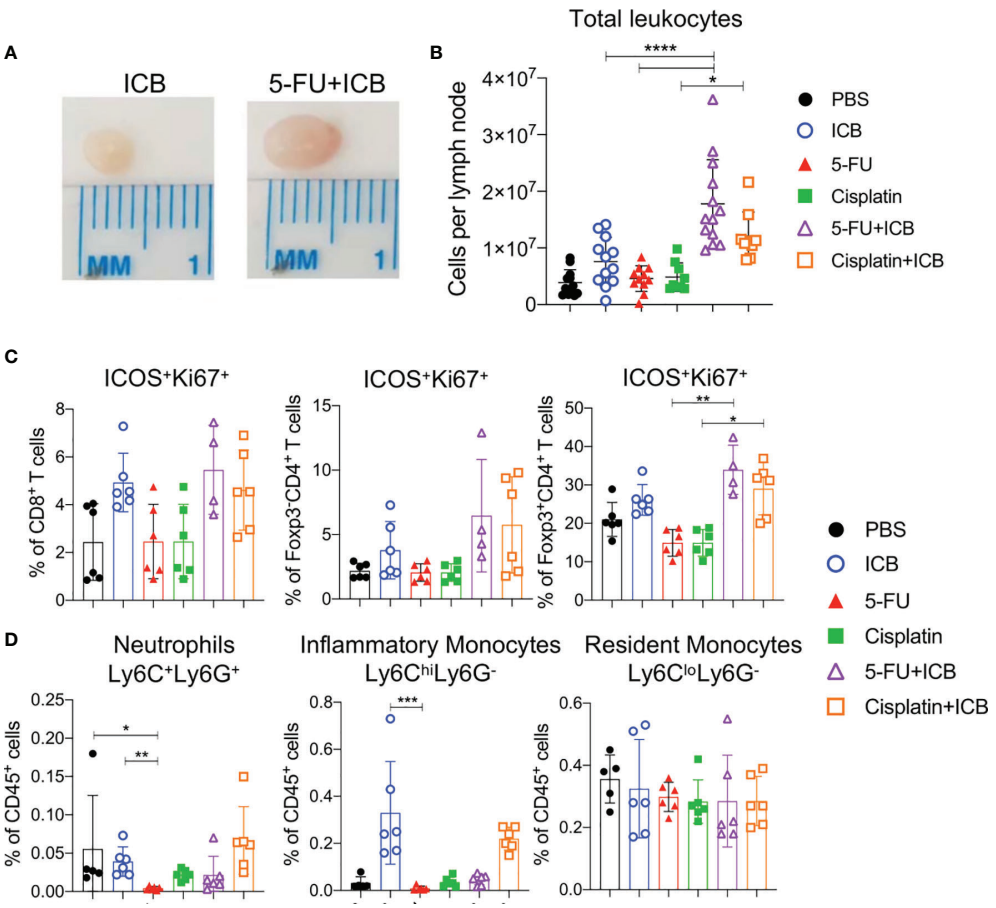

FIGURE 2 | 5-FU and cisplatin chemotherapy in combination with ICB causes expansion of T cells in tumor draining lymph nodes. **(A)** Representative images of tumor draining lymph nodes from AB1 tumor bearing mice after ICB (left) or 5-FU+ICB (right). **(B)** Absolute numbers of leukocytes, **(C)** proportions of activated ($ICOS^+Ki67^+$) $CD8^+$, $CD4^+Foxp3^-$ (helper) and $CD4^+Foxp3^+$ (regulatory; T_{regs}) T cells, and **(D)** proportions of neutrophils ($CD11b^+Ly6C^+Ly6G^+$), inflammatory monocytes ($CD11b^+Ly6C^{hi}Ly6G^-$), resident monocytes ($CD11b^+Ly6C^{lo}Ly6G^-$) in DLNs of different treatment groups. Data represented as mean ± SD, summary of three independent experiments. Mann-Whitney U test corrected for multiple comparisons; *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

schedule in AB1-HA tumor-bearing mice to test whether these cells were required for effective chemo-immunotherapy. Depleting $CD8^+$ T cells significantly abrogated the anti-tumor effect of both chemo-immunotherapy combinations (5-FU+ICB: $p < 0.0001$; cisplatin +ICB: $p = 0.026$) **(Figures 3E, F)**. In comparison, depleting $CD4^+$ T cells had no significant impact on the efficacy of 5-FU+ICB combination therapy ($P = 0.45$), but significantly improved survival in the cisplatin+ICB combination group ($p = 0.0014$) **(Figures 3E, F)**. These data demonstrate that 5-FU/cisplatin combined with ICB enhanced activation of intratumoral $CD8^+$ and $CD4^+$ T cells, and the anti-tumor response for these chemo-immunotherapy combinations were dependent on $CD8^+$ T cells.

Inflammatory T Cell-Driven Pathways Are Enriched in Additive Chemo-Immunotherapy Combinations

To further elucidate the molecular and cellular pathways inducing the robust anti-tumor responses in effective chemo-immunotherapies, we compared the gene expression profiles of additive chemo-immunotherapy combinations and monotherapy-treated tumors. Unsupervised hierarchical clustering of the top 200 most variable DEGs for each chemo-immunotherapy compared to monotherapy controls (**Figures 4A**, **S8A**) demonstrated that ICB alone, or in combination with chemotherapy was driving most of the differences in gene expression. 5-FU alone was also clearly separated from PBS and ICB treated tumors (**Figure 4A**) whereas cisplatin alone was similar to PBS treated tumors (**Figure S8A**).

To determine the biological relevance of the DEGs identified in the additive chemo-immunotherapy combinations, we examined the over-representation of pathways using KEGG and Reactome databases. We first compared the combination therapies with ICB monotherapy. A total of 330 genes were differentially expressed between 5-FU+ICB and ICB, which were associated with a downregulation of pathways involved in glucose metabolism and hypoxia by 5-FU+ICB (**Figures 4B**; **S8B**). Only 55 genes were differentially expressed between cisplatin+ICB and ICB, and pathway analysis did not identify a

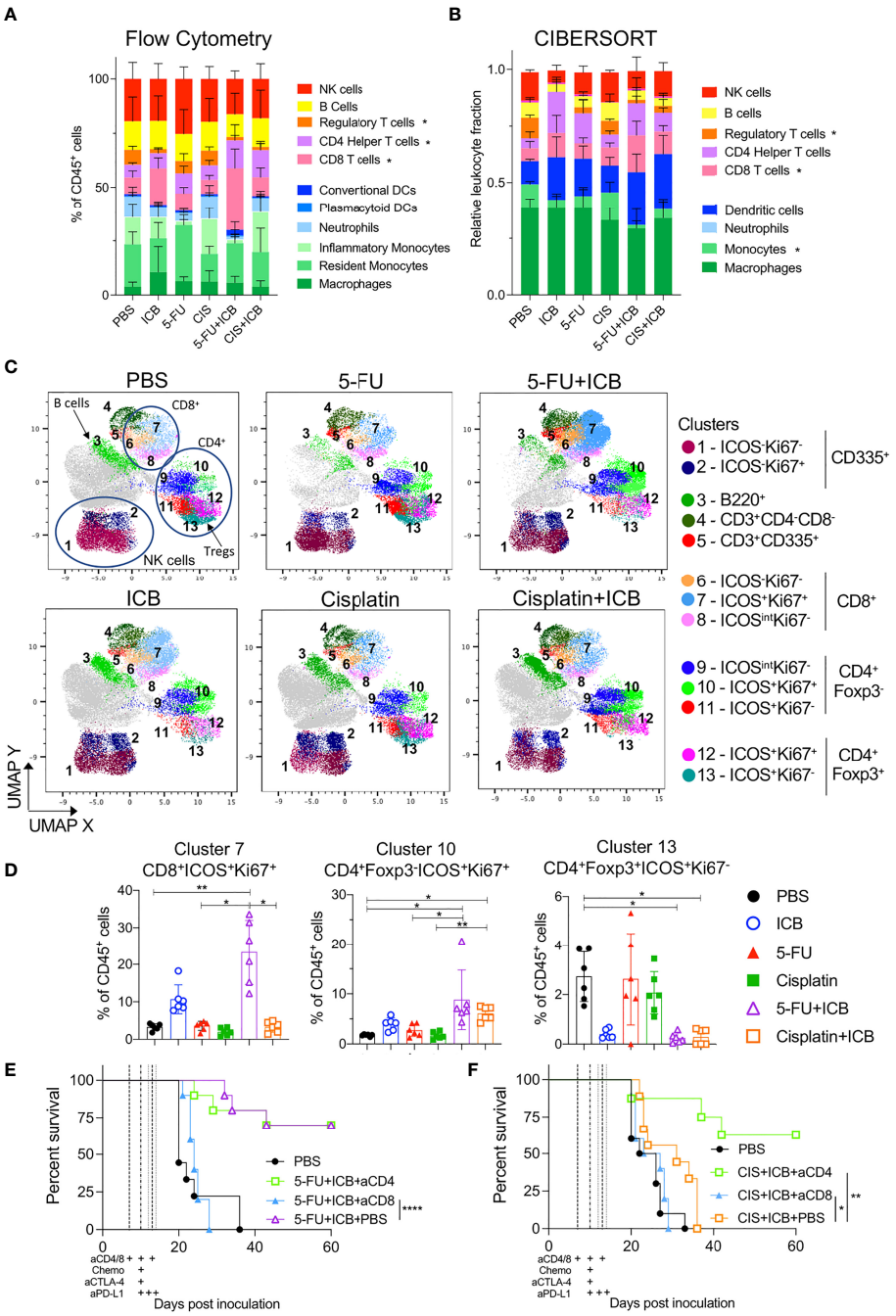

FIGURE 3 | Tumors of additive chemo-immunotherapy combinations are enriched for activated CD8$^+$ and CD4$^+$ T cells. **(A, B)** Summary of lymphoid and myeloid immune cell proportions in chemo-immunotherapy treated tumors analyzed using flow cytometry **(A)** and CIBERSORTx **(B)** from bulk RNAseq. * indicates $p < 0.05$ for that cell type between chemo-immunotherapy and PBS controls. **(C)** UMAP plots of clustered CD45$^+$ cells from flow cytometry data for each treatment group. Cells are colored by Phenograph clusters and annotated by expression of phenotypic markers in legend. Cells colored in grey had no expression of other phenotypic markers in panel and were excluded from analysis. **(D)** Frequencies of cells from clusters 7.10,13 in all chemotherapy and/or ICB treated tumors. **(E, F)** Survival curves of AB1-HA tumor bearing mice treated with 5-FU+ICB **(E)** and cisplatin+ICB **(F)** with or without anti-CD4 or anti-CD8 depletion antibodies. Dotted lines indicate when therapies were administered. Data shown as mean ± SD, flow cytometry data is summary of two independent experiments (n = 6 per group), RNAseq data (n = 5 per group except PBS and cisplatin; n = 4 per group), *in vivo* tumor growth data is summary of two independent experiments (n = 10 per group). Mann-Whitney U test corrected for multiple comparisons and Mantel-Cox survival test; *$P < 0.05$, **$P \leq 0.01$, ****$P \leq 0.0001$.

common biological pathway associated with these genes (data not shown).

We next compared each combination therapy with their respective chemotherapy alone and identified 779 DEGs comparing cisplatin+ICB with cisplatin monotherapy, and 536 DEGs between 5-FU+ICB and 5-FU monotherapy. Pathway analysis of DEGs demonstrated that both chemo-immunotherapy combinations significantly upregulated immune-related pathways

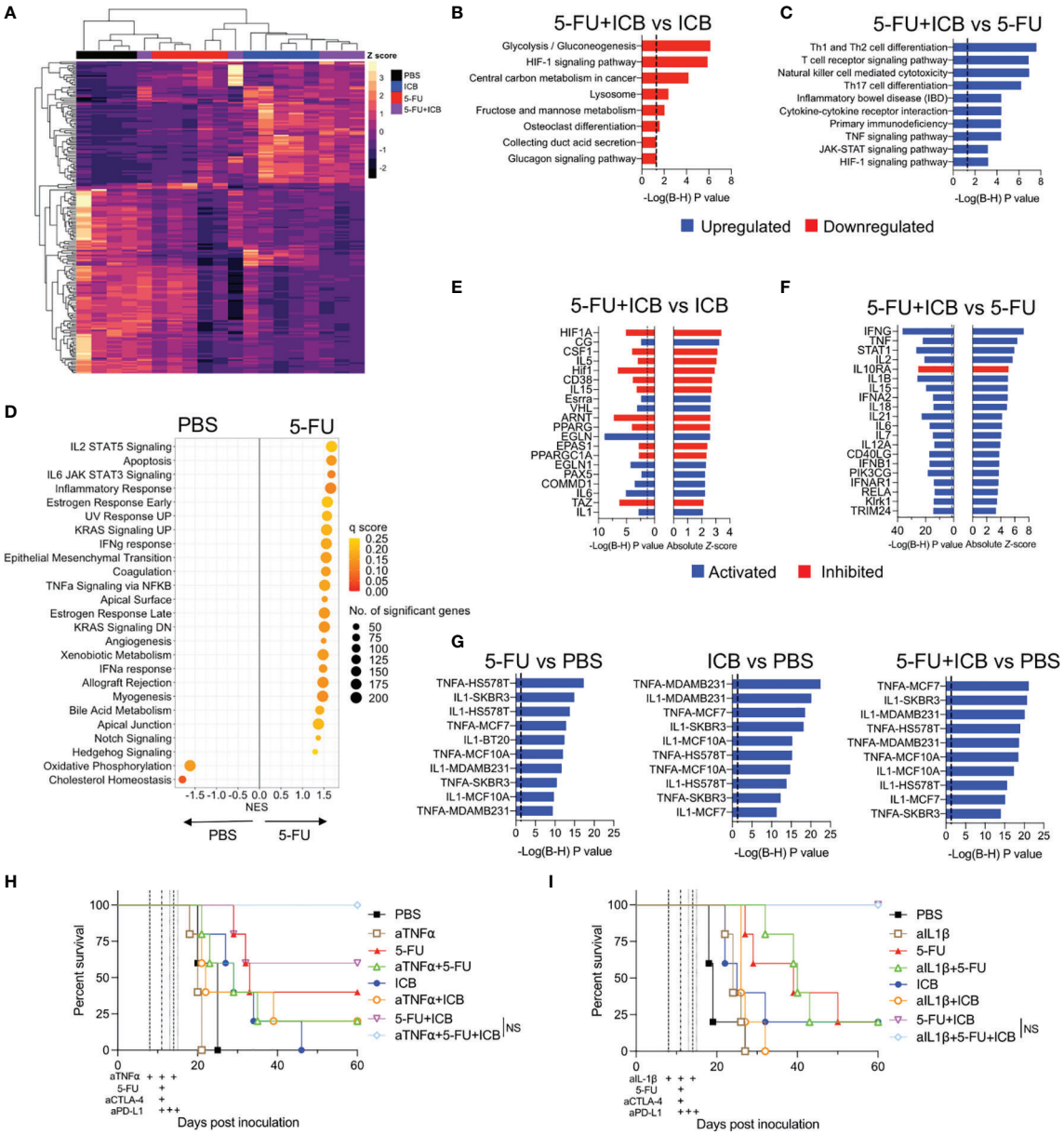

FIGURE 4 | 5-FU-based chemo-immunotherapy upregulates immune-associated pathways and downregulates hypoxia and glycolysis pathways. **(A)** Unsupervised hierarchical clustering of the top 200 differentially expressed genes from 5-FU+ICB treatment groups. **(B)** Downregulated KEGG pathways in 5-FU+ICB compared to ICB treated tumors. **(C)** Top 10 upregulated KEGG pathways in 5-FU+ICB when compared 5-FU. Multiple comparisons corrected using Bonferonni-Hochberg method. Significance denoted by $P < 0.05$. **(D)** GSEA displaying top hallmark gene sets significantly ($q < 0.25$) enriched in 5-FU compared to PBS. A positive normalized enrichment score (NES) indicates that specific gene set is enriched in a 5-FU treated tumor compared to PBS. **(E, F)** Graphs displaying the top 20 upstream regulators in 5-FU+ICB compared to ICB **(E)** or 5-FU **(F)** treated tumors. Upstream regulators are colored by the activation *Z*-score. Regulators with a *Z*-score ≥ 2 are activated and are displayed in blue. Regulators with a *Z*-score ≤ -2 are inhibited and are displayed in red. **(G)** Top 10 most significantly upregulated LINCS L1000 gene signatures in 5-FU, ICB and 5-FU+ICB in comparison to PBS treated tumors. Multiple comparisons corrected using Bonferonni-Hochberg method. Significance denoted by $P < 0.05$. **(H–I)** Survival curves of AB1-HA tumor bearing mice treated with anti-TNFα **(H)** or anti-IL-1β **(I)** blocking antibodies, 5-FU, ICB or 5-FU+ICB therapies. Data represents one experiment (n = 5 per group). Mantel-Cox survival test. B-H, Bonferonni-Hochberg.

involving immune cell differentiation, signaling and cytotoxicity compared to either respective chemotherapy alone, suggesting enhancement of the immunological activity of ICB (**Figures 4C**; **S8C–E**). These results were confirmed using GSEA (23), applying the curated Hallmark gene sets to the gene expression profiles (**Figure S8F**). Interestingly, 5-FU treated tumors were enriched with multiple immune related gene sets compared to PBS controls (**Figure 4D**), similar to the ICB gene expression profile reported previously (17). Together these results indicate that 5-FU may be further enhancing the immunostimulatory effects of ICB, generating a robust anti-tumor immune response seen for the combination therapy.

Having characterized molecular pathways that were associated with additive chemo-immunotherapy combinations, we next sought to identify key targets that could modulate the anti-tumor immune response. We focused on the 5-FU chemo-immunotherapy combination as it produced the most robust anti-tumor immune response *in vivo* (**Figure 1**). We performed upstream regulator analysis to identify key transcriptional regulators of molecular pathways enriched in 5-FU chemo-immunotherapy. In comparison to ICB, 5-FU+ICB induced a gene expression signature indicative of inhibition of upstream regulators involved in HIF1 signaling (HIF1A, CSF1, ARNT), peroxisome signaling (PPARG, PPARGC1A) and activation of upstream regulators IL-1 and IL-6 (**Figure 4E**). IFNγ, TNFα, IL-2, STAT1 and IL-1β were the top activated upstream regulators in 5-FU+ICB compared to 5-FU monotherapy (**Figure 4F**). We also analyzed the data using the LINCS L1000 connectivity map which measured the expression of over 3000 genes in eight different cell lines following exposure to defined ligands. The gene expression profiles of 5-FU, ICB and 5-FU+ICB treated tumors, were enriched for IL-1 and TNFα-induced genes (**Figure 4G**).

To test if TNFα or IL-1β cytokine signaling pathways were required to produce the robust anti-tumor immune response found when 5-FU is added to ICB, we administered TNFα or IL-1β blocking antibodies throughout the 5-FU+ICB treatment schedule in AB1-HA tumor bearing mice (**Figures 4H, I**). The efficacy of the 5-FU+ICB combination was unaffected when either pro-inflammatory cytokine was depleted. There was also no significant difference in survival for the 5-FU or ICB monotherapies when TNFα or IL-1β were blocked. (**Figure 4H**) (5-FU+ICB vs 5-FU+ICB+aTNFa, $P = 0.136$; 5-FU+ICB vs 5-FU+ICB+aIL-1β, $P > 0.999$). This indicates that whilst TNFα and IL-1 signaling were significantly enriched in 5-FU chemo-immunotherapy treated tumors, the robust anti-tumor response produced by this additive chemo-immunotherapy is likely to be dependent on the combination of multiple molecular pathways.

DISCUSSION

In this study, we analyzed the *in vivo* anti-tumor effects of 10 different chemotherapies in combination with anti-CTLA-4 and anti-PD-L1 ICB to identify effective chemo-immunotherapy combinations. We found that the addition of 5-FU or cisplatin to ICB significantly improved survival compared to either monotherapy alone in two murine cancer models. Importantly, no chemo-immunotherapy combination decreased overall survival compared to ICB alone, suggesting no antagonistic effects of tested chemotherapies.

Immunogenic chemotherapies such as vinorelbine, etoposide, cyclophosphamide and gemcitabine induced robust anti-tumor responses alone. While we only found 5-FU or cisplatin improved the efficacy of ICB, other studies have identified that vinorelbine and etoposide synergized with anti-CTLA-4 (27) and anti-PD-L1 (28) respectively. In addition, cyclophosphamide and gemcitabine have been previously shown to enhance $CD8^+$ T cell infiltration in tumors and deplete immunosuppressive cells (29, 30), but preclinical studies combining these chemotherapies with ICB have provided conflicting results (27–29, 31). These discrepancies may not only be due to different cancer models but also chemotherapy dosing and scheduling. For example, we previously established that multiple lower doses of gemcitabine (240 mg/kg) were synergistic with ICB in the AB1 tumor model (32), whereas MTD gemcitabine (700 mg/kg) did not provide additional benefit in this study. 5-FU at the previously reported MTD of 125 mg/kg (16) could not be administered with ICB without severe toxicity so a lower dose (75 mg/kg) was used which may have impacted the additive effect found with this combination. In addition, we investigated chemo-immunotherapy combinations in subcutaneous models of mesothelioma which may not fully recapitulate the tumor microenvironment in the pleural mesothelium. However, orthotopic models of mesothelioma are technically challenging and response rates to ICB and chemotherapy monotherapy in our subcutaneous models are similar to responses found in mesothelioma patients (33, 34).

We also administered ICB and MTD chemotherapy concurrently. Although staggering the ICB and chemotherapy doses have been explored previously (29, 35–37), it is difficult to separate the immunogenic effects of therapy from tumor size, particularly when one treatment has substantially reduced the tumor size before the addition of the next therapy. Administering chemotherapy before ICB could induce a highly inflammatory tumor microenvironment, sensitize tumor cells to cytotoxic T cell killing (38, 39), priming a tumor to be more responsive to ICB. Dosing and scheduling are potential factors that could affect the chemotherapy-induced immune response, and remain an important area of research, going forward.

Our study focused on the additive mechanisms of MTD 5-FU and cisplatin to ICB as they produced the most robust anti-tumor responses in our models. 5-FU has shown to be additive when combined with anti-PD-1/PD-L1 ICB in other pre-clinical models (35, 36, 40), whereas cisplatin chemotherapy synergized with anti-PD-1/PD-L1 or anti-CTLA-4 in some models, but not others (41, 42). Platinum-based chemotherapy has been shown to be a very effective combination with ICB in patients (10). In addition, multiple ongoing clinical trials are analyzing the efficacy of combination multi-modal chemotherapy, (including 5-FU and cisplatin) with ICB, particularly for patients with colorectal and bladder cancer

(NCT03202758, NCT02658214, NCT04241185, NCT03775265, NCT02912559). Increased numbers of activated $CD8^+$ T cells at the tumor site together with depletion of immunosuppressive cells (T_{regs} and MDSCs) were key immunological effects of 5-FU and cisplatin chemo-immunotherapy combinations in our study and others (40, 42).

Gene signatures associated with hypoxia and metabolism, in particular HIF-1α and glycolysis pathways were downregulated in tumors from mice treated with the 5-FU+ICB combination compared to monotherapy. HIF-1α signaling regulates chemotherapy-resistance, and the differentiation of immu nosuppressive myeloid-derived suppressor cells (MDSCs) (43). Both AB1 and AE17 tumors display hypoxic regions *in vivo* (44), and chemo-immunotherapy could have altered tumor hypoxia. It is also possible that combination 5-FU and ICB alter tumor immunosuppressive cells through HIF-1α mediated pathways, resulting in decreased MDSCs and T_{regs} observed in our study. Others have found that inhibition of the HIF-1α pathway improves the anti-tumor effect of 5-FU (45), and improves ICB responses (46) in preclinical models. There are numerous small molecule drugs that inhibit different parts of the HIF-1α signaling pathway, but the clinical efficacy of HIF-1α inhibitory drugs in cancer have been limited thus far (47).

Recent studies have also highlighted the importance of glucose metabolism in T cell activation and proliferation in response to a T cell receptor mediated stimulus. PD-1 and CTLA-4 signaling inhibit glycolysis in $CD4^+$ and $CD8^+$ T cells *in vitro*, preventing rapid proliferation and differentiation into effector cells (48). It is therefore counterintuitive that glycolysis pathways would be downregulated in the most efficacious chemo-immunotherapy combination from our study. However, a caveat of our study is that RNAseq of bulk tumors did not allow us to separate the metabolic effects of chemo-immunotherapy on tumor versus immune cells. The metabolic competition between tumor cells and T cells has been well described (49), and reduced glycolysis within tumors have been observed, particularly with PD-L1 blockade.

TNFα and IL-1β signaling associated genes were upregulated in 5-FU+ICB treated tumors. However, antibody neutralization experiments showed that these signaling pathways were not necessary for complete tumor regression. In fact, neutralization of TNFα further improved the efficacy of 5-FU+ICB. This is in line with multiple reports demonstrating that disruption of TNFα or IL-1R/IL-1β signaling by either blocking antibodies or deficient mouse models improves the anti-tumor immune response in combination with 5-FU (50, 51) or ICB (52, 53). These results highlight the complexity, and redundancy of different pro-inflammatory cytokines in mediating anti-tumor immunity. As TNFα and IL-1β inhibitors are now available to treat severe ICB induced immune related adverse events (54), it is encouraging that blocking these pathways did not diminish the anti-tumor responses of chemo-immunotherapy in preclinical models.

Our study provides a resource and starting point for future studies to interrogate the mechanisms of combination ICB and chemotherapy. 5-FU and cisplatin treated tumors had vastly different gene expression profiles, suggesting additive mechanisms could be different for other chemotherapies. 5-FU is currently not used clinically for mesothelioma, and the results with this chemotherapy may therefore be particularly applicable to other cancers. However, understanding additive mechanisms is important to develop novel strategies to phenocopy a responding tumor microenvironment, and improve anti-tumor responses of chemotherapy and/or immunotherapy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE188481.

ETHICS STATEMENT

The animal study was reviewed and approved by Harry Perkins Institute of Medical Research Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

NP and WA performed all mouse experiments and wrote manuscript. NP, WA, CT, and DH performed mouse experiments. NP and WC analysed RNA sequencing data. NP and AM analysed flow cytometry data. ID assisted with statistical analysis. LB provided critical reagents. RL, AN, AM, SF, JC, and WL interpreted experiments and critically revised the manuscript. JC and WL shared the design of the study, supervised the project and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.872295/full#supplementary-material

REFERENCES

1. Hellmann MD, Paz-Ares L, Bernabe Caro R, Zurawski B, Kim S-W, Carcereny Costa E, et al. Nivolumab Plus Ipilimumab in Advanced Non–Small-Cell Lung Cancer. *N Engl J Med* (2019) 381(21):2020–31. doi: 10.1056/NEJMoa1910231
2. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Rutkowski P, Lao CD, et al. Five-Year Survival With Combined Nivolumab and Ipilimumab in Advanced Melanoma. *N Engl J Med* (2019) 381(16):1535–46. doi: 10.1056/NEJMoa1910836
3. Galluzzi L, Humeau J, Buque A, Zitvogel L, Kroemer G. Immunostimulation With Chemotherapy in the Era of Immune Checkpoint Inhibitors. *Nat Rev Clin Oncol* (2020) 17(12):725–41. doi: 10.1038/s41571-020-0413-z
4. McDonnell AM, Lesterhuis WJ, Khong A, Nowak AK, Lake RA, Currie AJ, et al. Tumor-Infiltrating Dendritic Cells Exhibit Defective Cross-Presentation of Tumor Antigens, But Is Reversed by Chemotherapy. *Eur J Immunol* (2015) 45(1):49–59. doi: 10.1002/eji.201444722
5. Nowak AK, Lake RA, Marzo AL, Scott B, Heath WR, Collins EJ, et al. Induction of Tumor Cell Apoptosis *In Vivo* Increases Tumor Antigen Cross-Presentation, Cross-Priming Rather Than Cross-Tolerizing Host Tumor-Specific CD8 T Cells. *J Immunol* (2003) 170(10):4905–13. doi: 10.4049/jimmunol.170.10.4905
6. Rettig L, Seidenberg S, Parvanova I, Samaras P, Curioni A, Knuth A, et al. Gemcitabine Depletes Regulatory T-Cells in Human and Mice and Enhances Triggering of Vaccine-Specific Cytotoxic T-Cells. *Int J Cancer* (2011) 129 (4):832–8. doi: 10.1002/ijc.25756
7. Pircher A, Gamerith G, Amann A, Reinold S, Popper H, Gachter A, et al. Neoadjuvant Chemo-Immunotherapy Modifies CD4(+)CD25(+) Regulatory T Cells (Treg) in Non-Small Cell Lung Cancer (NSCLC) Patients. *Lung Cancer* (2014) 85(1):81–7. doi: 10.1016/j.lungcan.2014.04.001
8. Park SJ, Ye W, Xiao R, Silvin C, Padget M, Hodge JW, et al. Cisplatin and Oxaliplatin Induce Similar Immunogenic Changes in Preclinical Models of Head and Neck Cancer. *Oral Oncol* (2019) 95:127–35. doi: 10.1016/j.oraloncology.2019.06.016
9. Lesterhuis WJ, Punt CJ, Hato SV, Eleveld-Trancikova D, Jansen BJ, Nierkens S, et al. Platinum-Based Drugs Disrupt STAT6-Mediated Suppression of Immune Responses Against Cancer in Humans and Mice. *J Clin Invest* (2011) 121(8):3100–8. doi: 10.1172/JCI43656
10. Schmidt EV, Chisamore MJ, Chaney MF, Maradeo ME, Anderson J, Baltus GA, et al. Assessment of Clinical Activity of PD-1 Checkpoint Inhibitor Combination Therapies Reported in Clinical Trials. *JAMA Netw Open* (2020) 3(2):e1920833. doi: 10.1001/jamanetworkopen.2019.20833
11. Paz-Ares L, Ciuleanu TE, Cobo M, Schenker M, Zurawski B, Menezes J, et al. First-Line Nivolumab Plus Ipilimumab Combined With Two Cycles of Chemotherapy in Patients With Non-Small-Cell Lung Cancer (CheckMate 9LA): An International, Randomised, Open-Label, Phase 3 Trial. *Lancet Oncol* (2021) 22(2):198–211. doi: 10.1016/S1470-2045(20)30641-0
12. Paz-Ares L, Dvorkin M, Chen Y, Reinmuth N, Hotta K, Trukhin D, et al. Durvalumab Plus Platinum–Etoposide Versus Platinum–Etoposide in First-Line Treatment of Extensive-Stage Small-Cell Lung Cancer (CASPIAN): A Randomised, Controlled, Open-Label, Phase 3 Trial. *Lancet* (2019) 394 (10212):1929–39. doi: 10.1016/S0140-6736(19)32222-6
13. Nowak AK, Lesterhuis WJ, Kok PS, Brown C, Hughes BG, Karikios DJ, et al. Durvalumab With First-Line Chemotherapy in Previously Untreated Malignant Pleural Mesothelioma (DREAM): A Multicentre, Single-Arm, Phase 2 Trial With a Safety Run-in. *Lancet Oncol* (2020) 21(9):1213–23. doi: 10.1016/S1470-2045(20)30462-9
14. Davis MR, Manning LS, Whitaker D, Garlepp MJ, Robinson BW. Establishment of a Murine Model of Malignant Mesothelioma. *Int J Cancer* (1992) 52(6):881–6. doi: 10.1002/ijc.2910520609
15. Jackaman C, Bundell CS, Kinnear BF, Smith AM, Filion P, van Hagen D, et al. IL-2 Intratumoral Immunotherapy Enhances CD8+ T Cells That Mediate Destruction of Tumor Cells and Tumor-Associated Vasculature: A Novel Mechanism for IL-2. *J Immunol* (2003) 171(10):5051–63. doi: 10.4049/jimmunol.171.10.5051
16. Aston WJ, Hope DE, Nowak AK, Robinson BW, Lake RA, Lesterhuis WJ. A Systematic Investigation of the Maximum Tolerated Dose of Cytotoxic Chemotherapy With and Without Supportive Care in Mice. *BMC Cancer* (2017) 17(684). doi: 10.1186/s12885-017-3677-7
17. Zemek RM, De Jong E, Chin WL, Schuster IS, Fear VS, Casey TH, et al. Sensitization to Immune Checkpoint Blockade Through Activation of a STAT1/NK Axis in the Tumor Microenvironment. *Sci Transl Med* (2019) 11(501). doi: 10.1126/scitranslmed.aav7816
18. Bray NL, Pimentel H, Melsted P, Pachter L. Near-Optimal Probabilistic RNA-Seq Quantification. *Nat Biotechnol* (2016) 34(5):525–7. doi: 10.1038/nbt.3519
19. Soneson C, Love MI, Robinson MD. Differential Analyses for RNA-Seq: Transcript-Level Estimates Improve Gene-Level Inferences. *F1000Res* (2015) 4:1521. doi: 10.12688/f1000research.7563.1
20. Love MI, Huber W, Anders S. Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data With Deseq2. *Genome Biol* (2014) 15(12):550. doi: 10.1186/s13059-014-0550-8
21. Xie Z, Bailey A, Kuleshov MV, Clarke DJB, Evangelista JE, Jenkins SL, et al. Gene Set Knowledge Discovery With Enrichr. *Curr Protoc* (2021) 1(3):e90. doi: 10.1002/cpz1.90
22. Kramer A, Green J, Pollard JJr., Tugendreich S. Causal Analysis Approaches in Ingenuity Pathway Analysis. *Bioinformatics* (2014) 30(4):523–30. doi: 10.1093/bioinformatics/btt703
23. Subramanian A, Kuehn H, Gould J, Tamayo P, Mesirov JP. GSEA-P: A Desktop Application for Gene Set Enrichment Analysis. *Bioinformatics* (2007) 23(23):3251–3. doi: 10.1093/bioinformatics/btm369
24. de Mutsert R, Jager KJ, Zoccali C, Dekker FW. The Effect of Joint Exposures: Examining the Presence of Interaction. *Kidney Int* (2009) 75(7):677–81. doi: 10.1038/ki.2008.645
25. Cook AM, Lesterhuis WJ, Nowak AK, Lake RA. Chemotherapy and Immunotherapy: Mapping the Road Ahead. *Curr Opin Immunol* (2016) 39:23–9. doi: 10.1016/j.coi.2015.12.003
26. Vincent J, Mignot G, Chalmin F, Ladoire S, Bruchard M, Chevriaux A, et al. 5-Fluorouracil Selectively Kills Tumor-Associated Myeloid-Derived Suppressor Cells Resulting in Enhanced T Cell-Dependent Antitumor Immunity. *Cancer Res* (2010) 70(8):3052–61. doi: 10.1158/0008-5472.CAN-09-3690
27. Jure-Kunkel M, Masters G, Girit E, Dito G, Lee F, Hunt JT, et al. Synergy Between Chemotherapeutic Agents and CTLA-4 Blockade in Preclinical Tumor Models. *Cancer Immunol Immunother* (2013) 62(9):1533–45. doi: 10.1007/s00262-013-1451-5
28. Orecchioni S, Talarico G, Labanca V, Calleri A, Mancuso P, Bertolini F. Vinorelbine, Cyclophosphamide and 5-FU Effects on the Circulating and Intratumoural Landscape of Immune Cells Improve Anti-PD-L1 Efficacy in Preclinical Models of Breast Cancer and Lymphoma. *Br J Cancer* (2018) 118 (10):1329–36. doi: 10.1038/s41416-018-0076-z
29. Iida Y, Harashima N, Motoshima T, Komohara Y, Eto M, Harada M. Contrasting Effects of Cyclophosphamide on Anti-CTL-Associated Protein 4 Blockade Therapy in Two Mouse Tumor Models. *Cancer Sci* (2017) 108 (10):1974–84. doi: 10.1111/cas.13337
30. Suzuki E, Kapoor V, Jassar AS, Kaiser LR, Albelda SM. Gemcitabine Selectively Eliminates Splenic Gr-1+/CD11b+ Myeloid Suppressor Cells in Tumor-Bearing Animals and Enhances Antitumor Immune Activity. *Clin Cancer Res* (2005) 11(18):6713–21. doi: 10.1158/1078-0432.CCR-05-0883
31. Parra K, Valenzuela P, Lerma N, Gallegos A, Reza LC, Rodriguez G, et al. Impact of CTLA-4 Blockade in Conjunction With Metronomic

Chemotherapy on Preclinical Breast Cancer Growth. *Br J Cancer* (2017) 116 (3):324–34. doi: 10.1038/bjc.2016.429
32. Lesterhuis WJ, Salmons J, Nowak AK, Rozali EN, Khong A, Dick IM, et al. Synergistic Effect of CTLA-4 Blockade and Cancer Chemotherapy in the Induction of Anti-Tumor Immunity. *PloS One* (2013) 8(4):e61895. doi: 10.1371/journal.pone.0061895
33. Fear VS, Tilsed C, Chee J, Forbes CA, Casey T, Solin JN, et al. Combination Immune Checkpoint Blockade as an Effective Therapy for Mesothelioma. *Oncoimmunology* (2018) 7(10):e1494111. doi: 10.1080/2162402X.2018.1494111
34. Robinson C, Solin JN, Lee YG, Lake RA, Lesterhuis WJ. Mouse Models of Mesothelioma: Strengths, Limitations and Clinical Translation. *Lung Cancer Management* (2014) 3(5):397–410. doi: 10.2217/lmt.14.27
35. Wu Y, Deng Z, Wang H, Ma W, Zhou C, Zhang S. Repeated Cycles of 5-Fluorouracil Chemotherapy Impaired Anti-Tumor Functions of Cytotoxic T Cells in a CT26 Tumor-Bearing Mouse Model. *BMC Immunol* (2016) 17 (1):29. doi: 10.1186/s12865-016-0167-7
36. Saint-Jean M, Fronteau C, Peuvrel L, Khammari A, Varey E, Quereux G, et al. Chemotherapy Efficacy After First-Line Immunotherapy in 18 Advanced Melanoma Patients. *Med (Baltimore)* (2020) 99(29):e21329. doi: 10.1097/MD.0000000000021329
37. Zhao X, Kassaye B, Wangmo D, Lou E, Subramanian S. Chemotherapy But Not the Tumor Draining Lymph Nodes Determine the Immunotherapy Response in Secondary Tumors. *iScience* (2020) 23(5):101056. doi: 10.1016/j.isci.2020.101056
38. Bae SH, Park YJ, Park JB, Choi YS, Kim MS, Sin JI. Therapeutic Synergy of Human Papillomavirus E7 Subunit Vaccines Plus Cisplatin in an Animal Tumor Model: Causal Involvement of Increased Sensitivity of Cisplatin-Treated Tumors to CTL-Mediated Killing in Therapeutic Synergy. *Clin Cancer Res* (2007) 13(1):341–9. doi: 10.1158/1078-0432.CCR-06-1838
39. Ramakrishnan R, Assudani D, Nagaraj S, Hunter T, Cho HI, Antonia S, et al. Chemotherapy Enhances Tumor Cell Susceptibility to CTL-Mediated Killing During Cancer Immunotherapy in Mice. *J Clin Invest* (2010) 120(4):1111–24. doi: 10.1172/JCI40269
40. Cui S. Immunogenic Chemotherapy Sensitizes Renal Cancer to Immune Checkpoint Blockade Therapy in Preclinical Models. *Med Sci Monit* (2017) 23:3360–6. doi: 10.12659/MSM.902426
41. He X, Du Y, Wang Z, Wang X, Duan J, Wan R, et al. Upfront Dose-Reduced Chemotherapy Synergizes With Immunotherapy to Optimize Chemoimmunotherapy in Squamous Cell Lung Carcinoma. *J Immunother Cancer* (2020) 8(2). doi: 10.1136/jitc-2020-000807
42. Wei H, Zhao L, Li W, Fan K, Qian W, Hou S, et al. Combinatorial PD-1 Blockade and CD137 Activation has Therapeutic Efficacy in Murine Cancer Models and Synergizes With Cisplatin. *PloS One* (2013) 8(12):e84927. doi: 10.1371/journal.pone.0084927
43. Corzo CA, Condamine T, Lu L, Cotter MJ, Youn JI, Cheng P, et al. HIF-1alpha Regulates Function and Differentiation of Myeloid-Derived Suppressor Cells in the Tumor Microenvironment. *J Exp Med* (2010) 207(11):2439–53. doi: 10.1084/jem.20100587
44. Magkouta S, Pappas A, Pateras IS, Kollintza A, Moschos C, Vazakidou ME, et al. Targeting Tie-2/Angiopoietin Axis in Experimental Mesothelioma Confers Differential Responses and Raises Predictive Implications. *Oncotarget* (2018) 9(31):21783–96. doi: 10.18632/oncotarget.25004
45. Harada K, Ferdous T, Harada T, Ueyama Y. Metformin in Combination With 5-Fluorouracil Suppresses Tumor Growth by Inhibiting the Warburg Effect in Human Oral Squamous Cell Carcinoma. *Int J Oncol* (2016) 49(1):276–84. doi: 10.3892/ijo.2016.3523
46. Lequeux A, Noman MZ, Xiao M, Van Moer K, Hasmim M, Benoit A, et al. Targeting HIF-1 Alpha Transcriptional Activity Drives Cytotoxic Immune Effector Cells Into Melanoma and Improves Combination Immunotherapy. *Oncogene* (2021) 40(28):4725–35. doi: 10.1038/s41388-021-01846-x
47. Tang W, Zhao G. Small Molecules Targeting HIF-1alpha Pathway for Cancer Therapy in Recent Years. *Bioorg Med Chem* (2020) 28(2):115235. doi: 10.1016/j.bmc.2019.115235
48. Patsoukis N, Bardhan K, Chatterjee P, Sari D, Liu B, Bell LN, et al. PD-1 Alters T-Cell Metabolic Reprogramming by Inhibiting Glycolysis and Promoting Lipolysis and Fatty Acid Oxidation. *Nat Commun* (2015) 6:6692. doi: 10.1038/ncomms7692
49. Chang CH, Qiu J, O'Sullivan D, Buck MD, Noguchi T, Curtis JD, et al. Metabolic Competition in the Tumor Microenvironment Is a Driver of Cancer Progression. *Cell* (2015) 162(6):1229–41. doi: 10.1016/j.cell.2015.08.016
50. Bruchard M, Mignot G, Derangere V, Chalmin F, Chevriaux A, Vegran F, et al. Chemotherapy-Triggered Cathepsin B Release in Myeloid-Derived Suppressor Cells Activates the Nlrp3 Inflammasome and Promotes Tumor Growth. *Nat Med* (2013) 19(1):57–64. doi: 10.1038/nm.2999
51. Liu F, Ai F, Tian L, Liu S, Zhao L, Wang X. Infliximab Enhances the Therapeutic Effects of 5-Fluorouracil Resulting in Tumor Regression in Colon Cancer. *Onco Targets Ther* (2016) 9:5999–6008. doi: 10.2147/OTT.S109342
52. Kaplanov I, Carmi Y, Kornetsky R, Shemesh A, Shurin GV, Shurin MR, et al. Blocking IL-1beta Reverses the Immunosuppression in Mouse Breast Cancer and Synergizes With Anti-PD-1 for Tumor Abrogation. *Proc Natl Acad Sci U S A* (2019) 116(4):1361–9. doi: 10.1073/pnas.1812266115
53. Perez-Ruiz E, Minute L, Otano I, Alvarez M, Ochoa MC, Belsue V, et al. Prophylactic TNF Blockade Uncouples Efficacy and Toxicity in Dual CTLA-4 and PD-1 Immunotherapy. *Nature* (2019) 569(7756):428–32. doi: 10.1038/s41586-019-1162-y
54. Kang JH, Bluestone JA, Young A. Predicting and Preventing Immune Checkpoint Inhibitor Toxicity: Targeting Cytokines. *Trends Immunol* (2021) 42(4):293–311. doi: 10.1016/j.it.2021.02.006

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Advances in Immune Checkpoint Inhibitors for Advanced Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is usually diagnosed in an advanced stage and has become the second deadliest type of cancer worldwide. The systemic treatment of advanced HCC has been a challenge, and for decades was limited to treatment with tyrosine kinase inhibitors (TKIs) until the application of immune checkpoint inhibitors (ICIs) became available. Due to drug resistance and unsatisfactory therapeutic effects of monotherapy with TKIs or ICIs, multi-ICIs, or the combination of ICIs with antiangiogenic drugs has become a novel strategy to treat advanced HCC. Antiangiogenic drugs mostly include TKIs (sorafenib, lenvatinib, regorafenib, cabozantinib and so on) and anti-vascular endothelial growth factor (VEGF), such as bevacizumab. Common ICIs include anti-programmed cell death-1 (PD-1)/programmed cell death ligand 1 (PD-L1), including nivolumab, pembrolizumab, durvalumab, and atezolizumab, and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4), including tremelimumab and ipilimumab. Combination therapies involving antiangiogenic drugs and ICIs or two ICIs may have a synergistic action and have shown greater efficacy in advanced HCC. In this review, we present an overview of the current knowledge and recent clinical developments in ICI-based combination therapies for advanced HCC and we provide an outlook on future prospects.

Keywords: hepatocellular carcinoma, immune checkpoint inhibitors, tyrosine kinase inhibitors, vascular endothelial growth factor, tumor microenvironment

INTRODUCTION

Liver cancer is a global health burden with an increasing incidence and a leading cause of cancer-related deaths (1). Hepatocellular carcinoma (HCC) is the most common type of liver cancer, and 72% of cancer-related death cases are observed in Asia (2). Most cases (80-90%) of HCC can be considered prototypical inflammation-driven cancers for the backdrop of chronic liver injury/cirrhosis caused by hepatitis B virus (HBV) or hepatitis C virus (HCV) infections, alcohol abuse, obesity, and aflatoxin B1 (3). The high mortality of HCC is attributed to an advanced-stage

presentation and a high prevalence of liver dysfunction. For delayed diagnosis, postsurgical recurrence and metastasis, there is a poor 5-year survival rate of less than 50% (4).

The Barcelona Clinic Liver Cancer (BCLC) system is the most commonly recommended staging system for HCC. Based on the underlying liver function evaluated by the Child–Pugh score and the performance status, HCC patients can be divided into five classes, including BCLC stage 0, A, B, C and D (5). This classification is associated with the treatment strategies and prognosis of HCC. For early-stage (BCLC stage 0 and A) HCC patients, those with solitary nodules less than 3 cm or multiple nodules less than 3 cm limited in the liver with preserved liver function and without macrovascular invasion, curative approaches, such as surgical resection, ablation, and liver transplantation could be effective. For large, multinodular without vascular invasion intermediate-stage HCC (BCLC stage B), transcatheter arterial chemoembolization (TACE) is the preferred treatment option if liver function is preserved. Unfortunately, most patients are diagnosed at a relatively advanced stage (BCLC stage C) with a poor prognosis and a survival time of less than 1 year. In this state, tumors have expanded outside the liver or vascular invasion or liver dysfunction (6). Due to the strong and extensive resistance of chemotherapy, as well as the increasing toxicity for the underlying altered liver function, the use of cytotoxic agents is frequently restricted in HCC. Clinical trials using doxorubicin in combination with cytotoxic chemotherapy have proven that there are low response rates with no survival benefit (7). Therefore, systemic therapies are required at an advanced stage. For patients in the terminal stage (BCLC stage D) with poor liver function, supportive care is required when they are not considered suitable for transplantation (8).

The common pathophysiological features of hypervascularity and vascular abnormalities include sinusoidal capillarization and overexpression of proangiogenic growth factors, such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) in HCC. In recent decades, anti-angiogenesis has attracted attention as a potential therapeutic target (9). Sorafenib, an oral small molecule multityrosine kinase inhibitor (TKI) that can suppress angiogenesis, exerts an anticancer effect by inhibiting vascular endothelial growth factor receptor (VEGFR) and fibroblast growth factor receptor (FGFR) (10). In 2007, two phase III trials (one is the SHARP trial in Europe and the USA, one is the ORIENTAL trial in Asia-Pacific regions) showed promising results that sorafenib significantly prolonged the survival of advanced-stage HCC patients compared with the placebo (11, 12). Based on the results of these two clinical trials, sorafenib was recommended as a first-line targeted agent for advanced HCC worldwide in the 2008 NCCN guidelines (10). Even for transplant recipient patients with unresectable HCC, sorafenib is generally well-tolerated and associated with improved overall survival (OS) (13, 14). Lenvatinib, another oral small molecule multi-TKI that inhibits tumor angiogenesis and growth, was found to be no less effective than sorafenib. Hence, lenvatinib therapy became the second recommended first-line targeted molecular therapy in the 2019 NCCN guidelines (15). Other multitarget TKIs, regorafenib and cabozantinib, were recommended as second-line agents for HCC patients who progressed on sorafenib treatment in the 2017 and 2019 NCCN guidelines, respectively (15, 16). Ramucirumab, a recombinant IgG1 monoclonal antibody (mAb) and an inhibitor of VEGFR2, showed efficacy after sorafenib among advanced patients with elevated levels of α-fetoprotein (AFP) (17). In view of this, ramucirumab was included in the second-line therapy in the 2019 NCCN guidelines (15). However, low objective response rates (ORRs), an improvement in OS of only 2-3 months, resistance, and cancer progression after standard treatment, regardless of first- and second-line settings, were observed, and therefore, more efficacious therapeutics should be explored (18).

HCC is a chronic inflammation-induced type of cancer that expresses various antigens that can mediate immune responses. Over the past decade, immune-based therapies that modulate the balance of immune homeostasis have been increasingly explored and have shown beneficial outcomes in HCC (19). Immune checkpoints include coinhibitory receptors on T cells and their ligands on tumor cells and stromal cells in the tumor microenvironment (TME). Immune checkpoint inhibitors (ICIs) prevent the inactivation of T cells by blocking interactions between checkpoint proteins and their ligands, such as those mediated by programmed cell death-1 (PD-1)/programmed cell death ligand 1 (PD-L1), cytotoxic T-lymphocyte-associated protein 4 (CTLA4), T-cell immunoglobulin, mucin domain containing-3 (TIM3), and lymphocyte-activation gene 3 (LAG3), thereby exerting antitumor effects (20, 21). However, not all patients (especially in the era of pre-liver transplantation) with HCC respond to immunotherapy, and more importantly, the ORR is low, and OS does not significantly improve with single-agent immunotherapy (22, 23). Given these data, more effective combination therapies for the treatment of HCC are explored, including ICIs combined with other ICIs, TKIs, anti-VEGFs, and other agents (24). In recent years, the emergence of combination therapies using multi-ICIs or ICIs with antiangiogenics represents the main avenue for the treatment of advanced HCC (5, 25). The objective of this review is to focus on the current knowledge of ICI monotherapy or in combination with other ICIs or molecularly targeted therapies (TKIs or anti-VEGFs) in advanced HCC and to provide an outlook on future prospects.

IMMUNE MICROENVIRONMENT OF THE LIVER

The liver is an organ with metabolic function and immune regulatory function. Liver cells are commonly exposed to food antigens and gut pathogens in terms of the dual supply of arterial and portal systemic blood (26). Therefore, the liver not only regulates immune responses but also has the ability to maintain immune tolerance to self and foreign antigens. This tolerogenic environment is maintained by specialized immunocytes, including Kupffer cells (KCs), liver resident dendritic cells (DCs), liver sinusoidal endothelial cells (LSECs), hepatic

stellate cells (HSCs), natural killer (NK) cells, and innate T and B cells (27). Among them, KCs, DCs, HSCs, and LSECs are antigen-presenting cells (APCs). DCs (conventional APCs) exist in multiple subtypes with different functions. Under physiological conditions, in the hepatic microenvironment, DCs appear as a tolerogenic phenotype and can secrete an array of immunosuppressive cytokines, including interleukin 10 (IL-10), prostaglandin E2 (PGE2), and indoleamine 2,3-dioxygenase (IDO), which can promote regulatory T cell (T_{reg}, derived from naive CD4+ T cells) activation, thus playing an inhibitory role in innate immune responses (28). Under homeostatic conditions, non-conventional APCs (KCs, LSECs, and HSCs) in the liver are known to act as weak T-cell activators due to low expression of major histocompatibility complex (MHC) molecules and APC activation markers CD80 and CD86 (29). KCs eliminate high-affinity antigen-specific CD8+ T cells in the liver and express heightened amounts of IL-10 and transforming growth factor beta (TGF-β) to promote the activation of T_{regs} (**Figure 1**) (30, 31). In addition, a variety of immune checkpoint proteins limit T-cell hyperactivation in physiological circumstances. T cells express CTLA4, PD-1, LAG3, and TIM3, which interact with ligands on APCs (such as PD-L1) and play a key role in immune tolerance in the liver (32).

TUMOR MICROENVIRONMENT OF HCC

It is an immense challenge to produce immune tolerance or immune response by distinguishing between benign foreign antigens and pathogenic antigens. Failure to respond to HBV and HCV infections would markedly induce immunosuppression and impair immune surveillance, which increases the risk of chronic infections and ultimately gradually develops into HCC (33). The TME of HCC is composed of immune cells (cytotoxic CD4+ T cells, CD8+ T cells, and NK cells), abundant immunosuppressive cells, such as T_{regs}, myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), stromal cells, the extracellular matrix (ECM), blood vessels, tumor cells, and lymphatic vessels, which play an important role in tumor survival, proliferation, invasion, and metastasis (34). Immune cells recognize and kill cancer cells. Moreover, deficiencies and malfunctioning of immune cells can influence the balance of the TME and lead to an immunosuppressive microenvironment. Several factors,

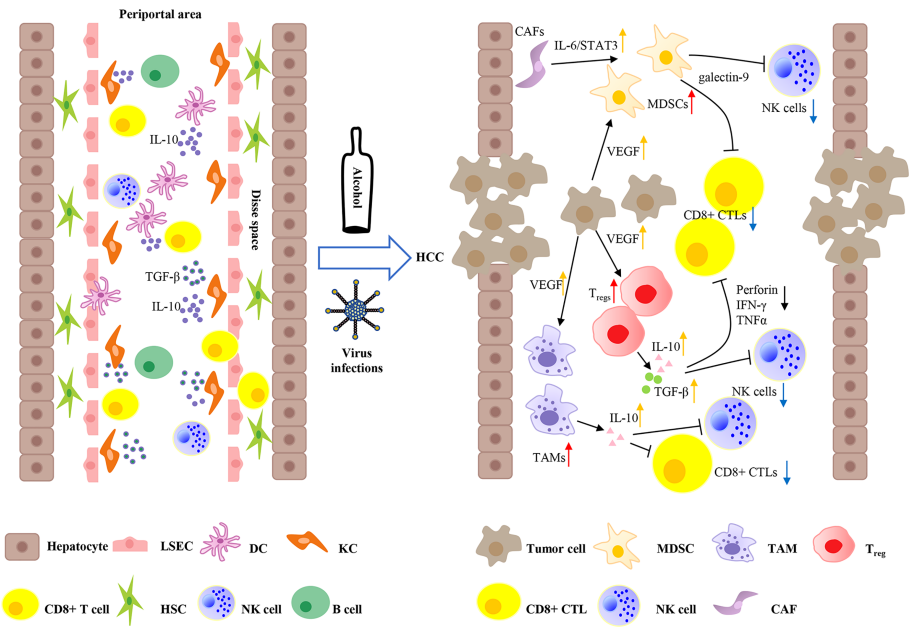

FIGURE 1 | Immune microenvironment of liver and tumor microenvironment of hepatocellular carcinoma. The liver not only regulates immune responses, but also maintains immune tolerance to self and foreign antigens. Liver sinusoidal endothelial cells (LSECs) line the liver sinusoid wall that controls the exchange of materials between hepatocytes and blood. Kupffer cells (KCs) and liver resident dendritic cells (DCs) can access to the Disse space to get in touch with hepatocytes and hepatic stellate cells (HSCs). KCs are key regulators of tolerance by expressing a large amount of IL-10 and transforming growth factor beta (TGF-β). Moreover, liver DCs produce elevated amounts of IL-10, resulting in immune tolerance. Continuous hepatitis B virus (HBV) or hepatitis C virus (HCV) infections, and alcohol abuse can lead eventually to the development of HCC. HCC is hypervascularity and overexpresses VEGF, which can recruit several inhibitory cells, such as myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and regulatory T cells (T_{regs}) to form an immunosuppressive microenvironment. In addition, HCC-related cancer-associated fibroblasts (CAFs) can induce the differentiation of MDSCs by IL-6/STAT3 signaling. T_{regs} can produce suppressive cytokines IL-10 and TGF-β to impair the inflammatory functions of CD8+ cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells through inhibiting tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and the release of perforin. Furthermore, T_{regs} and TAMs also secrete IL-10 to attenuate the capacity of CD8+ CTLs and NK cells. Moreover, MDSCs express TIM3 ligand galectin-9 and induce T-cell apoptosis.

including immunity suppression, chronic inflammation, and the decreased recognition of cancer cells have been suggested to play a role in promoting tumor antigen tolerance, which induces hepatocarcinogenesis (35). In a number of recent clinical trials, it was highlighted that the onset of HCC may be favored by alterations in cytokine levels as well as in immune cell function and number. IL-6 is a pleiotropic cytokine that exerts its biological effects mainly through the IL-6/STAT3 signaling pathway. IL-6 is abundantly present in the TME, and an abnormally activated IL-6/STAT3 signaling pathway can play a role in the occurrence and development of HCC by affecting tumor cell proliferation, migration, invasion, angiogenesis, and apoptosis (36, 37). IL-10 and TGF-β are important regulatory cytokines of hepatocytes. Moreover, in addition to overcoming the tumor suppressor effect of hepatocytes, the mechanism of action of tumor cell development involves other pathways related to IL-10 and TGF-β, such as epithelial-mesenchymal transition (EMT) and suppressing IFN-γ production, which contributes to tumor progression and metastasis (38). The TME is shaped by complex interactions between tumor cells and immune cells. HCC has a high degree of malignancy and the poor survival rate of patients is closely related to an imbalance of the immune microenvironment, the breakdown of immune system surveillance, and the suppression of host immune system responses. These components synergistically construct an immunosuppressive microenvironment in HCC *via* a variety of mechanisms (**Figure 1**) (19).

Immunosuppressive Cells in the TME of HCC

MHC I/II is usually functionally depleted in HCC, is unable to activate T cells, and downregulates the expression of the costimulatory molecular receptor B7 family (such as B7.1/B7.2), leading to immune escape, which is a prerequisite for tumorigenesis (39). Low expression of MHC-I (binding to cytotoxic CD8+ T cells) and high expression of MHC-II (binding to immunosuppressive CD4+ T cells) is the reason for immune escape in terms of the failure of antigen presentation related to HCC. The result is that a large number of immunosuppressive cells are recruited into the TME of HCC (40).

Regulatory T Cells (T_{regs})

T_{regs} play a pivotal role in antitumor suppression and are mainly derived from peripheral blood or resident naive CD4+ T lymphocytes, and are recruited by the CC chemokine receptor 6 (CCR6)-CC chemokine ligand 20 (CCL20) axis (41). The differentiation of T_{regs} from CD4+ T cells requires the action of cytokines IL-2 and TGF-β, followed by the production of the suppressive cytokines IL-10 and TGF-β by expressing the transcription factor Foxp3, which in turn promotes further differentiation and suppresses inflammatory functions (42, 43). Compared with normal liver tissue, the proportion and number of CD4+CD25+ T_{regs} are markedly increased in HCC. Among these, CD4+ CD25+ Foxp3+ subtype T_{regs} have been found to suppress CD8+ cytotoxic T lymphocyte (CTL) activation and disable the killing capacity of CTLs by inhibiting tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) and the release of granzyme A, B (GrA, B), and perforin (44, 45). Another mechanism is the disruption of antigen presentation by downregulation of CD80 and CD86 expression in DCs and direct lysis of APCs *via* GrA and GrB (46, 47).

Myeloid-Derived Suppressor Cells (MDSCs)

MDSCs are immature myeloid cells that originate from the bone marrow that are increased in HCC and upregulate the expression of immune suppressive factors to suppress antitumor immunity in HCC (48). HCC-related cancer-associated fibroblasts (CAFs), which are components of the extracellular matrix in the TME, can induce MDSC differentiation from peripheral blood monocytes *via* IL-6/STAT3 signaling (49). In a previously established mouse model, it was demonstrated that granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, VEGF, and other tumor-associated cytokines could promote the accumulation and migration of MDSCs. Recent evidence has shown that a cell cycle-related kinase (CCRK) unique to HCC can also induce MDSC infiltration into the TME by promoting the expression of IL-6 by activating the zeste homolog 2 (EZH2)/nuclear factor-κB (NF-κB) signaling pathway (50). In addition, local hypoxia (a crucial factor in the TME of solid tumors) is another key factor for the recruitment of MDSCs with the action of the chemokine (C-C motif) ligand 26 (CCL26)/CX3CR1 pathway (51). MDSCs exert continuous immune-suppressive effects by inducing CD4+ CD25+ Foxp3+ T_{regs}, damaging CD8+ T cells, expanding immune checkpoint signaling and inhibiting NK-cell cytotoxicity (52, 53). MDSCs express the TIM3 ligand galectin-9 and induce T-cell apoptosis (54). Furthermore, PD-L1 expression can be induced by MDSCs in concert with KCs in advanced HCC, which mediates the inhibition of NK-cell cytotoxicity (55).

Tumor-Associated Macrophages (TAMs)

TAMs are predominant tumor-infiltrating leucocytes and vary depending on the cancer type (56). In HCC, TAMs arrive from CCR2+ inflammatory monocytes after the induction of the HCC-derived cytokines IL-4, CCL2, CXCL12, and others. Based on the state of macrophage activation, TAMs can be divided into two polarizing phenotypes, M1 and M2. M1 is the classical phenotype and activated by interferon-α, β or γ (IFNα/β/γ), which induces antitumor immune responses. In contrast, M2 is the alternative phenotype and activated by IL-4 and IL-10, which stimulate tumor promotion and metastasis by various mechanisms (57, 58). This suggests the presence of both antitumorigenic (M1) and protumorigenic (M2) macrophages in HCC, and the balance of M1/M2 is regulated by various TME components. TAMs contribute to malignant progression and metastasis by the production of IL-6, epithelial-to-mesenchymal transition (EMT) and immunosuppression (59). TAMs are highly associated with immune checkpoint molecules, such as PD-1/PD-L1, CTLA4, and TIM3, to exert immune inhibitory regulation. TAMs in the tumor stroma of HCC secrete pivotal cytokines (e.g., NF-α, IL-6, IL-23) and expand IL-17-producing CD4+ T helper 17 cells (Th17), which inhibit antitumor

immunity by upregulating PD-1 and CTLA-4 (60). Moreover, TAMs can directly promote T_{reg} expansion *via* surface expression of PD-L1. In addition, TAMs in HCC promote the expression of TIM3 by TGF-β stimulation, thereby ultimately facilitating tumor progression and immune tolerance (61).

CD8+ Cytotoxic T Lymphocytes (CTLs)

Naive CD8+ T cells (without cytotoxic activity) can become CTLs when they receive a signal from costimulatory molecules and then have the ability to protect against APCs. CD8+ CTLs can recognize abnormal cells, such as tumor cells by cooperating with helper T1 cells (Th1) and mediate antitumor immune responses by releasing perforin, granzyme, and TNF-α to damage tumor cells (48). However, the efficacy of CD8+ CTLs in HCC is functionally limited through a variety of mechanisms. Hypoxia, in conditions of an acidic environment (overload of lactic acid and low pH), lack the help of CD4+ T cells, and overabundant immunoregulatory molecules (IL-10, VEGF, IDO), may be responsible for restricted CD8+ CTL-specific cytotoxic responses (62). Unlike other TME immunosuppressive cells, the infiltration of CD8+ CTLs can be reduced by liver fibrosis (a striking feature of HCC) by disrupting CD8+ T-cell recognition of platelet-derived CD44 (63). Most CTLs are exhausted after their effect, but some remain memory killer cells that respond to the same tumor cells quickly when they are encountered in the future. In HCC, TOX, a novel T-cell exhaustion transcription regulator, is heavily overexpressed in CD8+ T cells, thereby suppressing cytotoxic effector and memory function (64). Notably, immune checkpoint signaling has recently been found to remarkably induce CTL exhaustion. PD-1/PD-L1 signaling is a crucial driver of CTL exhaustion (inhibition of T-cell survival and growth) in HCC and plays a role by blocking T-cell receptor (TCR) sequences through the PI3K/AKT pathway. CTLA-4 is upregulated after the activation of T cells and acts as a competitive antagonist of CD80 and CD86 in APCs and inhibits downstream AKT signaling, thereby ultimately exerting inhibitory effects (65). Other drivers of T-cell exhaustion include TIM3 and LAG3, which are expressed on CD8+ T cells and T_{regs} in HCC and lead to hypofunctional CD8+ responses by reducing CTL capacity (66, 67).

Natural Killer (NK) Cells

NK cells are innate immune cells with a high frequency (~30%) in the liver and a low frequency in peripheral blood. Upon NK-cell activation triggered by virus-infected cells and tumor cells, NK-cells function rapidly without antigen presentation (68). NK cells are crucial in maintaining the balance of immune defense/tolerance. The antitumor effect of NK cells is induced by secreting several killer cytokines (e.g., IFN-γ and TNF-α) and chemokines and by inducing tumor cell apoptosis *via* the Fas/FasL pathway as well as the release of cytotoxic granules (mainly perforin and granzyme) (69). In HCC, increasing evidence has shown that hypoxia can dysfunction the antitumor immunity of NK cells by utilizing TME immunosuppressive components to influence the switch of activating/inhibiting NK receptors (NKRs). For example, AFP (known to be overexpressed in HCC), especially when extended, decreased the expression of natural killer group 2, member D (NKG2D), an activating NKR, and negatively regulated NK-cell viability (70, 71). Other modulators in the TME, such as $T_{regs,}$ release the cytokines IL-8, IL-10, and TGF-β to downregulate NKG2D ligand membrane expression in HSCs, which suggests tumor progression in HCC patients (72).

Extracellular Matrix (ECM) in TME of HCC

Chronic liver inflammation/injury causes liver fibrosis, which is characterized by the continuous accumulation of ECM-producing myofibroblasts and results in the gradual substitution of liver parenchyma by fibrous or scar tissue and liver cirrhosis (73). In the physiological liver, quiescent HSCs localize in the space of Disse but are activated in myofibroblasts and secrete ECM components in the pathological liver (74). CAFs are most important components that form the ECM and promote EMT (normal epithelial cells transform into mesenchymal cells) in the TME. CAFs mostly stem from HSCs or bone marrow (BM)-derived activated mesenchymal stem cells (MSCs). CAFs can alter stiffness of the ECM, secrete cytokines, including epidermal growth factor (EGF), TGF-β, and PDGF, and in turn promote tumorigenesis of the liver. Moreover, CAFs have been found to indirectly promote HCC through crosstalk with immunosuppressive cells (mostly MDSCs and T_{regs}) in the TME, and reduce immune surveillance (75). Specifically, MDSC production can be induced by CAFs through the IL-6/STAT3 signaling axis and secretion of stromal cell-derived factor (SDF)-1α. More recently, in several studies, it was demonstrated that MDSC differentiation from blood monocytes can be promoted by PGE2 secretion in a CD44-dependent manner (76). CAFs also caused T-cell hyporesponsiveness and an increased number of T_{regs}, followed by inhibition of T-cell-mediated cytotoxicity (77). In summary, CAFs play a critical role in contributing to the occurrence of liver fibrosis and the progression of HCC in the TME.

Cytokines in the TME of HCC

The abundance of cytokines in the TME of HCC can mediate intercellular crosstalk and have multiple other functions. Based on their function, these cytokines can be classified into two groups. One type involves immune response cytokines, including TNFα, IFN-γ, IL-1, and IL-17, and the other type involves immunosuppressive cytokines, including IL-10, IL-4, IL-8, and TGF-β (78, 79). IL-10 is produced by DCs, TAMs, T cells, and T_{regs} and is elevated in HCC, thereby directly impairing the function of NK cells and downstream CD8+ T cells. Moreover, IL-10 inhibits the stimulatory function of APCs and promotes elevation of PD-L1 in monocytes, thus exerting immune escape-promoting effects (56, 80). High expression of a large amount of TGF-β in the HCC TME is made possible by tumor cells, macrophages, and T_{regs}. It not only attenuates the activation of DCs but can also trigger the activation of T_{regs} and impair the effector functions of T cells and NK cells to inhibit antitumor efficacy (81). Additionally, TGF-β increases TIM3 expression on TAMs, subsequently facilitating immune tolerance through the TNF-α/NF-κB signaling pathway (61). IFN-γ and TNFα are two pivotal cytokines that play a role in antitumor immune

responses, while lower serum levels of these two cytokines were found in HCC. As mentioned above, the production of immunosuppressive cytokines, such as IL-10, TGF-β, and PD-L1 can suppress IFN-γ/TNFα production derived from NK cells or effector T cells (82).

ICIS IN HCC

As shown above, in the TME of HCC, immune checkpoint molecules (PD-1, PD-L1, CTLA4, TIM3, LAG3) are associated with immunosuppressive cells to promote tumor growth and immune escape. This novel finding indicates that there are strong reasons to treat HCC patients with immunotherapies, especially ICI therapy. Increasingly, monoclonal antibodies aimed at blocking these immune checkpoint molecules have attracted increased attention in the HCC landscape (**Figure 2** and **Table 1**) (83).

PD-1/PD-L1 Monotherapy

PD-1 is mainly expressed on activated CD4+ and CD8+ T cells and NK cells. PD-L1, the ligand for PD-1, is mainly expressed on APCs and HCC tumor cells. Coinhibitory signals are mediated by the binding of PD-1 and PD-L1 to suppress T-cell immunity. In HCC, it has been shown that the upregulation of PD-1 and PD-L1 induced by various cytokines contributes to the dysfunction of effector T cells, which eventually promotes tumor aggressiveness and recurrence (84, 85). Clinically, the CheckMate-040 study is a multicohort, open label, phase 1/2 trial on the anti-PD-1 antibody nivolumab in patients with advanced HCC. In the dose-escalation phase, a total of 48 advanced HCC patients were enrolled into 3 groups (virus-uninfected, HBV, HCV-infected). The objective response rate (ORR) was 15% (95% CI, 6-28), and the disease control rate (DCR) was 58% (95% CI 43-72). Furthermore, the median progression-free survival (PFS) was 3.4 months (95% CI, 1.6-6.9), and the median overall survival (OS) was 15.0 months (95% CI 9.6-20.2). Severe grade 3/4 treatment-related adverse events (TRAEs), including diarrhea and hepatitis, were observed in 12 (25%) out of 48 patients. In addition, in the dose-expansion phase, a total of 214 advanced HCC patients were enrolled into 4 cohorts, including uninfected sorafenib refractory (n = 57), uninfected sorafenib intolerance (n = 56), HCV infected (n = 50), and HBV infected (n = 51). The ORR was 20% (95% CI 15-26), the DCR was reported as 64% (95% CI, 50-71), and the median PFS was 4.0 months (95% CI, 2.9-5.4). The OS was not reached. Nivolumab may offer favorable efficacy with a manageable safety profile, and a phase 3 randomized trial compared with sorafenib is underway (86). In another phase 3 trial (CheckMate-459) 743 systemic therapy-naive patients with advanced HCC were recruited to verify the effects of nivolumab compared with sorafenib. The median OS was 16.4 months (95%

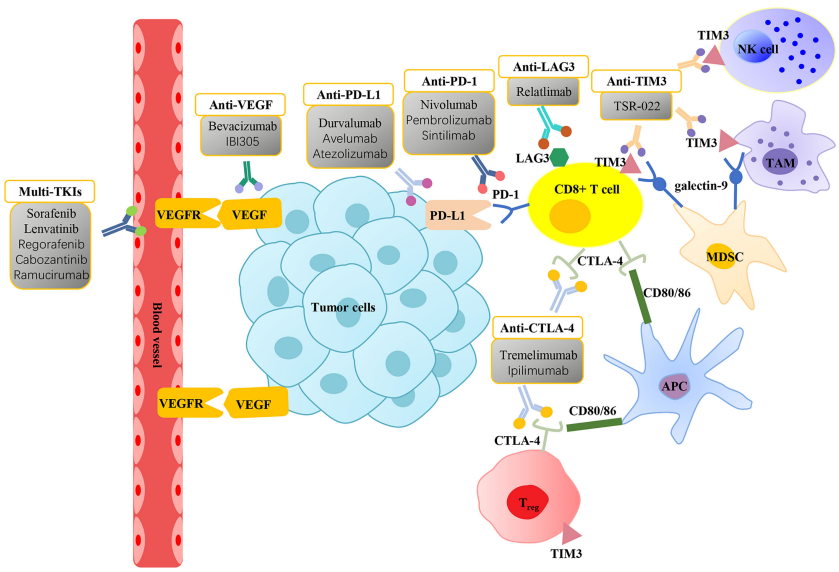

FIGURE 2 | Molecularly targeted therapies and immune checkpoint inhibitors for the treatment of hepatocellular carcinoma. Vascular endothelial growth factor (VEGF) is overexpressed in hepatocellular carcinoma (HCC) and interacts with vascular endothelial growth factor receptor (VEGFR) in the vascular endothelium to promote tumor growth. Molecularly targeted therapies focus on VEGF/VEGFR inhibitors, including multi-tyrosine kinase inhibitors (Multi-TKIs) and anti-VEGF can suppress angiogenesis and thus exert an anticancer effect. CD8+ T cells exhibit the expression of immune checkpoint molecules programmed cell death-1 (PD-1), cytotoxic T-lymphocyte antigen 4 (CTLA-4), mucin domain containing-3 (TIM3), and lymphocyte-activation gene 3 (LAG3) on their surface. High expression of CTLA-4 and TIM3 are displayed on the surface of T_{regs}. Tumor-associated macrophages (TAMs) and natural killer (NK) cells markedly express TIM3. Binding of PD-1 with its ligand programmed cell death ligand 1 (PD-L1) expressed on tumor cells promotes CD8+ T-cell apoptosis. CTLA-4 inhibits the proliferation of T cells and induces the activity of T_{regs} by binding to CD80/86 in antigen-presenting cells (APCs). The interaction between TIM3 and ligand galectin-9 on the surface of myeloid-derived suppressor cells (MDSCs) also induces T-cell apoptosis. Immune checkpoint inhibitors (ICIs) prevent the inactivation of T cells by blocking the interactions between immune checkpoint molecules with their ligands, thereby exerting antitumor effects.

TABLE 1 | Clinical trials with ICIs in HCC.

NCT	Number	Drug Type	Drug	Stage	ORR (%)	DCR (%)	mPFS (months)	mOS (months)	TRAEs (%)	First Posted (year)	Status
Monotherapy											
NCT01658878	48/214	Anti-PD-1	Nivolumab	Phase 1/2	15/20	58/64	3.4/4.0	15.0/NR	25.0	2012	Active, not recruiting
NCT02576509	743	Anti-PD-1	Nivolumab	Phase 3	NA	NA	NA	16.4	49.6	2015	Active, not recruiting
NCT02702414	104	Anti-PD-1	Pembrolizumab	Phase 2	17.0	62.0	4.9	12.9	25.0	2016	Active, not recruiting
NCT02702401	413	Anti-PD-1	Pembrolizumab	Phase 3	18.3	62.2	3 .0	13.9	52.7	2016	Completed
NCT01693562	40	Anti-PD-L1	Durvalumab	Phase 1/2	10.3	33.0	NA	13.2	20.0	2012	Completed
NCT03389126	30	Anti-PD-L1	Avelumab	Phase 2	10.0	73.3	4.4	14.2	19.4	2018	Completed
NCT01008358	21	Anti CTLA-4	Tremelimumab	Phase 2	NA	76.4	6.5	8.2	45	2009	Completed
NCT01853618	32	Anti CTLA-4	Tremelimumab	Phase 1	NA	NA	7.4	12.3	13.0	2013	Completed
ICIs Combinations											
NCT01658878	148	Anti-PD-1 + Anti CTLA-4	Nivolumab + Ipilimumab	Phase 1/2	31.0	49.0	NA	22.8	2.1	2012	Active, not recruiting
NCT03222076	27	Anti-PD-1 + Anti CTLA-4	Nivolumab + Ipilimumab	Phase 2	NA	NA	19.5	NA	43.0%	2017	Active, not recruiting
NCT02519348	332	Anti-PD-L1 + Anti CTLA-4	Durvalumab + Tremelimumab	Phase 1/2	24.0	NA	2.2	18.7	37.8	2015	Active, not recruiting
ICIs combined with Anti-angiogenesis											
NCT03006926	104	Anti-PD-1 + TKIs	Pembrolizumab + Lenvatinib	Phase 1	46.0	NA	9.3	22.0	67.0	2016	Active, not recruiting
NCT03299946	15	Anti-PD-1 + TKIs	Nivolumab + Cabozantinib	Phase 1	NA	NA	NA	NA	NA	2017	Active, not recruiting
NCT03755791	740	Anti-PD-L1 + TKIs	Atezolizumab + Cabozantinib	Phase 3	NA	NA	NA	NA	NA	2018	Recruiting
NCT03794440	595	PD-1 inhibitor + Anti-VEGF	Sintilimab + IBI305	Phase 2/3	NA	NA	4.6	NR	14.0	2019	Active, not recruiting
NCT02715531	223	Anti-PD-L1 + Anti-VEGF	Atezolizumab + Bevacizumab	Phase 1	20.0	NA	5.6	NR	5.0	2016	Completed
NCT03434379	501	Anti-PD-L1 + Anti-VEGF	Atezolizumab + Bevacizumab	Phase 3	27.3	NA	6.8	NR	61.1	2018	Active, not recruiting

ICIs, immune checkpoints inhibitors; ORR, objective response rate; DCR, disease control rate; mPFS, median progression free survival; mOS, median overall survival; TRAEs, treatment-related adverse events; PD-1, programmed cell death-1; PD-L1, programmed cell death ligand 1; CTLA4, cytotoxic T-lymphocyte-associated protein 4; TKIs, tyrosine kinase inhibitors; VEGF, vascular endothelial growth factor; NR, not reached; NA, not available.

CI, 13.9-18.4) for nivolumab and 14.7 months (95% CI, 11.9-17.2) for sorafenib. TRAEs were reported in 82 patients (22.3%) and 180 patients (49.6%) treated with nivolumab and sorafenib, respectively (87). In KEYNOTE-224, a phase 2 trial, the efficacy and safety of pembrolizumab (anti-PD-1 antibody) were evaluated in 104 HCC patients who had progressed or were intolerant to sorafenib. The ORR was recorded as 17% (95% CI, 11-26), and the DCR was 62% (95% CI, 52-71). The median PFS and OS were 4.9 months (95% CI, 3.4-7.2) and 12.9 months (95% CI, 9.7-15.5), respectively. Twenty-six (25%) grade 3-4 TRAEs were observed (88). In addition, in a randomized, multicenter phase 3 trial (KEYNOTE-240) the efficacy and safety of pembrolizumab compared with a placebo were assessed in 413 HCC patients after progression on sorafenib. The results indicated that the ORR and DCR of the pembrolizumab group were 18.3% (95% CI, 14.0-23.4) and 62.2%, respectively, which was significantly better than those of the control group (4.4% (95% CI, 1.6-9.4) and 53.3%, respectively. The median PFS and OS for pembrolizumab were 3.0 months (95% CI, 2.8-4.1) and 13.9 months (95% CI, 11.6-16.0) versus 2.8 months (95% CI, 1.6-

3.0) and 10.6 months (95% CI, 8.3-13.5) for the placebo, respectively. The incidence of TRAEs of grade 3 and above was 52.7% in the pembrolizumab group and 46.3% in the placebo group. Anti-PD-L1 monotherapy (durvalumab) was observed as part of a randomized expansion phase 1/2 study in 104 HCC patients who progressed or refused sorafenib treatment, with an ORR of 10.6% (95% CI, 5.4-18.1) and a median OS of 13.6 months (8.7 to 17.6) (89). At the 2017 American Society of Clinical Oncology (ASCO) annual meeting, an ongoing phase 1/2 trial that was aimed at evaluating the safety and clinical activity of durvalumab in advanced solid tumors showed promising antitumor activity and management safety in 40 patients with HCC (5). The fully human monoclonal anti-PD-L1 agent avelumab underwent further assessment in a phase 2, single-arm, single center in patients with advanced HCC who were previously treated with sorafenib (NCT03389126). Preliminary results were promising: ORR: 10.0%, DCR: 73.3%, median PFS: 4.4 months (95% CI, 2.9-5.9), median OS: 14.2 months (95% CI, 9.5-18.9). Avelumab was well tolerated with manageable toxicity, with 7 grade 3 TRAEs and no grade 4 TRAEs (90).

CTLA-4 Monotherapy

CTLA-4 is present on T_{regs} and activated T cells and is an inhibitory coreceptor that plays an important role in regulating the function of CD4+ T cells. In many types of solid cancer, including HCC, CTLA-4 suppresses the proliferation of T cells, promotes the production of the suppressive cytokines IL-10 and IDO, and induces T_{reg} activity (91, 92). Many clinical trials on anti-CTLA-4 are currently ongoing with promising results. The antitumor effect of blocking CTLA-4 with tremelimumab in the treatment of HCV-associated advanced HCC was demonstrated in a phase 2 trial (NCT01008358), involving 21 patients. A remarkable DCR of 76.4% was observed, and although there was no complete response, the partial response rate (PRR) was 17.6%. In the study, the efficacy of tremelimumab was investigated with a median PFS of 6.48 months (95% CI, 3.95-9.14) and a median OS of 8.2 months (95% CI, 4.64-21.34) and a manageable safety profile. In addition, tremelimumab has been shown to play an antiviral role, and a progressive course of decreased viral load was observed for almost 3 months in most patients (93). In another recent communication, a phase 1 trial (NCT01853618) was reported, evaluating tremelimumab in combination with ablation in 32 patients with advanced HCC. The study showed significant results, with a median PFS and OS of 7.4 months (95% CI, 4.7-19.4) and 12.3 (95% CI, 9.3-15.4), respectively. Four (13%) patients presented with grade 3/4 TRAEs (94).

ICI Combination

Most ICI combination trials in advanced HCC have previously shown efficacy. The combination of the anti-PD-1 antibody nivolumab and the anti-CTLA-4 antibody ipilimumab was first tested in the phase 1/2 CheckMate-040 trial (NCT01658878). Based on different dosages, 148 advanced HCC patients who were previously treated with sorafenib were randomized into three arms: (A) nivolumab 1 mg/kg + ipilimumab 3 mg/kg, (B) nivolumab 3 mg/kg + ipilimumab 1 mg/kg every 3 weeks (Q3 W), and (C) nivolumab 3 mg/kg + ipilimumab 1 mg/kg every 6 weeks (Q6 W). The primary endpoint ORR was 31.0% (95% CI, 18-45) in combination therapy compared to 15% (95% CI, 6-28) in nivolumab monotherapy. At 24 months, the DCR was 48.8%, and the OS was 40%. A promising effect on outcome was observed, especially in arm A, with a median OS of 22.8 months (95% CI 9.4 - not reached). Grade 3-4 TRAEs were reported in 5 out of 49 patients (10.2%) in arm A, 2 out of 49 patients (4.1%) in arm B, and 1 out of 48 patients (2.1%) in arm C (95). Based on these promising results, in March 2020, the FDA approved combination therapy (arm A) as a second-line treatment after sorafenib. Recently, an open-label, randomized phase 2 trial (NCT03222076) evaluated the efficacy of nivolumab monotherapy versus nivolumab plus ipilimumab in the treatment of HCC patients who could be treated by surgery. All 27 patients were classified into nivolumab monotherapy (n = 13) and nivolumab plus ipilimumab combination therapy (n = 14) groups. Feasible data were observed, with a median PFS of 19.53 months (95% CI, 2.33 - not estimable) in the combination group and 9.4 months (95% CI, 1.47 - not estimable) in the monotherapy group. However, in combination therapy, grade 3-4 TRAEs (6 of 14 (43.0%)) were higher than those of nivolumab alone (3 of 13 (23.0%)). Overall, nivolumab plus ipilimumab appeared to be safe and effective (96). Clinical data on durvalumab (anti-PD-L1) in combination with tremelimumab (anti-CTLA-4) were presented in a phase 1/2 study including 332 HCC patients. Four cohorts were assigned, including T300 + D (tremelimumab 300 mg + durvalumab), durvalumab or tremelimumab monotherapy, and T75 + D (tremelimumab 75 mg + durvalumab). The results showed that the ORR and median OS of the T300 + D cohort were 24.0% (95% CI, 14.9-35.3) and 18.7 months (95% CI, 10.8-27.3), respectively, which were better than the data obtained from monotherapy and T75 + D groups. However, the incidence of grade ≥ 3 TRAEs was the highest (37.8%) in the 4 groups (86). Recently, TIM3 has been shown to overcome resistance to PD-1 blockade (97). The results for a phase 2 trial assessing the efficacy and safety of anti-PD-1 and anti-TIM3 combination therapy (NCT03680508) (98) are still awaited. In addition, dual blockade of PD-1 with anti-LAG3 therapy is being conducted in a phase 1 trial (NCT01968109). However, the clinical values of TIM3 and LAG3 need to be further elucidated.

ICIs Combined With Anti-Angiogenesis

Additional strategies aimed at combining TKIs/anti-VEGFs with ICI therapy after ICI progression may represent future treatment options. A total of 104 patients were enrolled in a phase 1b, multicenter, open-label trial of lenvatinib (TKIs) plus pembrolizumab (anti-PD-1) in patients with unresectable HCC. Patients received lenvatinib (12 mg if ≥ 60 kg, 8 mg if < 60 kg) orally daily plus pembrolizumab 200 mg Q3 W intravenously on day 1 of a 3-week cycle. The ORR was 46.0% (95% CI, 36.0-56.3), with a median PFS of 9.3 months and a median OS of 22.0 months. Grade ≥3 TRAEs occurred in 67% of patients, and no new safety signals were observed (99). A cohort study was launched within the single arm phase 1b trial (NCT03299946) exploring the combination of cabozantinib (TKIs) and nivolumab (anti-PD-1)

in locally advanced HCC patients. A total of 15 patients were included in the study; 12 out of 15 patients (80%) underwent surgical resection after combination therapy, and 5 out of 15 patients (42%) had major pathologic responses (100). A COSMIC-312 phase 3 study trial of cabozantinib in combination with atezolizumab (anti-PD-L1) versus sorafenib is currently ongoing (NCT03755791) in treatment-naive HCC patients. Approximately 740 patients were randomized into 3 groups: cabozantinib plus atezolizumab (370 patients) and sorafenib or cabozantinib single-agent (185 patients). For the combination group, cabozantinib was administered orally (40 mg once daily) plus atezolizumab 1200 mg Q3 W intravenously (101). Currently, clinical trials are ongoing. Recently, an open-label, phase 2-3 trial (NCT03794440) was performed in 595 unresectable HBV-associated HCC patients in China. First, a phase 2 study was performed in 24 patients, and inspiring results were obtained. The ORR in the phase 2 part of the study was 25.0% (95% CI, 9.8-46.7), and TRAEs were observed in 7 out of 24 patients (29%). Subsequently, a randomized phase 3 trial was started because of its preliminary safety profile and effectiveness. The remaining 571 patients were randomly assigned to the sintilimab (PD-1 inhibitor) plus IBI305 (anti-VEGF agent bevacizumab biosimilar) group (n = 380) or sorafenib group (n = 191). This trial demonstrated that patients with sintilimab plus IBI305 combination treatment had a significantly longer median PFS (4.6 months (95% CI, 4.1-5.7)) and median OS (not reached) than patients in the sorafenib group (median PFS and OS were 2.8 months and 10.4 months, respectively) (102). In GO30140, an open-label, multicenter, phase 1b trial (NCT02715531), two unresectable HCC cohorts, groups A and F, from 26 academic centers were described. In group A, 104 patients were enrolled and treated with atezolizumab plus bevacizumab (anti-VEGF). In group F, 119 patients were enrolled and randomly assigned into 2 groups: atezolizumab combined with bevacizumab (n = 60) and atezolizumab monotherapy (n = 59). In group A and in the combination therapy subgroup in group F, all patients received 1200 mg atezolizumab and 15 mg/kg bevacizumab intravenously Q3 W. P patients in the other group of group F were given only 1200 mg atezolizumab intravenously Q3 W. The results showed that the ORR (20%, (95% CI, 11-32)) and median PFS (5.6 (95% CI, 3.6-7.4)) of patients in the atezolizumab plus bevacizumab group were superior to those of patients who received atezolizumab monotherapy (103). A total of 501 unresectable HCC patients who had not previously received systemic treatment were enrolled in a global, phase 3 clinical trial (IMbrave150, NCT03434379) and were randomly divided in a 2:1 ratio into two groups: atezolizumab plus bevacizumab therapy (336 patients) or sorafenib therapy (165 patients). Patients in the combined therapy arm were treated with a standard dose (1200 mg) of atezolizumab followed by a high dose of bevacizumab (15 mg/kg) Q3 W, and patients in the sorafenib arm orally received 400 mg twice daily. After treatment, according to the RECIST 1.1 criteria, we showed that the ORR was 27.3% (95% CI, 22.5-32.5) in the atezolizumab plus bevacizumab group and 11.9% (95% CI, 7.4-18.0) in the sorafenib group. A prognostic advantage of combination therapy over sorafenib was also observed. The median PFS was 6.8 months (95% CI, 5.7-8.3), which was significantly longer than the 4.3 months (95% CI, 4.0-5.6) in the sorafenib group. The median OS was 13.2 months (95% CI, 10.4 - not reached) with sorafenib but was not reached in the combined group. For safety, 201 patients (61.1%) with serious TRAEs (≥ grade 3) were observed in the atezolizumab plus bevacizumab arm, and 95 patients (60.9%) were observed in the sorafenib arm (104).

CHALLENGES IN COMBINATION THERAPY FOR HCC

Despite encouraging preliminary data generated using combination strategies of antiangiogenic therapy and ICIs of advanced HCC, challenges still represent a burden in HCC management.

Drug Resistance of Combination Therapy

One of the main challenges is drug resistance (primary or acquired), which remains the major cause of treatment failure. Drug resistance is complex and dynamic because abnormal behavior at any step can lead to drug resistance. In recent years, various molecular mechanisms underlying drug resistance have been investigated and identified (105). First, HCC is generally considered an "immune-cold" tumor, characterized by T-cell deficiency, infiltration of immunosuppressive cells (MDSCs, TAMs, T_{regs}) and poor antigen presentation, resulting in the ability to maintain immune tolerance and an inability to produce tumor immune responses (106). The characteristic of a "cold" HCC tumor is the common mechanism for primary resistance (107). Moreover, tumor heterogeneity is the other underlying mechanism involved in primary resistance. Unlike other primary tumors, multifocal lesions in the liver are common and although these tumors genetically originate from similar cells, they differ significantly from each other. It is not only the multifocal tumors that cause the heterogeneity of HCC but also the difference in patients for the differential expression of immune checkpoint molecules (108). Thus, it is critical to develop new ways to diminish primary drug resistance by transforming the "cold" tumor microenvironment into a "hot" tumor as well as circumventing tumor heterogeneity. In addition, the heterogeneity of the HCC TME also plays an important role in later acquired resistance. In previous studies, it has been shown that approximately a quarter of HCC (classified as immune class) has higher immune infiltration and higher PD-1/PD-L1 expression levels and thus has higher response rates to immunotherapy than the rest of HCCs (109, 110). However, a high response to treatment cannot be guaranteed for immune-suppressive cells, including MDSCs, TAMs, and T_{regs}, in the TME of HCC. These immune-suppressive components of the TME may contribute to T-cell exhaustion and immune checkpoint protein dysfunction, which further develop drug resistance to ICIs. Thus, a model that stratifies HCC patients according to the status of immune infiltration and immune checkpoint molecules may help to adequately select candidates for ICI therapy (111).

Intratumor heterogeneity is the key reason for sorafenib therapy resistance. In a previous study, it was demonstrated that sorafenib can induce the accumulation of autophagosomes in an *in vitro* HCC model. Several studies have reported that sorafenib induces an autophagic-protective response in HCC cells, resulting in drug resistance and affecting therapeutic efficacy. In addition, an imbalance between anti-apoptotic and pro-apoptotic proteins is associated with sorafenib resistance. Nonetheless, the exact underlying mechanism of sorafenib resistance needs to be further elucidated (112).

In the author's opinion, physicians need to consider individual differences in the treatment process and specify individual diagnosis and treatment plans to improve treatment efficacy. In addition, further studies on HCC immunity and molecular pathology are needed to elucidate the underlying mechanisms involved in the TME that may lead to the failure of immunotherapy.

Potential Biomarkers of Clinical Response in Combination Therapy

The potential of ICIs in combination with TKIs/anti-VEGFs for HCC has been widely recognized. Moreover, many clinical trials have indicated that not all HCC patients receiving combination treatment will achieve the desired efficacy. Biomarkers are good indicators for predicting and evaluating treatment response. Recently, a meta-analysis indicated that patients with high PD-L1 expression (>1% score) had longer survival than patients with <1% PD-L1 expression. Therefore, the PD-L1 status may be a potential predictive biomarker in the context of anti-PD-1/PD-L1 therapy (98). Moreover, one study showed that HCC patients with Wnt/CTNNB1 mutations were insensitive to anti-PD-1/PD-L1 therapy and had a worse prognosis than patients without mutations (113). Furthermore, relevant literature shows that CD28/B7 may be a biomarker for the clinical response to anti-PD-1 in mouse models and lung cancer patients. However, clinical data from HCC patients are insufficient (114). In another preclinical study, it was suggested that HCC patients with high AFP levels (≥400 ng/mL) are more likely to profit from combination therapy of ICIs and lenvatinib (115). Accordingly, there is a need to develop predictive biomarkers with high specificity and sensitivity to accurately identify HCC patients who most likely benefit from combination therapy. As mentioned above, comprehensive and systematic studies on the molecular level of HCC immunity are warranted.

TRAEs of Combination Therapy

TRAEs are one of the most concerning issues in clinical trials and not only affect the quality of life of patients but also affect treatment compliance. TRAEs are classified into five grades based on severity, and TRAEs of grade 3 or more are considered serious TRAEs. The most common TRAEs occur in the skin, gastrointestinal, liver, lung, and endocrine systems (116). Skin toxicity and gastrointestinal toxicity (diarrhea and colitis) were the first and second most common TRAEs, respectively. The incidence of skin and gastrointestinal toxicity in patients who were treated with anti-PD-1/PD-L1 is approximately 30.0% and 10.0-20.0%, respectively. For anti-CTLA-4 treatment, skin and gastrointestinal toxicity was as high as 40.0% and 30.0-40.0%, respectively. It has been suggested that anti-PD1/PDL1 results in fewer TRAEs than anti-CTLA-4. Moreover, a combination of anti-PD1 with anti-CTLA-4 showed an increased hepatic TRAEs in the early phase of treatment, although most improved after six weeks (117). Of note, fatal cardiotoxicity has been reported in patients who were treated with pembrolizumab or a combination of nivolumab and ipilimumab. Most TRAEs are reversible and controllable, but severe cardiac and autoimmune diseases should receive more attention for early recognition and intervention in the future (118).

In the author's opinion, the proportion of patients with advanced HCC complicated with HBV in China is high, and the associated TRAEs are more complex. Therefore, no matter which treatment method is chosen, special attention should be paid to a patients' underlying liver disease and other underlying diseases.

CONCLUSION

More than 70% of HCC patients are diagnosed at an intermediate or advanced stage (BCLC stage B, C or D) and require systemic therapy. The clinical efficacy of traditional TKI drugs (sorafenib, lenvatinib etc.) is still not satisfactory, although once brought patients hope. Thus, novel strategies are currently being developed, including ICIs, ICI combinations, and ICI combinations with antiangiogenics. More recently, the application of ICI-based combination therapy has become a growing field of study that has gradually displaced TKI monotherapy in advanced HCC. However, challenges remain, including drug resistance, predictive biomarkers of treatment effectiveness, and TRAEs in combination treatment. More safe and effective combination therapy strategies for advanced HCC should be developed, and further studies are needed.

AUTHOR CONTRIBUTIONS

CZ contributed to the study design. YC and HH were responsible for data collection and prepared the manuscript. XY and XF drafted and prepared the manuscript. All authors participated in the data interpretation, contributed to the manuscript writing with important intellectual content and approved the final version of the manuscript.

REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: Globocan Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* (2021) 71(3):209–49. doi: 10.3322/caac.21660
2. Ogasawara S, Choo SP, Li JT, Yoo C, Wang B, Lee D, et al. Evolving Treatment of Advanced Hepatocellular Carcinoma in the Asia-Pacific Region: A Review and Multidisciplinary Expert Opinion. *Cancers (Basel)* (2021) 13(11):2626. doi: 10.3390/cancers13112626
3. Sangro B, Sarobe P, Hervas-Stubbs S, Melero I. Advances in Immunotherapy for Hepatocellular Carcinoma. *Nat Rev*

Gastroenterol Hepatol (2021) 18(8):525–43. doi: 10.1038/s41575-021-00438-0
4. Yang JD, Hainaut P, Gores GJ, Amadou A, Plymoth A, Roberts LR. A Global View of Hepatocellular Carcinoma: Trends, Risk, Prevention and Management. *Nat Rev Gastroenterol Hepatol* (2019) 16(10):589–604. doi: 10.1038/s41575-019-0186-y
5. Hilmi M, Neuzillet C, Calderaro J, Lafdil F, Pawlotsky JM, Rousseau B. Angiogenesis and Immune Checkpoint Inhibitors as Therapies for Hepatocellular Carcinoma: Current Knowledge and Future Research Directions. *J Immunother Cancer* (2019) 7(1):333. doi: 10.1186/s40425-019-0824-5
6. Forner A, Reig M, Bruix J. Hepatocellular Carcinoma. *Lancet* (2018) 391 (10127):1301–14. doi: 10.1016/s0140-6736(18)30010-2
7. European Association for the Study of the Liver and European Association for the Study of the L. Easl Clinical Practice Guidelines: Management of Hepatocellular Carcinoma. *J Hepatol* (2018) 69(1):182–236. doi: 10.1016/j.jhep.2018.03.019
8. Tsochatzis EA, Bosch J, Burroughs AK. Liver Cirrhosis. *Lancet* (2014) 383 (9930):1749–61. doi: 10.1016/S0140-6736(14)60121-5
9. Morse MA, Sun W, Kim R, He AR, Abada PB, Mynderse M, et al. The Role of Angiogenesis in Hepatocellular Carcinoma. *Clin Cancer Res* (2019) 25 (3):912–20. doi: 10.1158/1078-0432.CCR-18-1254
10. Liu Z, Lin Y, Zhang J, Zhang Y, Li Y, Liu Z, et al. Molecular Targeted and Immune Checkpoint Therapy for Advanced Hepatocellular Carcinoma. *J Exp Clin Cancer Res* (2019) 38(1):447. doi: 10.1186/s13046-019-1412-8
11. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. Sorafenib in Advanced Hepatocellular Carcinoma. *N Engl J Med* (2008) 359(4):378–90. doi: 10.1056/NEJMoa0708857
12. Cheng AL, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, et al. Efficacy and Safety of Sorafenib in Patients in the Asia-Pacific Region With Advanced Hepatocellular Carcinoma: A Phase Iii Randomised, Double-Blind, Placebo-Controlled Trial. *Lancet Oncol* (2009) 10(1):25–34. doi: 10.1016/S1470-2045(08)70285-7
13. Abdelrahim M, Esmail A, Abudayyeh A, Murakami N, Saharia A, McMillan R, et al. Transplant Oncology: An Evolving Field in Cancer Care. *Cancers (Basel)* (2021) 13(19):4911. doi: 10.3390/cancers13194911
14. Abdelrahim M, Victor D, Esmail A, Kodali S, Graviss EA, Nguyen DT, et al. Transarterial Chemoembolization (Tace) Plus Sorafenib Compared to Tace Alone in Transplant Recipients With Hepatocellular Carcinoma: An Institution Experience. *Cancers (Basel)* (2022) 14(3):650. doi: 10.3390/cancers14030650
15. Benson AB, D'Angelica MI, Abbott DE, Abrams TA, Alberts SR, Anaya DA, et al. Guidelines Insights: Hepatobiliary Cancers, Version 2.2019. *J Natl Compr Canc Netw* (2019) 17(4):302–10. doi: 10.6004/jnccn.2019.0019
16. Benson AB3rd, D'Angelica MI, Abbott DE, Abrams TA, Alberts SR, Saenz DA, et al. Nccn Guidelines Insights: Hepatobiliary Cancers, Version 1.2017. *J Natl Compr Canc Netw* (2017) 15(5):563–73. doi: 10.6004/jnccn.2017.0059
17. Zhu AX, Kang YK, Yen CJ, Finn RS, Galle PR, Llovet JM, et al. Ramucirumab After Sorafenib in Patients With Advanced Hepatocellular Carcinoma and Increased Alpha-Fetoprotein Concentrations (Reach-2): A Randomised, Double-Blind, Placebo-Controlled, Phase 3 Trial. *Lancet Oncol* (2019) 20 (2):282–96. doi: 10.1016/S1470-2045(18)30937-9
18. Sharma R, Motedayen Aval L. Beyond First-Line Immune Checkpoint Inhibitor Therapy in Patients With Hepatocellular Carcinoma. *Front Immunol* (2021) 12:652007. doi: 10.3389/fimmu.2021.652007
19. Zongyi Y, Xiaowu L. Immunotherapy for Hepatocellular Carcinoma. *Cancer Lett* (2020) 470:8–17. doi: 10.1016/j.canlet.2019.12.002
20. Zhou G, Boor PPC, Bruno MJ, Sprengers D, Kwekkeboom J. Immune Suppressive Checkpoint Interactions in the Tumour Microenvironment of Primary Liver Cancers. *Br J Cancer* (2022) 126(1):10–23. doi: 10.1038/s41416-021-01453-3
21. He X, Xu C. Immune Checkpoint Signaling and Cancer Immunotherapy. *Cell Res* (2020) 30(8):660–9. doi: 10.1038/s41422-020-0343-4
22. Liu ZL, Liu JH, Staiculescu D, Chen J. Combination of Molecularly Targeted Therapies and Immune Checkpoint Inhibitors in the New Era of Unresectable Hepatocellular Carcinoma Treatment. *Ther Adv Med Oncol* (2021) 13:17588359211018026. doi: 10.1177/17588359211018026
23. Abdelrahim M, Esmail A, Saharia A, Abudayyeh A, Abdel-Wahab N, Diab A, et al. Utilization of Immunotherapy for the Treatment of Hepatocellular Carcinoma in the Peri-Transplant Setting: Transplant Oncology View. *Cancers (Basel)* (2022) 14(7):1760. doi: 10.3390/cancers14071760
24. Dong Y, Wong JSL, Sugimura R, Lam KO, Li B, Kwok GGW, et al. Recent Advances and Future Prospects in Immune Checkpoint (Ici)-Based Combination Therapy for Advanced Hcc. *Cancers (Basel)* (2021) 13 (8):1949. doi: 10.3390/cancers13081949
25. Rizzo A, Ricci AD, Gadaleta-Caldarola G, Brandi G. First-Line Immune Checkpoint Inhibitor-Based Combinations in Unresectable Hepatocellular Carcinoma: Current Management and Future Challenges. *Expert Rev Gastroenterol Hepatol* (2021) 15(11):1245–51. doi: 10.1080/17474124.2021.1973431
26. Johnston MP, Khakoo SI. Immunotherapy for Hepatocellular Carcinoma: Current and Future. *World J Gastroenterol* (2019) 25(24):2977–89. doi: 10.3748/wjg.v25.i24.2977
27. Leone P, Solimando AG, Fasano R, Argentiero A, Malerba E, Buonavoglia A, et al. The Evolving Role of Immune Checkpoint Inhibitors in Hepatocellular Carcinoma Treatment. *Vaccines (Basel)* (2021) 9(5):532. doi: 10.3390/vaccines9050532
28. El Dika I, Khalil DN, Abou-Alfa GK. Immune Checkpoint Inhibitors for Hepatocellular Carcinoma. *Cancer* (2019) 125(19):3312–9. doi: 10.1002/cncr.32076
29. Thomson AW, Knolle PA. Antigen-Presenting Cell Function in the Tolerogenic Liver Environment. *Nat Rev Immunol* (2010) 10(11):753–66. doi: 10.1038/nri2858
30. Elsegood CL, Tirnitz-Parker JE, Olynyk JK, Yeoh GC. Immune Checkpoint Inhibition: Prospects for Prevention and Therapy of Hepatocellular Carcinoma. *Clin Transl Immunol* (2017) 6(11):e161. doi: 10.1038/cti.2017.47
31. Guardascione M, Toffoli G. Immune Checkpoint Inhibitors as Monotherapy or Within a Combinatorial Strategy in Advanced Hepatocellular Carcinoma. *Int J Mol Sci* (2020) 21(17):6302. doi: 10.3390/ijms21176302
32. Pardoll DM. The Blockade of Immune Checkpoints in Cancer Immunotherapy. *Nat Rev Cancer* (2012) 12(4):252–64. doi: 10.1038/nrc3239
33. Li B, Yan C, Zhu J, Chen X, Fu Q, Zhang H, et al. Anti-Pd-1/Pd-L1 Blockade Immunotherapy Employed in Treating Hepatitis B Virus Infection-Related Advanced Hepatocellular Carcinoma: A Literature Review. *Front Immunol* (2020) 11:1037. doi: 10.3389/fimmu.2020.01037
34. Hu H, Chen Y, Tan S, Wu S, Huang Y, Fu S, et al. The Research Progress of Antiangiogenic Therapy, Immune Therapy and Tumor Microenvironment. *Front Immunol* (2022) 13:802846. doi: 10.3389/fimmu.2022.802846
35. Giraud J, Chalopin D, Blanc JF, Saleh M. Hepatocellular Carcinoma Immune Landscape and the Potential of Immunotherapies. *Front Immunol* (2021) 12:655697. doi: 10.3389/fimmu.2021.655697
36. Zheng X, Xu M, Yao B, Wang C, Jia Y, Liu Q. Il-6/Stat3 Axis Initiated Cafs *Via* Up-Regulating Timp-1 Which Was Attenuated by Acetylation of Stat3 Induced by Pcaf in Hcc Microenvironment. *Cell Signal* (2016) 28(9):1314–24. doi: 10.1016/j.cellsig.2016.06.009
37. Yin Z, Ma T, Lin Y, Lu X, Zhang C, Chen S, et al. Il-6/Stat3 Pathway Intermediates M1/M2 Macrophage Polarization During the Development of Hepatocellular Carcinoma. *J Cell Biochem* (2018) 119(11):9419–32. doi: 10.1002/jcb.27259
38. Yang R, Gao N, Chang Q, Meng X, Wang W. The Role of Ido, Il-10, and Tgf-Beta in the Hcv-Associated Chronic Hepatitis, Liver Cirrhosis, and Hepatocellular Carcinoma. *J Med Virol* (2019) 91(2):265–71. doi: 10.1002/jmv.25083
39. Shen X, Zhang L, Li J, Li Y, Wang Y, Xu ZX. Recent Findings in the Regulation of Programmed Death Ligand 1 Expression. *Front Immunol* (2019) 10:1337. doi: 10.3389/fimmu.2019.01337
40. Liu HT, Jiang MJ, Deng ZJ, Li L, Huang JL, Liu ZX, et al. Immune Checkpoint Inhibitors in Hepatocellular Carcinoma: Current Progresses and Challenges. *Front Oncol* (2021) 11:737497. doi: 10.3389/fonc.2021.737497
41. Chen KJ, Lin SZ, Zhou L, Xie HY, Zhou WH, Taki-Eldin A, et al. Selective Recruitment of Regulatory T Cell Through Ccr6-Ccl20 in Hepatocellular Carcinoma Fosters Tumor Progression and Predicts Poor Prognosis. *PloS One* (2011) 6(9):e24671. doi: 10.1371/journal.pone.0024671
42. Yamane H, Paul WE. Early Signaling Events That Underlie Fate Decisions of Naive Cd4(+) T Cells Toward Distinct T-Helper Cell Subsets. *Immunol Rev* (2013) 252(1):12–23. doi: 10.1111/imr.12032

43. Zou W. Regulatory T Cells, Tumour Immunity and Immunotherapy. *Nat Rev Immunol* (2006) 6(4):295–307. doi: 10.1038/nri1806
44. Yuan CH, Sun XM, Zhu CL, Liu SP, Wu L, Chen H, et al. Amphiregulin Activates Regulatory T Lymphocytes and Suppresses Cd8+ T Cell-Mediated Anti-Tumor Response in Hepatocellular Carcinoma Cells. *Oncotarget* (2015) 6(31):32138–53. doi: 10.18632/oncotarget.5171
45. Huang Y, Wang FM, Wang T, Wang YJ, Zhu ZY, Gao YT, et al. Tumor-Infiltrating Foxp3+ Tregs and Cd8+ T Cells Affect the Prognosis of Hepatocellular Carcinoma Patients. *Digestion* (2012) 86(4):329–37. doi: 10.1159/000342801
46. Shevach EM. Mechanisms of Foxp3+ T Regulatory Cell-Mediated Suppression. *Immunity* (2009) 30(5):636–45. doi: 10.1016/j.immuni.2009.04.010
47. Larmonier N, Marron M, Zeng Y, Cantrell J, Romanoski A, Sepassi M, et al. Tumor-Derived Cd4(+)Cd25(+) Regulatory T Cell Suppression of Dendritic Cell Function Involves Tgf-Beta and Il-10. *Cancer Immunol Immunother* (2007) 56(1):48–59. doi: 10.1007/s00262-006-0160-8
48. Oura K, Morishita A, Tani J, Masaki T. Tumor Immune Microenvironment and Immunosuppressive Therapy in Hepatocellular Carcinoma: A Review. *Int J Mol Sci* (2021) 22(11):5801. doi: 10.3390/ijms22115801
49. Deng Y, Cheng J, Fu B, Liu W, Chen G, Zhang Q, et al. Hepatic Carcinoma-Associated Fibroblasts Enhance Immune Suppression by Facilitating the Generation of Myeloid-Derived Suppressor Cells. *Oncogene* (2017) 36(8):1090–101. doi: 10.1038/onc.2016.273
50. Zhou J, Liu M, Sun H, Feng Y, Xu L, Chan AWH, et al. Hepatoma-Intrinsic Ccrk Inhibition Diminishes Myeloid-Derived Suppressor Cell Immunosuppression and Enhances Immune-Checkpoint Blockade Efficacy. *Gut* (2018) 67(5):931–44. doi: 10.1136/gutjnl-2017-314032
51. Chiu DK, Xu IM, Lai RK, Tse AP, Wei LL, Koh HY, et al. Hypoxia Induces Myeloid-Derived Suppressor Cell Recruitment to Hepatocellular Carcinoma Through Chemokine (C-C Motif) Ligand 26. *Hepatology* (2016) 64(3):797–813. doi: 10.1002/hep.28655
52. Nan J, Xing YF, Hu B, Tang JX, Dong HM, He YM, et al. Endoplasmic Reticulum Stress Induced Lox-1(+) Cd15(+) Polymorphonuclear Myeloid-Derived Suppressor Cells in Hepatocellular Carcinoma. *Immunology* (2018) 154(1):144–55. doi: 10.1111/imm.12876
53. Kondo Y, Shimosegawa T. Significant Roles of Regulatory T Cells and Myeloid Derived Suppressor Cells in Hepatitis B Virus Persistent Infection and Hepatitis B Virus-Related Hccs. *Int J Mol Sci* (2015) 16(2):3307–22. doi: 10.3390/ijms16023307
54. Fu Y, Liu S, Zeng S, Shen H. From Bench to Bed: The Tumor Immune Microenvironment and Current Immunotherapeutic Strategies for Hepatocellular Carcinoma. *J Exp Clin Cancer Res* (2019) 38(1):396. doi: 10.1186/s13046-019-1396-4
55. Hoechst B, Voigtlaender T, Ormandy L, Gamrekelashvili J, Zhao F, Wedemeyer H, et al. Myeloid Derived Suppressor Cells Inhibit Natural Killer Cells in Patients With Hepatocellular Carcinoma *Via* the Nkp30 Receptor. *Hepatology* (2009) 50(3):799–807. doi: 10.1002/hep.23054
56. Kuang DM, Zhao Q, Peng C, Xu J, Zhang JP, Wu C, et al. Activated Monocytes in Peritumoral Stroma of Hepatocellular Carcinoma Foster Immune Privilege and Disease Progression Through Pd-L1. *J Exp Med* (2009) 206(6):1327–37. doi: 10.1084/jem.20082173
57. Noy R, Pollard JW. Tumor-Associated Macrophages: From Mechanisms to Therapy. *Immunity* (2014) 41(1):49–61. doi: 10.1016/j.immuni.2014.06.010
58. Qian BZ, Pollard JW. Macrophage Diversity Enhances Tumor Progression and Metastasis. *Cell* (2010) 141(1):39–51. doi: 10.1016/j.cell.2010.03.014
59. Wan S, Kuo N, Kryczek I, Zou W, Welling TH. Myeloid Cells in Hepatocellular Carcinoma. *Hepatology* (2015) 62(4):1304–12. doi: 10.1002/hep.27867
60. Kuang DM, Peng C, Zhao Q, Wu Y, Chen MS, Zheng L. Activated Monocytes in Peritumoral Stroma of Hepatocellular Carcinoma Promote Expansion of Memory T Helper 17 Cells. *Hepatology* (2010) 51(1):154–64. doi: 10.1002/hep.23291
61. Yan W, Liu X, Ma H, Zhang H, Song X, Gao L, et al. Tim-3 Fosters Hcc Development by Enhancing Tgf-Beta-Mediated Alternative Activation of Macrophages. *Gut* (2015) 64(10):1593–604. doi: 10.1136/gutjnl-2014-307671
62. Ye LY, Chen W, Bai XL, Xu XY, Zhang Q, Xia XF, et al. Hypoxia-Induced Epithelial-To-Mesenchymal Transition in Hepatocellular Carcinoma Induces an Immunosuppressive Tumor Microenvironment to Promote Metastasis. *Cancer Res* (2016) 76(4):818–30. doi: 10.1158/0008-5472.CAN-15-0977
63. Guidotti LG, Inverso D, Sironi L, Di Lucia P, Fioravanti J, Ganzer L, et al. Immunosurveillance of the Liver by Intravascular Effector Cd8(+) T Cells. *Cell* (2015) 161(3):486–500. doi: 10.1016/j.cell.2015.03.005
64. Wang X, He Q, Shen H, Xia A, Tian W, Yu W, et al. Tox Promotes the Exhaustion of Antitumor Cd8(+) T Cells by Preventing Pd1 Degradation in Hepatocellular Carcinoma. *J Hepatol* (2019) 71(4):731–41. doi: 10.1016/j.jhep.2019.05.015
65. Chambers CA, Kuhns MS, Egen JG, Allison JP. Ctla-4-Mediated Inhibition in Regulation of T Cell Responses: Mechanisms and Manipulation in Tumor Immunotherapy. *Annu Rev Immunol* (2001) 19:565–94. doi: 10.1146/annurev.immunol.19.1.565
66. Anderson AC. Tim-3: An Emerging Target in the Cancer Immunotherapy Landscape. *Cancer Immunol Res* (2014) 2(5):393–8. doi: 10.1158/2326-6066.CIR-14-0039
67. Grosso JF, Kelleher CC, Harris TJ, Maris CH, Hipkiss EL, De Marzo A, et al. Lag-3 Regulates Cd8+ T Cell Accumulation and Effector Function in Murine Self- and Tumor-Tolerance Systems. *J Clin Invest* (2007) 117(11):3383–92. doi: 10.1172/JCI31184
68. Hasmim M, Messai Y, Ziani L, Thiery J, Bouhris JH, Noman MZ, et al. Critical Role of Tumor Microenvironment in Shaping Nk Cell Functions: Implication of Hypoxic Stress. *Front Immunol* (2015) 6:482. doi: 10.3389/fimmu.2015.00482
69. Shi FD, Ljunggren HG, La Cava A, Van Kaer L. Organ-Specific Features of Natural Killer Cells. *Nat Rev Immunol* (2011) 11(10):658–71. doi: 10.1038/nri3065
70. Kamimura H, Yamagiwa S, Tsuchiya A, Takamura M, Matsuda Y, Ohkoshi S, et al. Reduced Nkg2d Ligand Expression in Hepatocellular Carcinoma Correlates With Early Recurrence. *J Hepatol* (2012) 56(2):381–8. doi: 10.1016/j.jhep.2011.06.017
71. Vujanovic L, Stahl EC, Pardee AD, Geller DA, Tsung A, Watkins SC, et al. Tumor-Derived Alpha-Fetoprotein Directly Drives Human Natural Killer-Cell Activation and Subsequent Cell Death. *Cancer Immunol Res* (2017) 5(6):493–502. doi: 10.1158/2326-6066.CIR-16-0216
72. Langhans B, Alwan AW, Kramer B, Glassner A, Lutz P, Strassburg CP, et al. Regulatory Cd4+ T Cells Modulate the Interaction Between Nk Cells and Hepatic Stellate Cells by Acting on Either Cell Type. *J Hepatol* (2015) 62(2):398–404. doi: 10.1016/j.jhep.2014.08.038
73. Lee UE, Friedman SL. Mechanisms of Hepatic Fibrogenesis. *Best Pract Res Clin Gastroenterol* (2011) 25(2):195–206. doi: 10.1016/j.bpg.2011.02.005
74. Gabele E, Brenner DA, Rippe RA. Liver Fibrosis: Signals Leading to the Amplification of the Fibrogenic Hepatic Stellate Cell. *Front Biosci* (2003), 69–77. doi: 10.2741/887
75. Baglieri J, Brenner DA, Kisseleva T. The Role of Fibrosis and Liver-Associated Fibroblasts in the Pathogenesis of Hepatocellular Carcinoma. *Int J Mol Sci* (2019) 20(7):1723. doi: 10.3390/ijms20071723
76. Hochst B, Schildberg FA, Sauerborn P, Gabel YA, Gevensleben H, Goltz D, et al. Activated Human Hepatic Stellate Cells Induce Myeloid Derived Suppressor Cells From Peripheral Blood Monocytes in a Cd44-Dependent Fashion. *J Hepatol* (2013) 59(3):528–35. doi: 10.1016/j.jhep.2013.04.033
77. Zhao W, Su W, Kuang P, Zhang L, Liu J, Yin Z, et al. The Role of Hepatic Stellate Cells in the Regulation of T-Cell Function and the Promotion of Hepatocellular Carcinoma. *Int J Oncol* (2012) 41(2):457–64. doi: 10.3892/ijo.2012.1497
78. Estevez J, Chen VL, Podlaha O, Li B, Le A, Vutien P, et al. Differential Serum Cytokine Profiles in Patients With Chronic Hepatitis B, C, and Hepatocellular Carcinoma. *Sci Rep* (2017) 7(1):11867. doi: 10.1038/s41598-017-11975-7
79. Dondeti MF, El-Maadawy EA, Talaat RM. Hepatitis-Related Hepatocellular Carcinoma: Insights Into Cytokine Gene Polymorphisms. *World J Gastroenterol* (2016) 22(30):6800–16. doi: 10.3748/wjg.v22.i30.6800
80. Yi Y, He HW, Wang JX, Cai XY, Li YW, Zhou J, et al. The Functional Impairment of Hcc-Infiltrating Gammadelta T Cells, Partially Mediated by

Regulatory T Cells in a Tgfbeta- and Il-10-Dependent Manner. *J Hepatol* (2013) 58(5):977–83. doi: 10.1016/j.jhep.2012.12.015
81. Zhong M, Zhong C, Cui W, Wang G, Zheng G, Li L, et al. Induction of Tolerogenic Dendritic Cells by Activated Tgf-Beta/Akt/Smad2 Signaling in Rig-I-Deficient Stemness-High Human Liver Cancer Cells. *BMC Cancer* (2019) 19(1):439. doi: 10.1186/s12885-019-5670-9
82. Nagao M, Nakajima Y, Kanehiro H, Hisanaga M, Aomatsu Y, Ko S, et al. The Impact of Interferon Gamma Receptor Expression on the Mechanism of Escape From Host Immune Surveillance in Hepatocellular Carcinoma. *Hepatology* (2000) 32(3):491–500. doi: 10.1053/jhep.2000.16470
83. Faivre S, Rimassa L, Finn RS. Molecular Therapies for Hcc: Looking Outside the Box. *J Hepatol* (2020) 72(2):342–52. doi: 10.1016/j.jhep.2019.09.010
84. Shi F, Shi M, Zeng Z, Qi RZ, Liu ZW, Zhang JY, et al. Pd-1 and Pd-L1 Upregulation Promotes Cd8(+) T-Cell Apoptosis and Postoperative Recurrence in Hepatocellular Carcinoma Patients. *Int J Cancer* (2011) 128 (4):887–96. doi: 10.1002/ijc.25397
85. Wu K, Kryczek I, Chen L, Zou W, Welling TH. Kupffer Cell Suppression of Cd8+ T Cells in Human Hepatocellular Carcinoma Is Mediated by B7-H1/Programmed Death-1 Interactions. *Cancer Res* (2009) 69(20):8067–75. doi: 10.1158/0008-5472.CAN-09-0901
86. El-Khoueiry AB, Sangro B, Yau T, Crocenzi TS, Kudo M, Hsu C, et al. Nivolumab in Patients With Advanced Hepatocellular Carcinoma (Checkmate 040): An Open-Label, Non-Comparative, Phase 1/2 Dose Escalation and Expansion Trial. *Lancet* (2017) 389(10088):2492–502. doi: 10.1016/s0140-6736(17)31046-2
87. Yau T, Park J-W, Finn RS, Cheng A-L, Mathurin P, Edeline J, et al. Nivolumab Versus Sorafenib in Advanced Hepatocellular Carcinoma (Checkmate 459): A Randomised, Multicentre, Open-Label, Phase 3 Trial. *Lancet Oncol* (2022) 23(1):77–90. doi: 10.1016/S1470-2045(21)00604-5
88. Zhu AX, Finn RS, Edeline J, Cattan S, Ogasawara S, Palmer D, et al. Pembrolizumab in Patients With Advanced Hepatocellular Carcinoma Previously Treated With Sorafenib (Keynote-224): A Non-Randomised, Open-Label Phase 2 Trial. *Lancet Oncol* (2018) 19(7):940–52. doi: 10.1016/S1470-2045(18)30351-6
89. Kelley RK, Sangro B, Harris W, Ikeda M, Okusaka T, Kang Y-K, et al. Safety, Efficacy, and Pharmacodynamics of Tremelimumab Plus Durvalumab for Patients With Unresectable Hepatocellular Carcinoma: Randomized Expansion of a Phase I/Ii Study. *J Clin Oncol* (2021) 39(27):2991–3001. doi: 10.1200/JCO.20.03555
90. Lee DW, Cho EJ, Lee JH, Yu SJ, Kim YJ, Yoon JH, et al. Phase Ii Study of Avelumab in Patients With Advanced Hepatocellular Carcinoma Previously Treated With Sorafenib. *Clin Cancer Res* (2021) 27(3):713–8. doi: 10.1158/1078-0432.CCR-20-3094
91. Han Y, Chen Z, Yang Y, Jiang Z, Gu Y, Liu Y, et al. Human Cd14+ Ctla-4+ Regulatory Dendritic Cells Suppress T-Cell Response by Cytotoxic T-Lymphocyte Antigen-4-Dependent Il-10 and Indoleamine-2,3-Dioxygenase Production in Hepatocellular Carcinoma. *Hepatology* (2014) 59(2):567–79. doi: 10.1002/hep.26694
92. Kudo M. Immune Checkpoint Inhibition in Hepatocellular Carcinoma: Basics and Ongoing Clinical Trials. *Oncology* (2017) 92 Suppl 1:50–62. doi: 10.1159/000451016
93. Sangro B, Gomez-Martin C, de la Mata M, Inarrairaegui M, Garralda E, Barrera P, et al. A Clinical Trial of Ctla-4 Blockade With Tremelimumab in Patients With Hepatocellular Carcinoma and Chronic Hepatitis C. *J Hepatol* (2013) 59(1):81–8. doi: 10.1016/j.jhep.2013.02.022
94. Duffy AG, Ulahannan SV, Makorova-Rusher O, Rahma O, Wedemeyer H, Pratt D, et al. Tremelimumab in Combination With Ablation in Patients With Advanced Hepatocellular Carcinoma. *J Hepatol* (2017) 66(3):545–51. doi: 10.1016/j.jhep.2016.10.029
95. Yau T, Kang Y-K, Kim T-Y, El-Khoueiry AB, Santoro A, Sangro B, et al. Efficacy and Safety of Nivolumab Plus Ipilimumab in Patients With Advanced Hepatocellular Carcinoma Previously Treated With Sorafenib. *JAMA Oncol* (2020) 6(11):e204564. doi: 10.1001/jamaoncol.2020.4564
96. Kaseb AO, Hasanov E, Cao HST, Xiao L, Vauthey J-N, Lee SS, et al. Perioperative Nivolumab Monotherapy Versus Nivolumab Plus Ipilimumab in Resectable Hepatocellular Carcinoma: A Randomised, Open-Label, Phase 2 Trial. *Lancet Gastroenterol Hepatol* (2022) 7(7):208–18. doi: 10.1016/S2468-1253(21)00427-1
97. Koyama S, Akbay EA, Li YY, Herter-Sprie GS, Buczkowski KA, Richards WG, et al. Adaptive Resistance to Therapeutic Pd-1 Blockade Is Associated With Upregulation of Alternative Immune Checkpoints. *Nat Commun* (2016) 7:10501. doi: 10.1038/ncomms10501
98. Chu PY, Chan SH. Cure the Incurable? Recent Breakthroughs in Immune Checkpoint Blockade for Hepatocellular Carcinoma. *Cancers (Basel)* (2021) 13(21):5295. doi: 10.3390/cancers13215295
99. Finn RS, Ikeda M, Zhu AX, Sung MW, Baron AD, Kudo M, et al. Phase Ib Study of Lenvatinib Plus Pembrolizumab in Patients With Unresectable Hepatocellular Carcinoma. *J Clin Oncol* (2020) 38(26):2960–70. doi: 10.1200/JCO.20.00808
100. Ho WJ, Zhu Q, Durham J, Popovic A, Xavier S, Leatherman J, et al. Neoadjuvant Cabozantinib and Nivolumab Converts Locally Advanced Hcc Into Resectable Disease With Enhanced Antitumor Immunity. *Nat Cancer* (2021) 2(9):891–903. doi: 10.1038/s43018-021-00234-4
101. Kelley RK, Oliver JW, Hazra S, Benzaghou F, Yau T, Cheng A-L, et al. Cabozantinib in Combination With Atezolizumab Versus Sorafenib in Treatment-Naive Advanced Hepatocellular Carcinoma: Cosmic-312 Phase Iii Study Design. *Future Oncol* (2020) 16(21):1525–36. doi: 10.2217/fon-2020-0283
102. Ren Z, Xu J, Bai Y, Xu A, Cang S, Du C, et al. Sintilimab Plus a Bevacizumab Biosimilar (Ibi305) Versus Sorafenib in Unresectable Hepatocellular Carcinoma (Orient-32): A Randomised, Open-Label, Phase 2–3 Study. *Lancet Oncol* (2021) 22(7):977–90. doi: 10.1016/s1470-2045(21)00252-7
103. Lee MS, Ryoo B-Y, Hsu C-H, Numata K, Stein S, Verret W, et al. Atezolizumab With or Without Bevacizumab in Unresectable Hepatocellular Carcinoma (Go30140): An Open-Label, Multicentre, Phase 1b Study. *Lancet Oncol* (2020) 21(6):808–20. doi: 10.1016/s1470-2045(20)30156-x
104. Finn RS, Qin S, Ikeda M, Galle PR, Ducreux M, Kim TY, et al. Atezolizumab Plus Bevacizumab in Unresectable Hepatocellular Carcinoma. *N Engl J Med* (2020) 382(20):1894–905. doi: 10.1056/NEJMoa1915745
105. Huang A, Yang XR, Chung WY, Dennison AR, Zhou J. Targeted Therapy for Hepatocellular Carcinoma. *Signal Transduct Target Ther* (2020) 5(1):146. doi: 10.1038/s41392-020-00264-x
106. Rizzo A, Ricci AD. Pd-L1, Tmb, and Other Potential Predictors of Response to Immunotherapy for Hepatocellular Carcinoma: How Can They Assist Drug Clinical Trials? *Expert Opin Investig Drugs* (2022) 31(4):415–23. doi: 10.1080/13543784.2021.1972969
107. Nishina S, Hino K. Cd26/Dpp4 as a Therapeutic Target in Nonalcoholic Steatohepatitis Associated Hepatocellular Carcinoma. *Cancers (Basel)* (2022) 14(2):454. doi: 10.3390/cancers14020454
108. McGranahan N, Swanton C. Clonal Heterogeneity and Tumor Evolution: Past, Present, and the Future. *Cell* (2017) 168(4):613–28. doi: 10.1016/j.cell.2017.01.018
109. Kurebayashi Y, Ojima H, Tsujikawa H, Kubota N, Maehara J, Abe Y, et al. Landscape of Immune Microenvironment in Hepatocellular Carcinoma and Its Additional Impact on Histological and Molecular Classification. *Hepatology* (2018) 68(3):1025–41. doi: 10.1002/hep.29904
110. Zhang Q, Lou Y, Yang J, Wang J, Feng J, Zhao Y, et al. Integrated Multiomic Analysis Reveals Comprehensive Tumour Heterogeneity and Novel Immunophenotypic Classification in Hepatocellular Carcinomas. *Gut* (2019) 68(11):2019–31. doi: 10.1136/gutjnl-2019-318912
111. Lin Z, Lu D, Wei X, Wang J, Xu X. Heterogeneous Responses in Hepatocellular Carcinoma: The Achilles Heel of Immune Checkpoint Inhibitors. *Am J Cancer Res* (2020) 10(4):1085–102.
112. Atwa SM, Odenthal M, El Tayebi HM. Genetic Heterogeneity, Therapeutic Hurdle Confronting Sorafenib and Immune Checkpoint Inhibitors in Hepatocellular Carcinoma. *Cancers (Basel)* (2021) 13(17):4343. doi: 10.3390/cancers13174343
113. Harding JJ, Nandakumar S, Armenia J, Khalil DN, Albano M, Ly M, et al. Prospective Genotyping of Hepatocellular Carcinoma: Clinical Implications of Next-Generation Sequencing for Matching Patients to Targeted and Immune Therapies. *Clin Cancer Res* (2019) 25(7):2116–26. doi: 10.1158/1078-0432.CCR-18-2293
114. Kamphorst AO, Wieland A, Nasti T, Yang S, Zhang R, Barber DL, et al. Rescue of Exhausted Cd8 T Cells by Pd-1-Targeted Therapies Is Cd28-Dependent. *Science* (2017) 355(6332):1423–7. doi: 10.1126/science.aaf0683

115. Kudo M, Finn RS, Qin S, Han KH, Ikeda K, Piscaglia F, et al. Lenvatinib Versus Sorafenib in First-Line Treatment of Patients With Unresectable Hepatocellular Carcinoma: A Randomised Phase 3 Non-Inferiority Trial. *Lancet* (2018) 391(10126):1163–73. doi: 10.1016/S0140-6736(18)30207-1
116. Dolladille C, Ederhy S, Sassier M, Cautela J, Thuny F, Cohen AA, et al. Immune Checkpoint Inhibitor Rechallenge After Immune-Related Adverse Events in Patients With Cancer. *JAMA Oncol* (2020) 6(6):865–71. doi: 10.1001/jamaoncol.2020.0726
117. Sangro B, Chan SL, Meyer T, Reig M, El-Khoueiry A, Galle PR. Diagnosis and Management of Toxicities of Immune Checkpoint Inhibitors in Hepatocellular Carcinoma. *J Hepatol* (2020) 72(2):320–41. doi: 10.1016/j.jhep.2019.10.021
118. Liu Y, Wang H, Deng J, Sun C, He Y, Zhou C. Toxicity of Tumor Immune Checkpoint Inhibitors-More Attention Should Be Paid. *Transl Lung Cancer Res* (2019) 8(6):1125–33. doi: 10.21037/tlcr.2019.11.26

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Behavioral factors to modulate immunotherapy efficacy in cancer

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Immune checkpoint inhibitors, including anti-PD-1 and anti-CTLA-4 therapies, are used to (re)activate the immune system to treat cancer. Despite promising results, a large group of patients does not respond to checkpoint inhibition. In the vulnerability-stress model of behavioral medicine, behavioral factors, such as stress, exercise and classical pharmacological conditioning, predict cancer incidence, recurrence and the efficacy of conventional cancer treatments. Given the important role of the immune system in these processes, certain behavior may be promising to complement immune checkpoint inhibition therapy. Here, we discuss the preliminary evidence and suitability of three behavioral mechanisms, i.e. stress modulation, exercise and classical pharmacological conditioning for the benefit of immunotherapy. It is crucial to study the potential beneficial effects of behavioral strategies that support immunotherapeutic anti-tumor effects with rigorous experimental evidence, to exploit behavioral mechanisms in improving checkpoint inhibition efficacy.

KEYWORDS

behavioral medicine, cancer, immune checkpoint inhibition, exercise, stress, classical pharmacological conditioning

Introduction

Immune responses are the collective of biological processes aiming to protect an organism from pathogens, like bacteria, viruses and parasites (1). In addition, the immune system plays a pivotal role in limiting cancer formation through dedicated immunosurveillance mechanisms (2). Recent developments in immunotherapy have caused a revolution in the treatment of a number of malignancies, often drastically

improving disease outcome (3). In particular, immune checkpoint inhibition (ICI) can be applied to target key suppressors of the immune system in order to treat cancer (4). By blocking the interaction between tumor cells and immune checkpoints on T cells, such as CTLA-4 or PD-1/PDL1, the break on T cell inhibition is released, enabling activation, proliferation and the release of cytotoxins such as perforin and granzymes that eventually lead to apoptosis of tumor cells (5–7). Despite promising results of ICI, immunotherapy is currently applicable to only a small proportion of cancers, of which only a limited number of patients respond (8–10). In addition, ICI therapy has several severe side effects associated to autoimmunity (11). Therefore, patients and healthcare at large would benefit from strategies to improve the efficacy of these treatments (3, 12–14).

Behavior, and consequently behavioral interventions, have been shown to broadly affect the immune system (15–26), and as such may help to improve therapeutic efficacy. Previous studies have shown that behavioral therapies improved quality of life and energy levels in patients receiving chemotherapy, radiotherapy or hormonal therapy (27–33). Also, preclinical studies have shown effects of behavioral therapies on clinical outcomes such as tumor growth, which could be partially mediated by the immune system (34, 35). Hence, we argue that the application of behavioral therapeutic approaches is especially relevant for immunotherapy. Nonetheless, research on behavioral interventions in relation to immunotherapy is scarce, as opposed to more conventional cancer therapies. While many pre-clinical behavioral interventions appear to benefit anti-cancer treatment effectivity, only few directly influence the immune system and therefore may serve as complementary to checkpoint inhibition treatment. Based on the vulnerability-stress model (36), we propose three behavioral applications in immune checkpoint therapy: management of the stress response, exercise, and classical pharmacological conditioning (Figure 1). Modulation of these behaviors can directly impact cancer development, progression or survival.

Here, we will first review the behavioral factors affecting immune responses during cancer progression and treatment. Next, we discuss the potential benefits of behavioral interventions to support checkpoint inhibitor therapies.

Immune responses can incite or restrain cancer

The human immune system, often described as innate and adaptive immune responses (1, 38), is essential for the host's survival by offering protection against pathogens through, for instance, cytokines, lymphocytes and antibodies. Innate immune responses recognize abnormal cell surface molecules, applicable to a broad group of pathogens, like viruses and bacteria, but, importantly, also tumor cells. The main cellular actors of the innate immune response, such as natural killer (NK) cells, and phagocytes, do not require a previous encounter to elicit responses. In contrast, adaptive immunity requires an initial encounter with an agent to mount an enhanced counterattack upon future encounters, providing a long-term immunological memory of specific pathogens. Adaptive immune responses consist of specialized lymphocytes, like T-lymphocytes (T cells). T cells, subdivided into the $CD4^+$ T (helper) cells and $CD8^+$ T (killer) cells, recognize peptide antigens that are presented on the cell surface *via* MHC molecules (3). T helper or killer cells respectively produce immune modulating cytokines or directly kill pathogenic cells by secreting perforin and granzymes (6). Perforin translocates to the target cell and binds to its cell membrane to cause pore formation (39). The pores allow diffusion of the granzymes into the target cell, activating cell death (39), and thereby complementing the innate immune system in clearing infected or tumor cells.

As a result of ongoing immunosurveillance the immune system can influence tumor onset, growth and therapy (2, 40). Paradoxically, immune responses may unintendedly shift from being tumor suppressive to supportive, for instance through inflammation. Acute and chronic inflammation both have distinct cellular profiles (41). Acute inflammation is characterized by a high presence of neutrophils, whereas chronic inflammation is featured by the presence of macrophages and lymphocytes (42). Both types of inflammation display inappropriate (dis)engagement of the immune system resulting in tissue remodeling and destruction, and even DNA alterations due to oxidative stress (43). As such, inflammation may also become uncontrolled, predisposing to tumorigenesis (44). Several cancer types, such as colorectal-, liver-, stomach- and bladder cancer may arise from sites of infection or chronic inflammation (45). In these tumors, albeit not exclusively, cancer cells engage with immune cells into an inflammatory tumor microenvironment, a prerequisite for most tumors (46). In inflammatory bowel disease, cancers are found predominantly at the sites of inflammation and chronic intake of anti-inflammatory medications has been shown to decrease the incidence of these cancers (47, 48), by putting a halt to the continuous recruitment of inflammatory cells that destroy the homeostasis of local tissues. During inflammation, the immune system releases reactive oxygen and nitrogen species (ROS and RNS), thereby causing DNA damage in proliferating cells (49). Oxidation is the most abundant type of DNA damage and is also able to inactivate DNA in a non-specific way, leading to accumulation of DNA lesions, genomic instability and cancer (50). An inflammatory microenvironment is not only essential to tumor onset, but also to tumor progression by

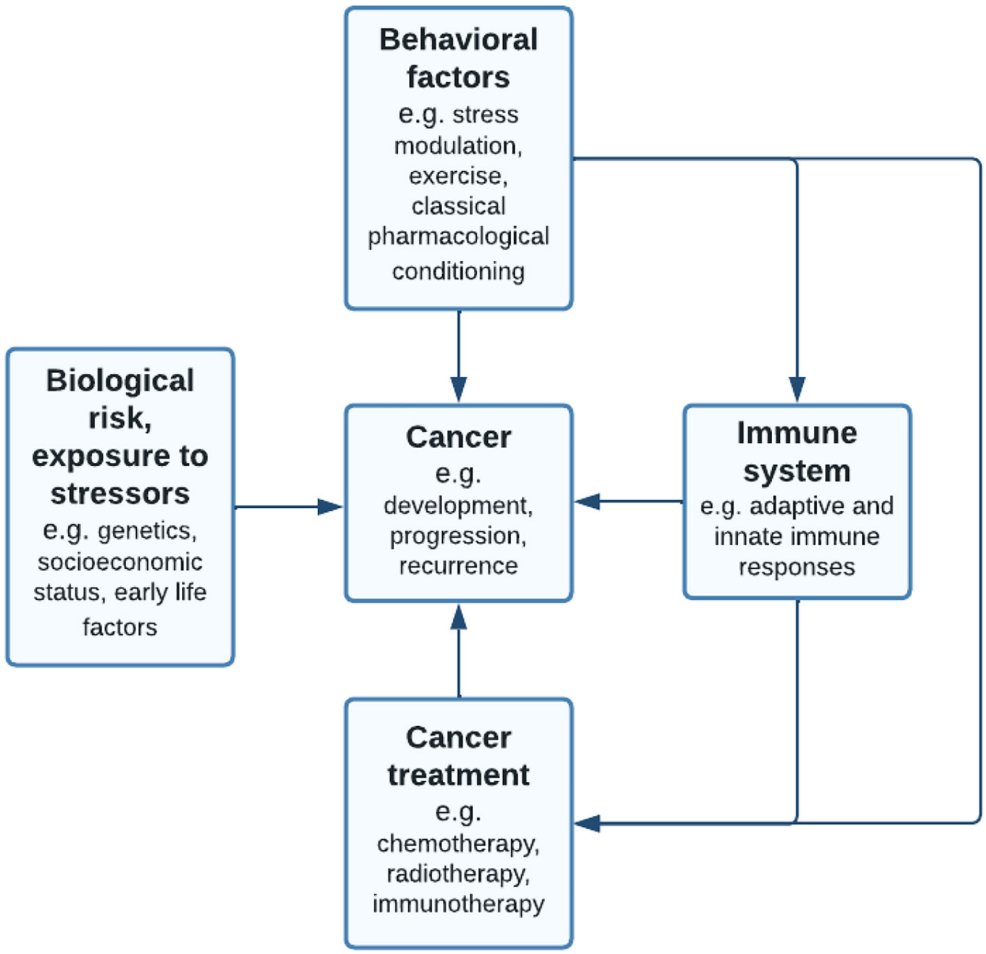

FIGURE 1
Adapted vulnerability-stress model (36): pathways linking behavioral factors to cancer. The behavioral medicine (37) model suggests that while individuals inherit biological risks such as genetic predisposition to cancer, this vulnerability requires interaction with stressors such as chronic traumatic events. Next to this, lifestyle behavior or behavioral interventions may influence cancer development, progression or recurrence, mediated by the immune system. Behavioral factors such as stress modulation, exercise and classical pharmacological conditioning are suggested to influence both conventional cancer treatments, such as chemotherapy, as well as immune checkpoint inhibition therapies.

sustaining tumor cell proliferation. This can be exerted by for instance tumor-associated macrophages with an M2-like profile (51), releasing angiogenic factors enhancing vascularization and thus promoting tumor growth (52). Subsequently, inflammation can also play a major role in the prognosis and treatment of cancer. For example, cancer therapy can trigger inflammatory responses by causing trauma and tissue injury, thereby stimulating tumor re-emergence and resistance to therapy (53). Hence, the use of anti-inflammatory agents, such as non-steroidal anti-inflammatory drugs, can be beneficial for the prognosis of patients (54, 55).

Behavior modulates immune responses

Behavior and the immune system are interrelated. Exemplary is the behavioral immune system, which is the psychological mechanism that allow individuals to detect parasites or pathogens in their environment and avoid contact with the objects or individuals carrying them (56, 57). The behavioral immune system aims to avert infections through preventive behavior; therefore, behavior directly influences the immune system. Next to this, the immune system is responsive to behavioral factors such as stressors, exercise, and classical

pharmacological conditioning. Those behavioral factors may modulate immune responses singularly as delineated below, however, they may also influence each other, e.g. exercise can reduce psychosocial stress (58).

Both physiological stress and psychosocial stress, i.e. exposure to a physical or social stressor like pain or social exclusion respectively, evoke a physiological stress response (59). Being confronted with stressors modulates the immune system by triggering the fight-or-flight response, which is a physiological reaction to a perceived harmful event (60). The physiological stress response depends on several factors, such as the stressor itself (e.g. duration), the host (e.g. age) and external factors (e.g. the environment) (61). Stressor perception induce the secretion of stress-related molecules, such as catecholamines and cortisol *via* the sympathetic nervous system or the hypothalamic-pituitary-adrenocortical (HPA) axis. Sympathetic fibers descend from the brain into lymphoid tissues, for example the thymus and the spleen, which release different substances that can bind to white blood cells, e.g. (nor) epinephrine (62). The HPA axis releases adrenal hormones that bind to white blood cells, regulating their distribution and function (62). In addition, managing stressful events may be demanding for individuals, leading them to engage in maladaptive behaviors as alcohol abuse or changes in sleeping patterns, which can modify immune system processes. Over 300 studies have investigated the different psychological challenges capable of modifying features of the immune responses, illustrating that the more a stressor becomes chronic, the more the immune system is compromised (62).

Analogously, a bout of exercise may lead to mobilization of different immune cells, including leukocytes, increased T cell activity and increased immune activity in general (63–65). Exercise is defined as physical activity that is planned, structured, repetitive and purposeful to improve or maintain physical fitness or health (66). It is generally accepted that prolonged exhaustive exercise training can depress immunity, while regular moderate intensity exercise is beneficial (67). The latter is illustrated by for instance decreased biomarkers of inflammation (for example c-reactive protein) in physically active as opposed to sedentary individuals (68).

Classical pharmacological conditioning is the third example of a behavioral mechanism that has been shown to influence the immune system. Classical conditioning is a learning process in which an initially neutral stimulus elicits a learned physiological response through repeated pairing of the stimulus and the physiological response. Ivan Pavlov first discovered these learned reflexes in 1927 (69) by training dogs to salivate at the presentation of a conditioned stimulus: the sound of a bell. Conditioning or learning is relevant for any human behavior and is therefore applied broadly, for example in psychoanalysis focused on social behavior. Here, we refer to the pharmacological form of classical conditioning, which is an instrumental learning paradigm that uses a medication as an unconditioned stimulus of which the physiological response is mimicked in response to a conditioned stimulus. This learning paradigm has later been applied to immune responses, in which an immune modulating medication is used as the physiological reaction, resulting in reduced immune medication dosages and maintained treatment efficacy in response to a stimulus (15). For instance, renal transplant patients, who received immunosuppressive treatment, were treated with a learned immunosuppressive placebo response, that was linked to a gustatory (conditioned) stimulus (70). When re-exposed to the conditioned stimulus, the T cell proliferative capacity was reduced in comparison to T cell functions under routine drug intake. Thus, classical pharmacological conditioning increased the medication efficacy.

Behavioral factors associated to cancer onset

Numerous behavioral factors, of which stressors, exercise and conditioning are three examples, have been associated with the onset of cancer (71–74). According to the vulnerability-stress model, vulnerabilities (e.g. genetics) and stressors (e.g. life events) lead to certain behaviors (e.g. lifestyle) and physiological responses (e.g. immune responses) and can influence disease and clinical outcomes (36). Here, we focus on the above-mentioned behavioral factors that can be targeted in behavioral interventions and are exemplary of a spectrum of analogous behaviors. Long lasting stressors or a lack of physical exercise can substantially reduce tumor growth as shown in both epidemiological and in animal models, possibly mediated by immune cell modulation (34, 35). Classical pharmacological conditioning may be used to assist cancer treatment.

Stressors

Confrontation with stressors can affect the immune system and increase cancer occurrence in human (75). The immune system mediates the relationship between stressors and cancer occurrence (76). Exposure to stressors is found to be accompanied by pro-inflammatory responses in animal and human research (77), which may stimulate tumor growth. Furthermore, stress related molecules, such as cortisol or the catecholamines (nor)epinephrine, can regulate diverse signaling pathways through their specific receptors that enhance the proliferative and invasive abilities of cancer cells in relation with the tumor microenvironment (78, 79). Cortisol or glucocorticoids have a pivotal role in regulating stress reactivity of organ systems (80) through glucocorticoid-receptor-mediated modulation of target genes (34). Glucocorticoids can activate survival genes that protect cancer cells from the effects of chemotherapy (81), and were shown to influence virus activation

including human papillomaviruses and other cancer-associated viruses (34). Instead, to counteract the stimulating effects of (nor) epinephrine on tumor growth, the administration of beta blockers that interfere with the physiological stress response was associated with a lower incidence of prostate cancer in a population based study (82).

The influence of stressors on cancer incidence is also hypothesized to be moderated by socioeconomic status. Disparities in socioeconomic status are associated to inequalities in behavioral factors such as physical inactivity, obesity, smoking, diet, alcohol and drug use, screening and treatment uptake (83). These health-impairing behaviors are thought to be stress-related behaviors and a lower socioeconomic status has been associated with higher levels of distress (84). Higher cancer occurrence is found in groups with lower socioeconomic status (83, 85). Even though confrontation with long lasting stressors may be one of the factors that plays a role in these processes, research should detangle the mechanisms with which stress modulates cancer occurrence and therapy response.

Exercise

In humans, pooled analyses of epidemiological studies showed that more physical activity during leisure time was associated with a decreased risk of 10 different type of cancers, independent of body mass index (BMI) (86). Overall, physical activity is associated with a 7%-20% lower cancer risk in individuals, with the strongest impact on colorectal and breast cancer (74, 87). One of the mechanisms by which physical activity may reduce risk of cancer occurrence is a reduction in chronic, low-grade inflammation and improved immune surveillance and function (88, 89). Cumulative evidence of both animal and humans studies shows that exercise modulates local and systemic inflammatory processes by altering both the number and function of circulating cells of the innate immune system (neutrophils, monocytes and NK cells), and of the adaptive immune system (T and B cells) (89). Exercise may also reduce the visceral fat mass, which is accompanied by less adipokine secretion and less macrophage infiltration into the adipose tissue, thereby reducing inflammation (90). Exercise also activates the HPA axis, initiating cortisol release, which in the case of exercise can contribute to an effective anti-inflammatory systemic host environment by downregulating cytokines as tumor necrosis factor (TNF)-α. The aforementioned physiological events activated by exercise are only examples among many other processes that have been detailed in a number of reviews (89–91). For example, exercise is accompanied by a higher level of catecholamines, such as (nor)epinephrine, which were related to similar anti-inflammatory effects as cortisol (90).

Classical pharmacological conditioning

Different from psychosocial stress or exercise, which can also be part of lifestyle, classical pharmacological conditioning is always an intervention and, therefore, there are no epidemiological studies investigating this behavioral mechanism in relation to cancer onset.

Behavioral factors enhance conventional cancer treatments

The behavior factors that we specified in our model have shown to influence conventional cancer treatments, such as chemotherapy, radiotherapy, and hormonal treatment. The immune system is one of the main links thought to connect behavioral factors to cancer therapy (92).

Stress modulation in conventional cancer therapies

There are indications that pharmacological stress modulation can improve cancer progression. The physiological stress response seems to drive therapeutic resistance in murine tumor models (93, 94). The cellular and molecular microenvironment of cancer includes (peripheral) nerves that can modulate behavior or malignant cells, promoting tumor growth and illustrating the cross-talk between the neuroimmune system and cancer progression (35). Regulation of the tumor microenvironment by the sympathetic nervous system has been demonstrated in animal studies (95). Intratumoral neurotransmitters and neuropeptides have regulatory roles in the physiological and pathological functions of tissues, and emerging data suggest that cancer cells may take advantage of neurotransmitters-initiated signaling pathways to activate uncontrolled proliferation (96). For example, norepinephrine and epinephrine activate β-adrenoreceptors expressed on both cancer and immune cells thereby promoting growth of malignancies and inflammation. Moreover, these catecholamines can induce an endothelial cell metabolic switch mediated by β-adrenoreceptors resulting in increased tumor vascularization (96). The β-adrenergic pathway may be suppressed by beta blockers and as an example, it was shown that propranolol, a medication of the β-blocker class, was used to complement the treatment of several types of cancer, directly blocking cancer cell proliferation induced by epinephrine *in vitro* (97). This experimental evidence is supported by clinical studies that combine propranolol with other agents to stop metastasis (98), and epidemiological evidence showing that of 24,238 patients, the 12,119 propranolol users (for over six months) had lower risk of head and neck, esophagus, stomach,

colon and prostate cancers (99). On the other hand, preclinical administration of dexamethasone, a synthetic glucocorticoid, induced chemotherapy and resistance in breast cancer, as well as *in vitro* tumor samples and cancer cell lines (100–102). This evidence underlines the importance of the HPA-pathway modulation in conventional cancer treatments.

While psychological interventions seem to influence immunity (103), evidence in relation with cancer remains very limited. Studies using psychological interventions in patients with cancer often did not assess treatment efficacy or health-related quality of life (104). The results of interventions on psychological wellbeing, such as cognitive behavioral therapy, in cancer treatment are variable finding mostly effects on outcomes such as anxiety, and fatigue (105). However, these interventions, e.g. cognitive behavioral therapy and mindfulness, cannot be tested in preclinical models.

Exercise and conventional cancer therapies

There is both pre-clinical and clinical evidence for a relation between exercise and the immune system and effectiveness of exercise during chemotherapy or radiotherapy (16–26). In patients, exercise has been associated with reduced side effects of cancer and its treatment (27, 28). Thereby, exercise improved the physical and mental health and the overall self-reported quality of life of patients (27–29). Patient-reported outcome measures were complemented by immunological readouts, including the number of NK cells, expression of IL-6, or TNF-α production (16–26). In observational and randomized controlled exercise trials - both increases and decreases in defined immune markers were reported. For example, immune markers of NK cells differed: exercise had an inhibitory effect on the absolute number of NK cells in patients with breast cancer (106), an augmenting effect on NK cells percentages in patients with lung cancer (107), whereas no effect in another cohort of patients with breast cancer and other solid tumors was observed (17, 22). Comparably, IL-6 expression differed after exercise interventions, showing either a decrease in some studies (24–26), but no effects (17, 20, 23, 108), or an increase in other studies (16, 18, 109). Given the diverse effects that exercise has on the number of immune cells in patients with cancer, for instance in NK cells, it has been suggested that exercise may instead affect the cytotoxic activity of the immune cells, mirroring the effects exercise has on healthy individuals through inflammatory response pathways (110, 111). The large variation in the type of exercise interventions ranging from aerobic to resistance training may explain differences in exercise responses (26, 106, 107).

To date, most causal evidence of exercise on the anti-tumor efficacy of cancer treatment comes from animal studies (112, 113). A large advantage of these *in vivo* experiments is that there is little variation in interventions; i.e. most studies examined the effects of voluntary running. These experiments indicate that physical exercise modulates factors that are inherent to cancer treatment sensitivity, including the tumor microenvironment, e.g. hypoxia, tumor cell metabolism and tumor perfusion, next to having profound effects on immune cell populations (112–114). Illustrating these results, voluntary running tumor bearing mice experienced reduced tumor growth in diverse cancer models, e.g. lung cancer or myeloma, and displayed higher NK cell mobilization compared to sedentary control groups (113, 115, 116).

Classical pharmacological conditioning of conventional cancer therapies

Conditioned effects in cancer patients during therapy were shown on outcomes such as nausea and immune modulation (117–120). Patients who were given a beverage prior to adjuvant chemotherapy experienced more nausea at later time points when they were confronted with the beverage alone compared to patients who did not receive a beverage before the therapy (118), and the other way around, e.g. a conditioning paradigm was applied to reduce nausea (120). Similarly, pediatric cancer patients undergoing chemotherapy showed increased natural killer cell activity and interferon-γ levels upon arrival at the hospital, when previously confronted with two cycles of chemotherapy in the hospital (117). These results may be seen as an indication that conditioned effects in cancer patients are possible on immune cell populations, however, the effects on cancer outcomes and therapy response remain to be investigated.

The mechanisms of how the immune system is conditioned are largely unknown. An association between the conditioned stimulus and the immune response needs to be established in the brain and conditioning thus relies on the interaction between the central nervous system and the immune system (15). Some murine studies demonstrated that lesioning of the insular cortex and central nucleus of the amygdala obstructs immunological conditioning, suggesting that these areas mediate conditioning (121, 122). Other murine studies hypothesize that continuous administration of substances, such as antigens, may be disruptive for the hosts' homeostasis, and that conditioning may be a favorable alternative given a decreased substance administration (123). By linking the immune reaction with the central nervous system, it is assumed that the effect of the substance might be achieved without the disruptive effects of the substance (123). Furthermore, it is thought that different pharmacological conditioning paradigms rely on different mechanisms, given that the physiological reaction mimics diverse medication effects (124, 125). Despite little evidence on the pathways of this associative learning process, the conditioning paradigm has

been successfully used in numerous rodent studies of nonconventional therapies, echoing the effects of cyclosporine A, opioids, lipopolysaccharide (LPS), lithium chloride, anti-lymphocyte serum, ovalbumin and bovine serum albumin using taste and odor as conditioned stimuli (126–132).

Immunotherapy may be supported by behavioral factors

Immune suppressive mechanisms in cancer hamper effective immune responses (133). By therapeutically assisting anti-cancer immune responses, tumor growth and progression may be counteracted and cure can be promoted. Promising targets for immunological therapies are immune checkpoint proteins, which are used as a break in the immune system and consequently block over-activation of the immune system preventing autoimmunity (3). In addition, checkpoint signals are required for optimal T-cell recognition and generation of long-lasting T cell memory responses (3). One of the most well-known checkpoint proteins is Cytotoxic T lymphocyte antigen-4, or CTLA-4 (134), which is considered critical for maintenance of T cell homeostasis and tolerance (135). T cell activation requires engagement of the T cell antigen receptor-CD3 complex and ligation of costimulatory receptors, such as CD28, that bind to CD80 (B7.1) and CD86 (B7.2) on antigen-presenting cells. CTLA-4 is transported to the immunologic synapse when there is a potent or long-lasting stimulus (*via* the T cell receptor) (136), and outcompetes binding of CD28 to CD80 and CD86, hereby acting as negative regulator of proliferation and effector function of T cells (137). When tumor cells express ligands (e.g. CD80, CD86) for CTLA-4, T cell activity is inhibited after binding, hereby evading clearance by the immune system (138). Monoclonal antibodies, i.e. checkpoint inhibitors, can block CTLA-4, allowing activation of T cells and killing of tumor cells (7).

Programmed death (PD)-1 is another immune checkpoint molecule involved in regulating the balance between immune activation and tolerance, similar to CTLA-4 (134). Its ligands PD-L1 (B7-H1) and PD-L2 (B7-H2) are expressed on antigen presenting cells, but can be expressed on tumor cells as well, resulting in an immunosuppressive tumor microenvironment. Therefore, also anti-PD-1 or anti-PD-L1 monoclonal antibodies promote T cell-mediated tumor cell death (139, 140).

Immune checkpoint inhibitors (i.e. Ipilimumab (anti-CTLA-4), Nivolumab (anti-PD-1), Pembrolizumab (anti-PD-1), Atezolizumab (anti-PD-L1)) have been approved or are studied in clinical trials to treat multiple types of cancer of which melanoma and lung cancer respond best to therapy. Next to this, there are currently several antibodies and small molecules in development, targeting other immune checkpoints such as TIM3, CD39, B7H3, CD73, LAG3, and more (141–145).

Unfortunately, large groups of patients do not respond to or benefit from immunotherapy (8–10). It is not completely clear yet why some patients respond and others do not, which may have to do with tumor-intrinsic qualities. For instance, it was shown that high microsatellite instability (MSI) results into a high number of mutations and increased number of tumor-infiltrating lymphocytes (146, 147). As such, patients with an MSI-tumor are suitable candidates for immunotherapy. Nonetheless, even in tumors with high MSI observed response rates range between 30% and 50% (147), indicating that there are other factors that come into play besides the genetic and immunologic aspects of the tumor. Similarly, predictive biomarkers, like tumor-cell PD-L1 expression, are used to stratify the immunotherapy responders from the non-responders (148). However, PD-L1 testing alone is insufficient for patient selection in most malignancies and immune responses are not uniform across all malignancies. It is estimated that in the US 38% of patients with cancer are eligible for ICI therapy, given the molecular profile of their tumor, but only up to 11% respond to the ICI therapy (10). The remaining 27% of patients were eligible but did not respond, indicating the necessity for better predictive biomarkers, next to the need of increasing treatment sensitivity. Another drawback of checkpoint inhibition is that it is not cost effective in certain malignancies, with an economic benefit for choosing chemotherapy to treat i.e. recurrent or metastatic head and neck cancers and non-small cell lung cancers (12). Furthermore, both CTLA-4 and PD-1 blockade can have severe immune related autoimmune complications, for example side effects on the gastrointestinal tract, brain, thyroid, lungs and skin (3, 13, 14). In the light of the diverse drawbacks, efforts are needed to improve and support immunotherapy, enhance its anti-cancer effects and decrease the side effects.

Behavioral factors have been shown to influence both the immune system and cancer treatment and may therefore possibly offer opportunities to improve immunotherapy. Given that behavioral factors can influence the effectiveness of conventional chemo- and radiotherapy, and that the immune system is thought to modulate this effect, immunotherapy may offer an ideal opportunity for behavioral intervention (Figure 2).

Physiological stress modulation and immune checkpoint inhibition

Few preliminary studies investigated the impact of stress modulation with pharmacological interventions on checkpoint inhibitors efficacyalthough there are indications that stress influences immunotherapy. For instance, social disruption stress compromised a vaccine based immunotherapy (poly(d,l-lactide-co-glycolide) microsphere) in a murine melanoma model

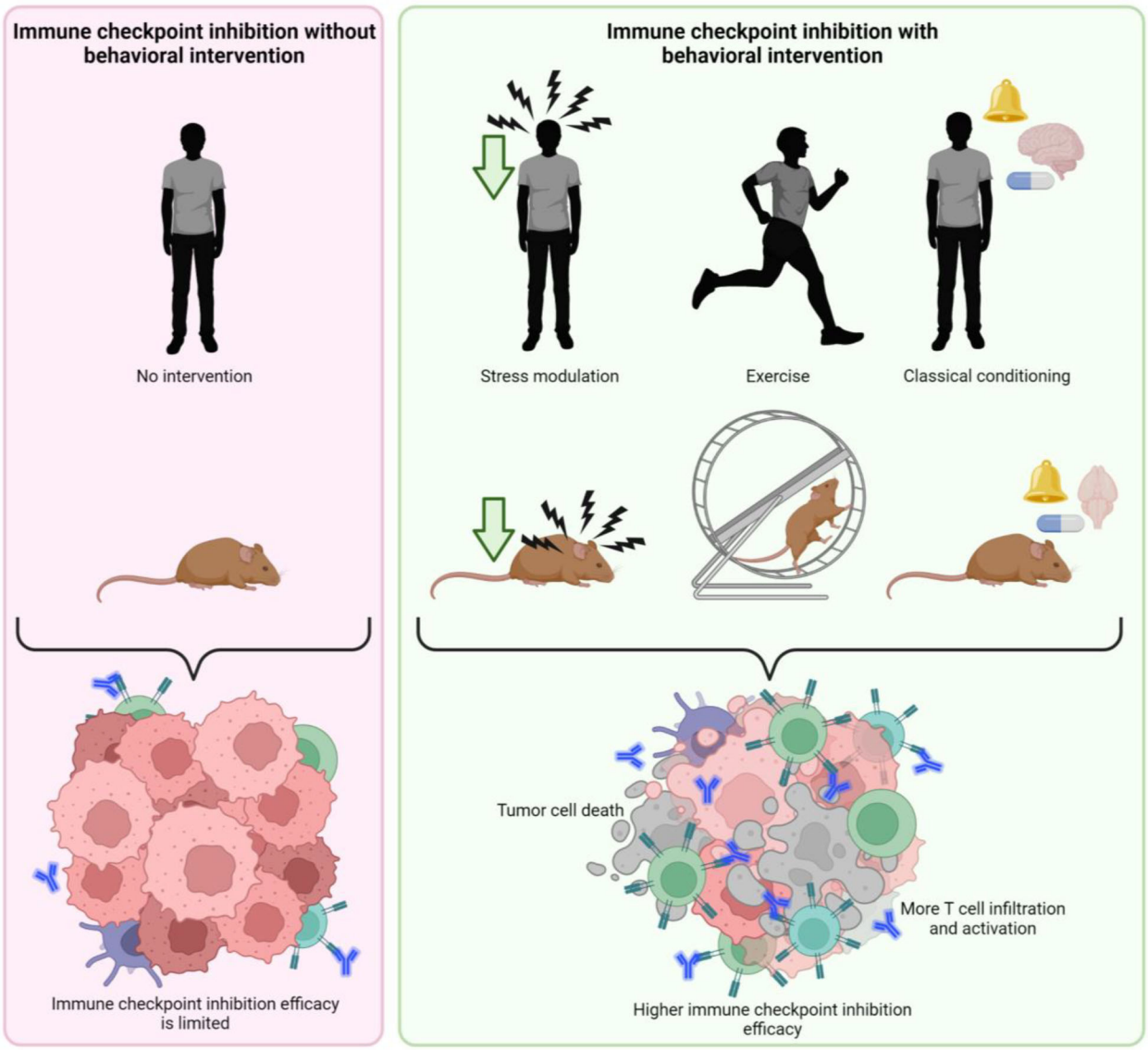

FIGURE 2
Putative effect of behavioral interventions on effectivity of immune checkpoint inhibition therapy. Behavioral interventions such as stress modulation, exercise and classical pharmacological conditioning may enhance the effectivity of immune checkpoint inhibition, promoting tumor cell death by higher T cell infiltration and activation created with BioRender.com.

through impairing $CD8^+$ T cell responses (149). Also, either behavioral stress or surgical stress, weakened the inhibition of metastasis by immunostimulating agents (CpG class C and glucopyranosyl lipid-A stable emulsion) (150, 151). Studies linking stress to cancer immunotherapy assume the involvement of the of HPA-axis, for example by glucocorticoid-induced expression of the immunosuppressive transcription factor TSC22D3 of dendritic cells (152).

With regard to ICI in a retrospective analysis of 109 medical records of non-small lung cancer patients, treated with either ICI therapy or ICI in combination with chemotherapy, the 28 patients who were concomitantly prescribed any beta blocker had a longer progression free survival with a hazard ratio of 0.58 (153). A possible association between beta blocker use and improved progression free survival in non-small-cell lung cancer patients treated with ICI, should however be confirmed in clinical randomized controlled trials. In humans, there are no randomized controlled trials using psychological interventions to complement immune checkpoint inhibition cancer therapy.

An indication of a relation between stress exposure and the efficacy of anti-PD-L1 was found in a mouse study (154). In this study researchers applied daily chronic unpredictable mild stressors, such as water or food deprivation, tail pinching, bodily restraint et cetera, for a total of 28 days. The effect of anti-PD-L1 therapy was attenuated in the stressed groups, coinciding with a decrease in $CD8^+$ lymphocytes and increase of regulatory T cells at tumor sites. In line with this, chronic cold stress strongly reduced anti-PD-1 efficacy in breast cancer and melanoma mouse models (155). An experimental group that experienced cold-induced stress (by being housed in an environment of 22 degrees Celsius instead of the thermoneutral temperature of 30 degrees Celsius) had larger tumors but this effect was counterbalanced by β-blocker treatment. The enhancement of anti-PD-1 efficacy by co-treatment with propranolol treatment was likely to be $CD8^+$ dependent, i.e. there was an increased frequency of effector $CD8^+$ T cells in subcutaneous breast cancer tumors and melanoma. The effects of propranolol treatment did not persist in T cell-deficient mice, suggesting that the β-adrenergic system influences T cell activity (155, 156). Similar results were found in mouse models of fibrosarcoma and colon cancers, where reduced tumor growth as well as enhanced response to anti-CTLA-4 therapy was observed after blocking the β-adrenergic receptor with propranolol (156). Here, propranolol treatment resulted in a reduction of tumor angiogenesis, increased T-cell infiltration, but a decrease in myeloid derived suppressor cells, as well as modifications on tumor associated macrophages, together leading to a tumor-suppressive environment (156). Despite these promising preliminary results, we are unaware of any ongoing clinical trials in humans.

Exercise and immune checkpoint inhibition

Several murine studies investigated the synergistic effects of exercise and immune checkpoint inhibitors on cancer treatment (157–160). Higher tumor necrosis and less apoptosis was found in a patient-derived xenograft model of non-small cell lung carcinoma when anti-PD-1 treatment was combined with exercise, indicating that excise may improve anti-PD-1 effectivity (157). Another study demonstrated that aerobic exercise, i.e. daily 30 minutes treadmill exposure, sensitized pancreatic tumors to anti-PD-1 therapy, which resulted in anti-tumor immunity though IL-15Rα$^+$ $CD8^+$ T cells and decreased tumor growth (160). Two other *in vivo* studies found no synergistic effects of the combination of exercise and immune checkpoint inhibition. However, one study observed an increase in $CD8^+$ T cells in orthotopically implanted breast tumors when anti-PD-1 was combined with voluntary wheel running, as compared to the tumors of mice without voluntary running regime (158, 159). These preliminary preclinical results offer a stepping-stone to translate exercise interventions to the clinic.

Classical pharmacological conditioning and immune checkpoint inhibition

To the best of our knowledge there are no published studies investigating the effects of a learned immune reaction on the efficacy of immune checkpoint inhibition. However, a recent study showed that conditioning of rapamycin-induced immunomodulation reduced tumor growth effectively in a murine glioblastoma model (161). In this study, the mTOR inhibitor Rapamycin was repeatedly paired with a novel gustatory stimulus. The experimental group, receiving only 10% of the initial drug dose together with the gustatory stimulus during the testing phase, showed similar tumor inhibition as the control group receiving 100% of the drug dose. the tumor growth inhibition was driven by a central and peripheral upregulation of pro-inflammatory markers and a decrease in anti-inflammatory cytokines such as IL-10. Similarly, older studies showed that conditioning of immunotherapy was more effective in delaying tumor growth in mice than immunotherapy alone (162, 163). For example, in a syngeneic *in vivo* study, the unconditioned stimulus was the injection of immunostimulating DBA/2 spleen cells, and the conditioned stimulus was camphor odor. When conditioned mice were re-exposed to the odor of camphor only, tumor growth was still delayed compared to non-conditioned mice (162). These experiments suggest that the immune system of the mice consistently mimicked the effect of the immune modulator when presented with the conditioned stimulus, which influenced health outcomes, demonstrating the feasibility of conditioning immune responses (123, 161–167). Therefore, conditioned effects of immunomodulatory inhibitors may be suitable also for immune checkpoint inhibition. Of note, both mechanistic animal studies and controlled human studies in healthy subjects and patients are necessary to understand whether learned immunity is a promising addition to immunotherapy. In patients, the conditioning paradigm could be applied using specific stimuli, for instance combining the use of checkpoint inhibitors with a distinctive stimulus: a taste, sound or smell. This setting may serve for reducing medication dosages, i.e. checkpoint inhibition could be given in reduced quantities or placebo medication could be administered intermittently. Potentially, the use of mechanisms that harness mimicking placebo effects could reduce healthcare costs associated to the high expenses of several medications, including immune checkpoint inhibition.

Several ongoing clinical trials in patients study how behavioral factors may affect the quality of life and other cancer-related outcomes during ICI (Table 1). To the best of our knowledge there are no ongoing clinical trials on stress modulation or classical pharmacological conditioning in checkpoint inhibition, but various studies use exercise as intervention. The results of these studies are yet unknown and most studies focus on feasibility of the intervention as primary outcome measure. Therefore, the mechanisms of behavior on cancer outcomes in patients undergoing immune checkpoint inhibition therapy remain unknown.

Conclusion

Behavioral factors are associated with the onset and therapy response of cancer. Behavioral interventions such as modulation of psychosocial stress, exercise, and classical pharmacological conditioning, have therefore been used to reduce toxicity and

TABLE 1 Ongoing studies researching behavioral factors and immune checkpoint inhibition therapy.

#	Study	Tumor	Intervention	N	Primary (1) and secondary (2) outcomes	Location	ID
1	Exercise to Boost Response to ICI	Cutaneous melanoma, cutaneous squamous cell carcinoma, Merkel cell carcinoma	30 minutes arm ergometer/pedal ergometer/treadmill exercise up to 12 times and 12 months prior to each administration of standard of care checkpoint blockade immunotherapy across all cycles	32	1: Feasibility of the exercise intervention 2: Tumor Immunological Biomarkers	Moffitt Cancer Center Tampa, Florida, USA	NCT05358938
2	Exercise as a Supportive Measure for Patients Undergoing ICI	Melanoma	60 minutes group, machine based resistance and endurance exercise (moderate-to-high-intensity), 2 times a week for 12 weeks	40	1: Feasibility of the exercise intervention 2: Quality of life (EORTC QLQ-C30, version 3.0), fatigue (MFI), sleep Quality (PSQI), depression (CES-D), physical Activity (SQUASH), cardiopulmonary fitness (maximal aerobic capacity (VO_2peak) *via* a maximal incremental cycling test), muscle strength (isometric and isokinetic with the Isomed 2000® diagnostic module), pain (BPI)	Heidelberg University Clinic, Heidelberg, DE	NCT03171064
3	Low-moderate Intensity Pedaling During Immunotherapy Administration	Skin, kidney, bladder cancer	30 minutes on a pedal ergometer (low-moderate intensity) concurrent to ICI infusion for maximum 12 weeks	10	1: Feasibility of pedaling measured by the number of completed pedaling sessions and the ability of patients to meet pedaling intensity goals. 2: Quality of life scores (Quality of Life Questionnaire - Core 30), treatment response biomarkers (checkpoint inhibitors, functional T and B cell subsets, pro and anti-inflammatory monocyte subsets, and soluble inflammatory mediators), CT-derived sarcopenia rates	Rush University Medical Center, Chicago, Illinois, USA	NCT04127318
4	Combined Aerobic and Resistance Exercise Training in Metastatic Renal Cell Carcinoma	Renal cell carcinoma	12 weeks home based, combined aerobic and resistance exercise training plan	16	1: Feasibility of the exercise intervention 2: Change in Health Related Quality of Life (FACT-G), the incidence of grade 3-5 toxicities as per CTCAE 5.0	Johns Hopkins, University/ Sidney Kimmel Cancer Center, Baltimore, Maryland, USA	NCT05103722
5	i-Move	Melanoma	12-week semi-supervised individualized exercises: moderate intensity aerobic exercise (walking and cycling, 20-45 min, 3-5 times a week), resistance training exercises (2-3 times a week) and stretching	30	1: Feasibility of the exercise intervention 2: Fatigue (FACIT F), functioning (PROMIS), symptoms (Edmonton Symptom Assessment Scale), quality of life (SF-36), adherence (Godin Leisure-time Physical Activity Questionnaire), physical fitness and functioning (30 s chair stand test, the 6 min walk test, the arm curl test and the Australia-modified Karnofsky Performance Scale)	Peter MacCallum Cancer Centre, Melbourne, Australia	ACTRN 12619000952145

EORTC QLQ-C30, European Organization for the Research and Treatment of Cancer Quality of Life Questionnaire C30; MFI, Multidimensional Fatigue Inventory; PSQI, Pittsburgh Sleep Quality Index; CES-D, Center for Epidemiological Studies Depression Scale; SQUASH, Short QUestionnaire to ASsess Health-enhancing Physical Activity; BPI, Brief Pain Inventory; FACT-G, Functional Assessment of Cancer Therapy – General; CTCAE 5.0, Common Terminology Criteria for Adverse Events 5.0; FACIT F, Functional assessment of chronic illness therapy-fatigue; PROMIS, Patient-Reported Outcomes Measurement Information System V2.0; SF-36, Short Form 36 Health Survey Questionnaire.

potentially improve conventional cancer therapy outcomes. In Figure 3, we summarize how behavioral factors might affect the individual immune cell types. With the rise of various immunotherapies that counteract the immune suppressing interactions between tumors and the immune system, there are ample opportunities for non-invasive behavioral interventions to improve immunotherapeutic results. Hence, it is of paramount importance to rigorously examine the potential advantageous effects of behaviors that may support tumor cell clearance by the immune system activated by ICI therapy. The efficacy of these behavioral factors remains to be tested both in animal models, to investigate underlying mechanisms, and in patients, to explore their suitability for the benefit of cancer therapy.

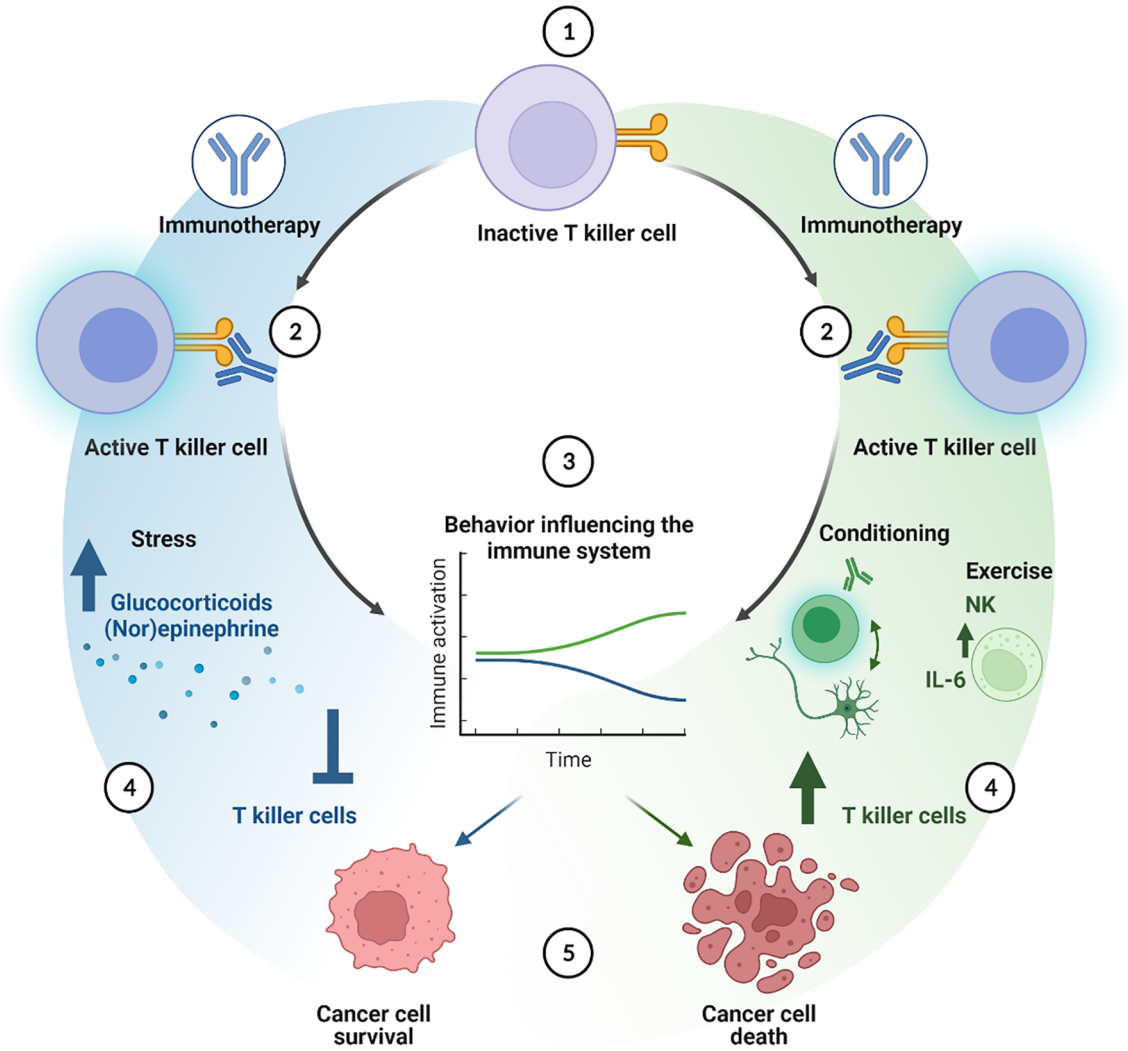

FIGURE 3
Cellular cascade of additive effects of behavioral interventions on immune checkpoint inhibition therapy. Immune checkpoint inhibition activates T killer cells (1 and 2). Behavioral factors or interventions can either improve (green line) or decrease (blue line) the effects of the immune therapy (3). Stress hampers the immune system through for instance the effects of glucocorticoids and (nor)epinephrine, inhibiting T killer cell function and numbers, thereby enhancing cancer cell survival (4 and 5, blue cascade). Classical pharmacological conditioning can enhance T killer cell activity and numbers mediated by brain-immune communication. Exercise may increase the amount of NK cells, induce IL-6 secretion, altogether improving T killer cell activation, leading to a better anti-tumor response (4 and 5, green cascade) created with BioRender.com.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

All authors have made substantial contributions to the conception or design of the work; have drafted and revised the work; and have provided approval for the publication of the content.

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Conflict of interest

LV received consultancy fees from Bayer, MSD, Genentech, Servier and Pierre Fabre, AE received consultancy fees from L'Oreal, but these had no relation to the content of this publication. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Gonzalez H, Hagerling C, Werb Z. Roles of the immune system in cancer: from tumor initiation to metastatic progression. *Genes Dev* (2018) 32(19-20):1267–84. doi: 10.1101/gad.314617.118

2. Stagg J, Johnstone RW, Smyth MJ. From cancer immunosurveillance to cancer immunotherapy. *Immunol Rev* (2007) 220(1):82–101. doi: 10.1111/j.1600-065X.2007.00566.x

3. Waldman AD, Fritz JM, Lenardo MJ. A guide to cancer immunotherapy: from T cell basic science to clinical practice. *Nat Rev Immunol* (2020) 20(11):651–68. doi: 10.1038/s41577-020-0306-5

4. Johnson DB, Nebhan CA, Moslehi JJ, Balko JM. Immune-checkpoint inhibitors: Long-term implications of toxicity. *Nat Rev Clin Oncol* (2022) 19 (4):254–67. doi: 10.1038/s41571-022-00600-w

5. Zhang H, Dai Z, Wu W, Wang Z, Zhang N, Zhang L, et al. Regulatory mechanisms of immune checkpoints PD-L1 and CTLA-4 in cancer. *J Exp Clin Cancer Res* (2021) 40(1):1–22. doi: 10.1186/s13046-021-01987-7

6. Chang H-F, Bzeih H, Chitirala P, Ravichandran K, Sleiman M, Krause E, et al. Preparing the lethal hit: interplay between exo-and endocytic pathways in cytotoxic T lymphocytes. *Cell Mol Life Sci* (2017) 74(3):399–408. doi: 10.1007/s00018-016-2350-7

7. Webb ES, Liu P, Baleeiro R, Lemoine NR, Yuan M, Wang Y. Immune checkpoint inhibitors in cancer therapy. *J Biomed Res* (2018) 32(5):317. doi: 10.7555/JBR.31.20160168

8. Xu Z, Park Y, Liu K, Zhu B. Treating non-responders: pitfalls and implications for cancer immunotherapy trial design. *J Hematol Oncol* (2020) 13 (1):1–11. doi: 10.1186/s13045-020-0847-x

9. Sharma P, Hu-Lieskovan S, Wargo JA, Ribas A. Primary, adaptive, and acquired resistance to cancer immunotherapy. *Cell.* (2017) 168(4):707–23. doi: 10.1016/j.cell.2017.01.017

10. Haslam A, Gill J, Prasad V. Estimation of the percentage of US patients with cancer who are eligible for immune checkpoint inhibitor drugs. *JAMA network Open* (2020) 3(3):e200423–e. doi: 10.1001/jamanetworkopen.2020.0423

11. Wojtukiewicz MZ, Rek MM, Karpowicz K, Górska M, Polityńska B, Wojtukiewicz AM, et al. Inhibitors of immune checkpoints–PD-1, PD-L1, CTLA-4–new opportunities for cancer patients and a new challenge for internists and general practitioners. *Cancer Metastasis Rev* (2021) 40(3):949–82. doi: 10.1007/s10555-021-09976-0

12. Verma V, Sprave T, Haque W, Simone CB, Chang JY, Welsh JW, et al. A systematic review of the cost and cost-effectiveness studies of immune checkpoint inhibitors. *J immunotherapy cancer.* (2018) 6(1):1–15. doi: 10.1186/s40425-018-0442-7

13. Martins F, Sofiya L, Sykiotis GP, Lamine F, Maillard M, Fraga M, et al. Adverse effects of immune-checkpoint inhibitors: epidemiology, management and surveillance. *Nat Rev Clin Oncol* (2019) 16(9):563–80. doi: 10.1038/s41571-019-0218-0

14. Kumar V, Chaudhary N, Garg M, Floudas CS, Soni P, Chandra AB. Current diagnosis and management of immune related adverse events (irAEs) induced by immune checkpoint inhibitor therapy. *Front Pharmacol* (2017) 8:49. doi: 10.3389/fphar.2017.00049

15. Hadamitzky M, Lückemann L, Pacheco-López G, Schedlowski M. Pavlovian conditioning of immunological and neuroendocrine functions. *Physiol Rev* (2020) 100(1):357–405. doi: 10.1152/physrev.00033.2018

16. Perry C, Herishanu Y, Hazan-Halevy I, Kay S, Bdolach N, Naparstek E, et al. Reciprocal changes in regulatory T cells and Th17 helper cells induced by exercise in patients with chronic lymphocytic leukemia. *Leukemia lymphoma.* (2012) 53 (9):1807–10. doi: 10.3109/10428194.2012.656634

17. Saxton JM, Scott EJ, Daley AJ, Woodroofe MN, Mutrie N, Crank H, et al. Effects of an exercise and hypocaloric healthy eating intervention on indices of psychological health status, hypothalamic-pituitary-adrenal axis regulation and immune function after early-stage breast cancer: a randomised controlled trial. *Breast Cancer Res* (2014) 16(2):1–10. doi: 10.1186/bcr3643

18. Zimmer P, Baumann FT, Bloch W, Schenk A, Koliamitra C, Jensen P, et al. Impact of exercise on pro inflammatory cytokine levels and epigenetic modulations of tumor-competitive lymphocytes in non-Hodgkin-Lymphoma patients-randomized controlled trial. *Eur J haematology.* (2014) 93(6):527–32. doi: 10.1111/ejh.12395

19. Ladha AB, Courneya KS, Bell GJ, Field CJ, Grundy P. Effects of acute exercise on neutrophils in pediatric acute lymphoblastic leukemia survivors: a pilot study. *J Pediatr hematology/oncology.* (2006) 28(10):671–7. doi: 10.1097/01.mph.0000243644.20993.54

20. Hutnick NA, Williams NI, Kraemer WJ, Orsega-Smith E, Dixon RH, Bleznak AD, et al. Exercise and lymphocyte activation following chemotherapy for breast cancer. *Med Sci sports exercise.* (2005) 37(11):1827. doi: 10.1249/01.mss.0000175857.84936.1a

21. Hanson ED, Sakkal S, Que S, Cho E, Spielmann G, Kadife E, et al. Natural killer cell mobilization and egress following acute exercise in men with prostate cancer. *Exp Physiol* (2020) 105(9):1524–39. doi: 10.1113/EP088627

22. Glass O, Inman B, Broadwater G, Courneya K, Mackey J, Goruk S, et al. Effect of aerobic training on the host systemic milieu in patients with solid tumours: an exploratory correlative study. *Br J cancer.* (2015) 112(5):825–31. doi: 10.1038/bjc.2014.662

23. Bartlett DB, Hanson ED, Lee JT, Wagoner CW, Harrell EP, Sullivan SA, et al. The effects of 16 weeks of exercise training on neutrophil functions in breast cancer survivors. *Front Immunol* (2021) 4465. doi: 10.3389/fimmu.2021.733101

24. Hojan K, Kwiatkowska-Borowczyk E, Leporowska E, Milecki P. Inflammation, cardiometabolic markers, and functional changes in men with prostate cancer. *A randomized Controlled trial a.* (2017) 12:25–35. doi: 10.20452/pamw.3888

25. Battaglini CL, Hackney A, Garcia R, Groff D, Evans E, Shea T. The effects of an exercise program in leukemia patients. *Integr Cancer therapies.* (2009) 8(2):130–8. doi: 10.1177/1534735409334266

26. Alizadeh AM, Isanejad A, Sadighi S, Mardani M, Hassan ZM. High-intensity interval training can modulate the systemic inflammation and HSP70 in the breast cancer: a randomized control trial. *J Cancer Res Clin Oncol* (2019) 145 (10):2583–93. doi: 10.1007/s00432-019-02996-y

27. Campbell KL, Winters-Stone KM, Wiskemann J, May AM, Schwartz AL, Courneya KS, et al. Exercise guidelines for cancer survivors: consensus statement from international multidisciplinary roundtable. *Med Sci Sports Exercise.* (2019) 51 (11):2375–90. doi: 10.1249/MSS.0000000000002116

28. Van Vulpen JK, Sweegers MG, Peeters PH, Courneya KS, Newton RU, Aaronson NK, et al. Moderators of exercise effects on cancer-related fatigue: a meta-analysis of individual patient data. *Med Sci sports exercise.* (2020) 52(2):303. doi: 10.1249/MSS.0000000000002154

29. Buffart LM, Kalter J, Sweegers MG, Courneya KS, Newton RU, Aaronson NK, et al. Effects and moderators of exercise on quality of life and physical function in patients with cancer: an individual patient data meta-analysis of 34 RCTs. *Cancer Treat Rev* (2017) 52:91–104. doi: 10.1016/j.ctrv.2016.11.010

30. Rummans TA, Clark MM, Sloan JA, Frost MH, Bostwick JM, Atherton PJ, et al. Impacting quality of life for patients with advanced cancer with a structured multidisciplinary intervention: a randomized controlled trial. *J Clin Oncol* (2006) 24(4):635–42. doi: 10.1200/JCO.2006.06.209

31. Stanton AL, Ganz PA, Kwan L, Meyerowitz BE, Bower JE, Krupnick JL, et al. Outcomes from the moving beyond cancer psychoeducational, randomized, controlled trial with breast cancer patients. *J Clin Oncol* (2005) 23(25):6009–18. doi: 10.1200/JCO.2005.09.101

32. Baider L, Peretz T, Hadani PE, Koch U. Psychological intervention in cancer patients: a randomized study. *Gen Hosp Psychiatry* (2001) 23(5):272–7. doi: 10.1016/S0163-8343(01)00158-X

33. Andersen BL, Farrar WB, Golden-Kreutz D, Emery CF, Glaser R, Crespin T, et al. Distress reduction from a psychological intervention contributes to improved health for cancer patients. *Brain behavior immunity.* (2007) 21(7):953–61. doi: 10.1016/j.bbi.2007.03.005

34. Antoni MH, Lutgendorf SK, Cole SW, Dhabhar FS, Sephton SE, McDonald PG, et al. The influence of bio-behavioural factors on tumour biology: pathways and mechanisms. *Nat Rev Cancer.* (2006) 6(3):240–8. doi: 10.1038/nrc1820

35. Shurin MR, Shurin GV, Zlotnikov SB, Bunimovich YL. The neuroimmune axis in the tumor microenvironment. *J Immunol* (2020) 204(2):280–5. doi: 10.4049/jimmunol.1900828

36. Steptoe A. Invited review: The links between stress and illness. *J psychosomatic Res* (1991) 35(6):633–44. doi: 10.1016/0022-3999(91)90113-3

37. Pomerleau OF. Behavioral medicine: The contribution of the experimental analysis of behavior to medical care. *Am Psychol* (1979) 34(8):654. doi: 10.1037/0003-066X.34.8.654

38. Kurtz J. Memory in the innate and adaptive immune systems. *Microbes Infection.* (2004) 6(15):1410–7. doi: 10.1016/j.micinf.2004.10.002

39. Voskoboinik I, Whisstock JC, Trapani JA. Perforin and granzymes: function, dysfunction and human pathology. *Nat Rev Immunol* (2015) 15 (6):388–400. doi: 10.1038/nri3839

40. Bellomo N, Delitala M. From the mathematical kinetic, and stochastic game theory to modelling mutations, onset, progression and immune competition of cancer cells. *Phys Life Rev* (2008) 5(4):183–206. doi: 10.1016/j.plrev.2008.07.001

41. Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, et al. Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget.* (2018) 9(6):7204. doi: 10.18632/oncotarget.23208

42. Ma B, Whiteford JR, Nourshargh S, Woodfin A. Underlying chronic inflammation alters the profile and mechanisms of acute neutrophil recruitment. *J pathology.* (2016) 240(3):291–303. doi: 10.1002/path.4776

43. De Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. *Nat Rev cancer.* (2006) 6(1):24–37. doi: 10.1038/nrc1782

44. Greten FR, Grivennikov SI. Inflammation and cancer: triggers, mechanisms, and consequences. *Immunity.* (2019) 51(1):27–41. doi: 10.1016/j.immuni.2019.06.025

45. Coussens LM, Werb Z. Inflammation and cancer. *Nature.* (2002) 420 (6917):860–7. doi: 10.1038/nature01322

46. Ritter B, Greten FR. Modulating inflammation for cancer therapy. *J Exp Med* (2019) 216(6):1234–43. doi: 10.1084/jem.20181739

47. Choi P, Zelig M. Similarity of colorectal cancer in crohn's disease and ulcerative colitis: implications for carcinogenesis and prevention. *Gut.* (1994) 35 (7):950–4. doi: 10.1136/gut.35.7.950

48. Shacter E, Weitzman SA. Chronic inflammation and cancer. *Oncol (Williston Park NY).* (2002) 16(2):217–26. 29; discussion 30.

49. Maeda H, Akaike T. Nitric oxide and oxygen radicals in infection, inflammation, and cancer. *Biochem C/C Biokhimiia.* (1998) 63:854–65. doi: 10.1007/978-1-4615-5081-5_18

50. Digifico E, Balinzo S, Belgiovine C. The dark side of the force: When the immune system is the fuel of tumor onset. *Int J Mol Sci* (2021) 22(3):1224. doi: 10.3390/ijms22031224

51. Sumitomo R, Hirai T, Fujita M, Murakami H, Otake Y, Huang CL. M2 tumor–associated macrophages promote tumor progression in non–small–cell lung cancer. *Exp Ther Med* (2019) 18(6):4490–8. doi: 10.3892/etm.2019.8068

52. Sica A, Allavena P, Mantovani A. Cancer related inflammation: the macrophage connection. *Cancer letters.* (2008) 267(2):204–15. doi: 10.1016/j.canlet.2008.03.028

53. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell.* (2010) 140(6):883–99. doi: 10.1016/j.cell.2010.01.025

54. Rayburn ER, Ezell SJ, Zhang R. Anti-inflammatory agents for cancer therapy. *Mol Cell Pharmacol* (2009) 1(1):29. doi: 10.4255/mcpharmacol.09.05

55. Zappavigna S, Cossu AM, Grimaldi A, Bocchetti M, Ferraro GA, Nicoletti GF, et al. Anti-inflammatory drugs as anticancer agents. *Int J Mol Sci* (2020) 21 (7):2605. doi: 10.3390/ijms21072605

56. Schaller M, Park JH. The behavioral immune system (and why it matters). *Curr Dir psychol science.* (2011) 20(2):99–103. doi: 10.1177/0963721411402596

57. Schaller M. Parasites, behavioral defenses, and the social psychological mechanisms through which cultures are evoked. *psychol Inquiry.* (2006) 17 (2):96–101. doi: 10.1207/s15327965pli1702_2

58. Milani RV, Lavie CJ. Reducing psychosocial stress: a novel mechanism of improving survival from exercise training. *Am J Med* (2009) 122(10):931–8. doi: 10.1016/j.amjmed.2009.03.028

59. Kogler L, Müller VI, Chang A, Eickhoff SB, Fox PT, Gur RC, et al. Psychosocial versus physiological stress–meta-analyses on deactivations and activations of the neural correlates of stress reactions. *Neuroimage.* (2015) 119:235–51. doi: 10.1016/j.neuroimage.2015.06.059

60. Cannon WB. *Bodily changes in pain, hunger, fear, and rage*. Boston: CH Branford (1953) p. 20–36.

61. Cruces J, Venero C, Pereda-Peeez I, de la Fuente M. The effect of psychological stress and social isolation on neuroimmunoendocrine communication. *Curr Pharm Design.* (2014) 20(29):4608–28. doi: 10.2174/1381612820666140130205822

62. Segerstrom SC, Miller GE. Psychological stress and the human immune system: a meta-analytic study of 30 years of inquiry. *psychol bulletin.* (2004) 130 (4):601. doi: 10.1037/0033-2909.130.4.601

63. Gustafson MP, Wheatley-Guy CM, Rosenthal AC, Gastineau DA, Katsanis E, Johnson BD, et al. Exercise and the immune system: Taking steps to improve responses to cancer immunotherapy. *J ImmunoTherapy Cancer* (2021) 9(7): e001872. doi: 10.1136/jitc-2020-001872

64. Fiuza-Luces C, Valenzuela PL, Castillo-García A, Lucia A. Exercise benefits meet cancer immunosurveillance: Implications for immunotherapy. *Trends Cancer* (2021) 7(2):91–3. doi: 10.1016/j.trecan.2020.12.003

65. Idorn M, Hojman P. Exercise-dependent regulation of NK cells in cancer protection. *Trends Mol Med* (2016) 22(7):565–77. doi: 10.1016/j.molmed.2016.05.007

66. Caspersen CJ, Powell KE, Christenson GM. Physical activity, exercise, and physical fitness: definitions and distinctions for health-related research. *Public Health Rep.* (1985) 100(2):126–31.

67. Simpson RJ, Kunz H, Agha N, Graff R. Exercise and the regulation of immune functions. *Prog Mol Biol Trans science.* (2015) 135:355–80. doi: 10.1016/bs.pmbts.2015.08.001

68. Swardfager W, Herrmann N, Cornish S, Mazereeuw G, Marzolini S, Sham L, et al. Exercise intervention and inflammatory markers in coronary artery disease: a meta-analysis. *Am Heart J* (2012) 163(4):666–76. e3. doi: 10.1016/j.ahj.2011.12.017

69. Pavlov IP, Gantt W. *Lectures on conditioned reflexes: Twenty-five years of objective study of the higher nervous activity (behaviour) of animals.* (1928) (New York: International Publishers).

70. Kirchhof J, Petrakova L, Brinkhoff A, Benson S, Schmidt J, Unteroberdörster M, et al. Learned immunosuppressive placebo responses in renal transplant patients. *Proc Natl Acad Sci* (2018) 115(16):4223–7. doi: 10.1073/pnas.1720548115

71. Kerr J, Anderson C, Lippman SM. Physical activity, sedentary behaviour, diet, and cancer: an update and emerging new evidence. *Lancet Oncol* (2017) 18(8): e457–e71. doi: 10.1016/S1470-2045(17)30411-4

72. Friedenreich CM, Shaw E, Neilson HK, Brenner DR. Epidemiology and biology of physical activity and cancer recurrence. *J Mol Med* (2017) 95(10):1029–41. doi: 10.1007/s00109-017-1558-9

73. Patel AV, Friedenreich CM, Moore SC, Hayes SC, Silver JK, Campbell KL, et al. American College of sports medicine roundtable report on physical activity, sedentary behavior, and cancer prevention and control. *Med Sci sports exercise.* (2019) 51(11):2391. doi: 10.1249/MSS.0000000000002117

74. McTiernan A, Friedenreich CM, Katzmarzyk PT, Powell KE, Macko R, Buchner D, et al. Physical activity in cancer prevention and survival: a systematic review. *Med Sci sports exercise.* (2019) 51(6):1252. doi: 10.1249/MSS.0000000000001937

75. Oh H-M, Son C-G. The risk of psychological stress on cancer recurrence: A systematic review. *Cancers.* (2021) 13(22):5816. doi: 10.3390/cancers13225816

76. Eckerling A, Ricon-Becker I, Sorski L, Sandbank E, Ben-Eliyahu S. Stress and cancer: mechanisms, significance and future directions. *Nat Rev Cancer.* (2021) 21(12):767–85. doi: 10.1038/s41568-021-00395-5

77. Eisenberger NI, Moieni M, Inagaki TK, Muscatell KA, Irwin MR. In sickness and in health: the co-regulation of inflammation and social behavior. *Neuropsychopharmacology.* (2017) 42(1):242–53. doi: 10.1038/npp.2016.141

78. Jin Shin K, Jin Lee Y, Ryoul Yang Y, Park S, Suh P-G, Yung Follo M, et al. Molecular mechanisms underlying psychological stress and cancer. *Curr Pharm design.* (2016) 22(16):2389–402. doi: 10.2174/1381612822666160226144025

79. Liu H-m, Li C, Cao B, Jiang Y, Han L, Xu R, et al. The molecular mechanism of chronic stress affecting the occurrence and development of breast cancer and potential drug therapy. *Trans Oncol* (2022) 15(1):101281. doi: 10.1016/j.tranon.2021.101281

80. Charmandari E, Tsigos C, Chrousos G. Endocrinology of the stress response 1. *Annu Rev Physiol* (2005) 67(1):259–84. doi: 10.1146/annurev.physiol.67.040403.120816

81. Herr I, Ucur E, Herzer K, Okouoyo S, Ridder Rd, Krammer PH, et al. Glucocorticoid cotreatment induces apoptosis resistance toward cancer therapy in carcinomas. *Cancer Res* (2003) 63(12):3112–20.

82. Perron L, Bairati I, Harel F, Meyer F. Antihypertensive drug use and the risk of prostate cancer (Canada). *Cancer causes control.* (2004) 15(6):535–41. doi: 10.1023/B:CACO.0000036152.58271.5e

83. Singh GK, Jemal A. Socioeconomic and racial/ethnic disparities in cancer mortality, incidence, and survival in the united states, 1950–2014: over six decades of changing patterns and widening inequalities. *J Environ Public Health* (2017) 2017:2819372. doi: 10.1155/2017/2819372

84. Baum A, Garofalo J, Yali AM. Socioeconomic status and chronic stress: does stress account for SES effects on health? *Ann New York Acad Sci* (1999) 896(1):131–44. doi: 10.1111/j.1749-6632.1999.tb08111.x

85. Tabuchi T. Cancer and socioeconomic status. In: *Social determinants of health in non-communicable diseases*. Singapore: Springer (2020). p. 31–40.

86. Moore SC, Lee I-M, Weiderpass E, Campbell PT, Sampson JN, Kitahara CM, et al. Association of leisure-time physical activity with risk of 26 types of cancer in 1.44 million adults. *JAMA Internal Med* (2016) 176(6):816–25. doi: 10.1001/jamainternmed.2016.1548

87. Liu L, Shi Y, Li T, Qin Q, Yin J, Pang S, et al. Leisure time physical activity and cancer risk: evaluation of the WHO's recommendation based on 126 high-quality epidemiological studies. *Br J sports Med* (2016) 50(6):372–8. doi: 10.1136/bjsports-2015-094728

88. Koelwyn GJ, Wennerberg E, Demaria S, Jones LW. Exercise in regulation of inflammation-immune axis function in cancer initiation and progression. *Oncol (Williston Park NY).* (2015) 29(12):908–22.

89. Mathur N, Pedersen BK. Exercise as a mean to control low-grade systemic inflammation. *Mediators Inflammation* (2008) 2008:1–6. doi: 10.1155/2008/109502

90. Gleeson M, Bishop NC, Stensel DJ, Lindley MR, Mastana SS, Nimmo MA. The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. *Nat Rev Immunol* (2011) 11(9):607–15. doi: 10.1038/nri3041

91. Walsh NP, Gleeson M, Shephard RJ, Gleeson M, Woods JA, Bishop NC, et al. Position statement. part one: Immune function and exercise. *Exercise Immunol Rev* (2011) 17:6–63.

92. Miller AH, Ancoli-Israel S, Bower JE, Capuron L, Irwin MR. Neuroendocrine-immune mechanisms of behavioral comorbidities in patients with cancer. *J Clin oncology: Off J Am Soc Clin Oncol* (2008) 26(6):971. doi: 10.1200/JCO.2007.10.7805

93. Eng JW-L, Reed CB, Kokolus KM, Pitoniak R, Utley A, Bucsek MJ, et al. Housing temperature-induced stress drives therapeutic resistance in murine tumour models through β2-adrenergic receptor activation. *Nat Commun* (2015) 6(1):1–13. doi: 10.1038/ncomms7426

94. Cole SW, Sood AK. Molecular pathways: β-adrenergic signaling in cancer. *Clin Cancer Res* (2012) 18(5):1201–6. doi: 10.1158/1078-0432.CCR-11-0641

95. Cole SW, Nagaraja AS, Lutgendorf SK, Green PA, Sood AK. Sympathetic nervous system regulation of the tumour microenvironment. *Nat Rev Cancer.* (2015) 15(9):563–72. doi: 10.1038/nrc3978

96. Jiang S-H, Hu L-P, Wang X, Li J, Zhang Z-G. Neurotransmitters: emerging targets in cancer. *Oncogene.* (2020) 39(3):503–15. doi: 10.1038/s41388-019-1006-0

97. Coelho M, Moz M, Correia G, Teixeira A, Medeiros R, Ribeiro L. Antiproliferative effects of β-blockers on human colorectal cancer cells. *Oncol Rep* (2015) 33(5):2513–20. doi: 10.3892/or.2015.3874

98. Pantziarka P, Bouche G, Sukhatme V, Meheus L, Rooman I, Sukhatme VP. Repurposing drugs in oncology (ReDO)–propranolol as an anti-cancer agent. *ecancermedicalscience.* (2016) 10:680. doi: 10.3332/ecancer.2016.680

99. Chang P-Y, Huang W-Y, Lin C-L, Huang T-C, Wu Y-Y, Chen J-H, et al. Propranolol reduces cancer risk: a population-based cohort study. *Medicine* (2015) 94(27):e1097. doi: 10.1097/MD.0000000000001097

100. Obradović M, Hamelin B, Manevski N, Couto JP, Sethi A, Coissieux M-M, et al. Glucocorticoids promote breast cancer metastasis. *Nature.* (2019) 567 (7749):540–4. doi: 10.1038/s41586-019-1019-4

101. Zhang C, Wenger T, Mattern J, Ilea S, Frey C, Gutwein P, et al. Clinical and mechanistic aspects of glucocorticoid-induced chemotherapy resistance in the majority of solid tumors. *Cancer Biol Ther* (2007) 6(2):278–87. doi: 10.4161/cbt.6.2.3652

102. Zhang C, Beckermann B, Kallifatidis G, Liu Z, Rittgen W, Edler L, et al. Corticosteroids induce chemotherapy resistance in the majority of tumour cells from bone, brain, breast, cervix, melanoma and neuroblastoma. *Int J Oncol* (2006) 29(5):1295–301. doi: 10.3892/ijo.29.5.1295

103. Shields GS, Spahr CM, Slavich GM. Psychosocial interventions and immune system function: a systematic review and meta-analysis of randomized clinical trials. *JAMA Psychiatry* (2020) 77(10):1031–43. doi: 10.1001/jamapsychiatry.2020.0431

104. Owen JE, Klapow JC, Hicken B, Tucker DC. Psychosocial interventions for cancer: review and analysis using a three-tiered outcomes model. *Psycho-Oncology: J Psychological Soc Behav Dimensions Cancer.* (2001) 10(3):218–30. doi: 10.1002/pon.509

105. Teo I, Krishnan A, Lee GL. Psychosocial interventions for advanced cancer patients: a systematic review. *Psycho-oncology.* (2019) 28(7):1394–407. doi: 10.1002/pon.5103

106. Schmidt T, Jonat W, Wesch D, Oberg H-H, Adam-Klages S, Keller L, et al. Influence of physical activity on the immune system in breast cancer patients during chemotherapy. *J Cancer Res Clin Oncol* (2018) 144(3):579–86. doi: 10.1007/s00432-017-2573-5

107. Liu J, Chen P, Wang R, Yuan Y, Wang X, Li C. Effect of tai chi on mononuclear cell functions in patients with non-small cell lung cancer. *BMC complementary Altern Med* (2015) 15(1):1–8. doi: 10.1186/s12906-015-0517-7

108. Hvid T, Lindegaard B, Winding K, Iversen P, Brasso K, Solomon TP, et al. Effect of a 2-year home-based endurance training intervention on physiological function and PSA doubling time in prostate cancer patients. *Cancer Causes Control.* (2016) 27(2):165–74. doi: 10.1007/s10552-015-0694-1

109. Galvao D, Nosaka K, Taaffe D, Peake J, Spry N, Suzuki K, et al. Endocrine and immune responses to resistance training in prostate cancer patients. *Prostate Cancer prostatic diseases.* (2008) 11(2):160–5. doi: 10.1038/sj.pcan.4500991

110. Kruijsen-Jaarsma M, Révész D, Bierings MB, Buffart LM, Takken T. Effects of exercise on immune function in patients with cancer: a systematic review. *Exercise Immunol Rev* (2013) 19:120–43.

111. Toffoli EC, Sweegers MG, Bontkes HJ, Altenburg TM, Verheul HM, van der Vliet HJ, et al. Effects of physical exercise on natural killer cell activity during (neo) adjuvant chemotherapy: a randomized pilot study. *Physiol Rep* (2021) 9(11): e14919. doi: 10.14814/phy2.14919

112. Ashcraft KA, Warner AB, Jones LW, Dewhirst MW. Exercise as adjunct therapy in cancer. *Seminars in Radiation Oncology.* (2019) 29(1):16–24.

113. Pedersen L, Idorn M, Olofsson GH, Lauenborg B, Nookaew I, Hansen RH, et al. Voluntary running suppresses tumor growth through epinephrine-and IL-6-dependent NK cell mobilization and redistribution. *Cell Metab* (2016) 23(3):554–62. doi: 10.1016/j.cmet.2016.01.011

114. Pedersen L, Christensen JF, Hojman P. Effects of exercise on tumor physiology and metabolism. *Cancer J* (2015) 21(2):111–6. doi: 10.1097/PPO.0000000000000096

115. Higgins KA, Park D, Lee GY, Curran WJ, Deng X. Exercise-induced lung cancer regression: Mechanistic findings from a mouse model. *Cancer.* (2014) 120 (21):3302–10. doi: 10.1002/cncr.28878

116. Garritson J, Krynski L, Haverbeck L, Haughian JM, Pullen NA, Hayward R. Physical activity delays accumulation of immunosuppressive myeloid-derived suppressor cells. *PloS One* (2020) 15(6):e0234548. doi: 10.1371/journal.pone.0234548

117. Stockhorst U, Spennes-Saleh S, Körholz D, Göbel U, Schneider ME, Steingrüber H-J, et al. Anticipatory symptoms and anticipatory immune responses in pediatric cancer patients receiving chemotherapy: features of a classically conditioned response? *Brain behavior Immun* (2000) 14(3):198–218. doi: 10.1006/brbi.1999.0581

118. Bovbjerg DH, Redd WH, Jacobsen PB, Manne SL, Taylor KL, Surbone A, et al. An experimental analysis of classically conditioned nausea during cancer chemotherapy. *Psychosomatic Med* (1992) 54(6):623–37. doi: 10.1097/00006842-199211000-00001

119. Stockhorst U, Steingrueber H-J, Enck P, Klosterhalfen S. Pavlovian conditioning of nausea and vomiting. *Autonomic Neurosci* (2006) 129(1-2):50–7. doi: 10.1016/j.autneu.2006.07.012

120. Stockhorst U, Wiener JA, Klosterhalfen S, Klosterhalfen W, Aul C, Steingrüber H-J. Effects of overshadowing on conditioned nausea in cancer patients: an experimental study. *Physiol behavior.* (1998) 64(5):743–53. doi: 10.1016/s0031-9384(98)00135-8

121. Ramírez-Amaya V, Alvarez-Borda Bn, Ormsby CE, Martínez RD, Pérez-Montfort R, Bermúdez-Rattoni F. Insular cortex lesions impair the acquisition of conditioned immunosuppression. *Brain behavior immunity.* (1996) 10(2):103–14. doi: 10.1006/brbi.1996.0011

122. Ramírez-Amaya V, Bermudez-Rattoni F. Conditioned enhancement of antibody production is disrupted by insular cortex and amygdala but not hippocampal lesions. *Brain behavior immunity.* (1999) 13(1):46–60. doi: 10.1006/brbi.1998.0547

123. Hiramoto RN, Hiramoto NS, Rish ME, Soong S-J, Miller DM, Ghanta VK. Role of immune cells in the pavlovian conditioning of specific resistance to cancer. *Int J Neurosci* (1991) 59(1-3):101–17. doi: 10.3109/00207459108985453

124. Lavond DG, Kim JJ, Thompson RF. Mammalian brain substrates of aversive classical conditioning. *Annu Rev Psychol* (1993) 44(1):317–42. doi: 10.1146/annurev.ps.44.020193.001533

125. Thompson RF. Neural mechanisms of classical conditioning in mammals. *Philos Trans R Soc London Ser B: Biol Sci* (1990) 329(1253):161–70. doi: 10.1098/rstb.1990.0161

126. Coussons ME, Dykstra LA, Lysle DT. Pavlovian conditioning of morphine-induced alterations of immune status. *J neuroimmunology.* (1992) 39(3):219–30. doi: 10.1016/0165-5728(92)90256-K

127. Dark K, Peeke HV, Ellman G, Salfi M. Behaviorally conditioned histamine release: Prior stress and conditionability and extinction of the response. *Ann New York Acad Sci* (1987) 496(1):578–82. doi: 10.1111/j.1749-6632.1987.tb35816.x

128. Exton MS, von Hörsten S, Vöge J, Westermann J, Schult M, Nagel E, et al. Conditioned taste aversion produced by cyclosporine a: concomitant reduction in lymphoid organ weight and splenocyte proliferation. *Physiol behavior.* (1998) 63 (2):241–7. doi: 10.1016/S0031-9384(97)00432-0

129. Husband A, Lin W, Madsen G, King M. A conditioning model for immunostimulation: Enhancement of the antibody response to ovalbumin by behavioral conditioning in rats. *Psychoimmunology CNS-Immune Interactions: CRC Press;* (2019) p:139–48. doi: 10.1201/9780429279164-13

130. Janz LJ, Green-Johnson J, Murray L, Vriend CY, Nance DM, Greenberg AH, et al. Pavlovian conditioning of LPS-induced responses: effects on corticosterone, splenic NE, and IL-2 production. *Physiol behavior.* (1996) 59 (6):1103–9. doi: 10.1016/0031-9384(95)02171-X

131. Kelley K, Dantzer R, Mormede P, Salmon H, Aynaud J-M. Conditioned taste aversion suppresses induction of delayed-type hypersensitivity immune reactions. *Physiol behavior.* (1985) 34(2):189–93. doi: 10.1016/0031-9384(85)90104-0

132. Kusnecov AW, Sivyer M, King M, Husband A, Cripps A, Clancy R. Behaviorally conditioned suppression of the immune response by antilymphocyte serum. *J Immunol* (1983) 130(5):2117–20.

133. Kroemer G, Senovilla L, Galluzzi L, André F, Zitvogel L. Natural and therapy-induced immunosurveillance in breast cancer. *Nat Med* (2015) 21 (10):1128–38. doi: 10.1038/nm.3944

134. Weber J ed. (2010). Immune checkpoint proteins: a new therapeutic paradigm for cancer–preclinical background: CTLA-4 and PD-1 blockade. Seminars in Oncology. 37(5):430–9

135. Brunet J-F, Denizot F, Luciani M-F, Roux-Dosseto M, Suzan M, Mattei M-G, et al. A new member of the immunoglobulin superfamily–CTLA-4. *Nature.* (1987) 328(6127):267–70. doi: 10.1038/328267a0

136. Linsley PS, Bradshaw J, Greene J, Peach R, Bennett KL, Mittler RS. Intracellular trafficking of CTLA-4 and focal localization towards sites of TCR engagement. *Immunity.* (1996) 4(6):535–43. doi: 10.1016/S1074-7613(00)80480-X

137. Contardi E, Palmisano GL, Tazzari PL, Martelli AM, Fala F, Fabbi M, et al. CTLA-4 is constitutively expressed on tumor cells and can trigger apoptosis upon ligand interaction. *Int J cancer.* (2005) 117(4):538–50. doi: 10.1002/ijc.21155

138. Centanni M, Moes DJA, Trocóniz IF, Ciccolini J, van Hasselt JC. Clinical pharmacokinetics and pharmacodynamics of immune checkpoint inhibitors. *Clin pharmacokinetics.* (2019) 58(7):835–57. doi: 10.1007/s40262-019-00748-2

139. Hirano F, Kaneko K, Tamura H, Dong H, Wang S, Ichikawa M, et al. Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. *Cancer Res* (2005) 65(3):1089–96. doi: 10.1158/0008-5472.1089.65.3

140. Alsaab HO, Sau S, Alzhrani R, Tatiparti K, Bhise K, Kashaw SK, et al. PD-1 and PD-L1 checkpoint signaling inhibition for cancer immunotherapy: mechanism, combinations, and clinical outcome. *Front Pharmacol* (2017) 8:561. doi: 10.3389/fphar.2017.00561

141. Chocarro L, Bocanegra A, Blanco E, Fernández-Rubio L, Arasanz H, Echaide M, et al. Cutting-edge: Preclinical and clinical development of the first approved lag-3 inhibitor. *Cells.* (2022) 11(15):2351. doi: 10.3390/cells11152351

142. Kanchan RK, Doss D, Khan P, Nasser MW, Mahapatra S. To kill a cancer: Targeting the immune inhibitory checkpoint molecule, B7-H3. *Biochim Biophys Acta (BBA)-Reviews Cancer* (2022) 188783. doi: 10.1016/j.bbcan.2022.188783

143. Moesta AK, Li X-Y, Smyth MJ. Targeting CD39 in cancer. *Nat Rev Immunol* (2020) 20(12):739–55. doi: 10.1038/s41577-020-0376-4

144. Nocentini A, Capasso C, Supuran CT. Small-molecule CD73 inhibitors for the immunotherapy of cancer: A patent and literature review (2017–present). *Expert Opin Ther Patents.* (2021) 31(10):867–76. doi: 10.1080/13543776.2021.1923694

145. Wolf Y, Anderson AC, Kuchroo VK. TIM3 comes of age as an inhibitory receptor. *Nat Rev Immunol* (2020) 20(3):173–85. doi: 10.1038/s41577-019-0224-6

146. Bonneville R, Krook MA, Kautto EA, Miya J, Wing MR, Chen H-Z, et al. Landscape of microsatellite instability across 39 cancer types. *JCO Precis Oncol* (2017) 1:1–15. doi: 10.1200/PO.17.00073

147. Sahin IH, Akce M, Alese O, Shaib W, Lesinski GB, El-Rayes B, et al. Immune checkpoint inhibitors for the treatment of MSI-H/MMR-D colorectal cancer and a perspective on resistance mechanisms. *Br J cancer.* (2019) 121 (10):809–18. doi: 10.1038/s41416-019-0599-y

148. Gibney GT, Weiner LM, Atkins MB. Predictive biomarkers for checkpoint inhibitor-based immunotherapy. *Lancet Oncol* (2016) 17(12):e542–e51. doi: 10.1016/S1470-2045(16)30406-5

149. Sommershof A, Scheuermann L, Koerner J, Groettrup M. Chronic stress suppresses anti-tumor TCD8+ responses and tumor regression following cancer immunotherapy in a mouse model of melanoma. *Brain behavior immunity.* (2017) 65:140–9. doi: 10.1016/j.bbi.2017.04.021

150. Levi B, Matzner P, Goldfarb Y, Sorski L, Shaashua L, Melamed R, et al. Stress impairs the efficacy of immune stimulation by CpG-c: Potential neuroendocrine mediating mechanisms and significance to tumor metastasis and the perioperative period. *Brain behavior immunity.* (2016) 56:209–20. doi: 10.1016/j.bbi.2016.02.025

151. Matzner P, Sorski L, Haldar R, Shaashua L, Benbenishty A, Lavon H, et al. Deleterious synergistic effects of distress and surgery on cancer metastasis: abolishment through an integrated perioperative immune-stimulating stress-inflammatory-reducing intervention. *Brain Behavior Immunity.* (2019) 80:170–8. doi: 10.1016/j.bbi.2019.03.005

152. Yang H, Xia L, Chen J, Zhang S, Martin V, Li Q, et al. Stress–glucocorticoid–TSC22D3 axis compromises therapy-induced antitumor immunity. *Nat Med* (2019) 25(9):1428–41. doi: 10.1038/s41591-019-0566-4

153. Oh MS, Guzner A, Wainwright DA, Mohindra NA, Chae YK, Behdad A, et al. The impact of beta blockers on survival outcomes in patients with non–small-cell lung cancer treated with immune checkpoint inhibitors. *Clin Lung cancer.* (2021) 22(1):e57–62. doi: 10.1016/j.cllc.2020.07.016

154. Zhou Q, Qian Z, Ding W, Jiang G, Sun C, Xu K. Chronic psychological stress attenuates the efficacy of anti-PD-L1 immunotherapy for bladder cancer in immunocompetent mice. *Cancer Invest* (2021) 39(6-7):571–81. doi: 10.1080/07357907.2021.1943746

155. Bucsek MJ, Qiao G, MacDonald CR, Giridharan T, Evans L, Niedzwecki B, et al. β-adrenergic signaling in mice housed at standard temperatures suppresses an effector phenotype in CD8+ T cells and undermines checkpoint inhibitor therapy. *Cancer Res* (2017) 77(20):5639–51. doi: 10.1158/0008-5472.CAN-17-0546

156. Fjæstad KY, Rømer AMA, Goitea V, Johansen AZ, Thorseth M-L, Carretta M, et al. Blockade of β-adrenergic receptors reduces cancer growth and enhances the response to anti-CTLA4 therapy by modulating the tumor microenvironment. *Oncogene* (2022) 41(9):1364–1375. doi: 10.1038/s41388-021-02170-0

157. Martín-Ruiz A, Fiuza-Luces C, Rincón-Castanedo C, Fernández-Moreno D, Gálvez BG, Martínez-Martínez E, et al. Benefits of exercise and immunotherapy in a murine model of human non-small-cell lung carcinoma. *Exercise Immunol Rev* (2020) 26:100–15.

158. Buss LA, Williams T, Hock B, Ang AD, Robinson BA, Currie MJ, et al. Effects of exercise and anti-PD-1 on the tumour microenvironment. *Immunol Letters.* (2021) 239:60–71. doi: 10.1016/j.imlet.2021.08.005

159. Bay ML, Unterrainer N, Stagaard R, Pedersen KS, Schauer T, Staffeldt MM, et al. Voluntary wheel running can lead to modulation of immune checkpoint molecule expression. *Acta Oncologica.* (2020) 59(12):1447–54. doi: 10.1080/0284186X.2020.1817550

160. Kurz E, Hirsch CA, Dalton T, Shadaloey SA, Khodadadi-Jamayran A, Miller G, et al. Exercise-induced engagement of the IL-15/IL-15Rα axis promotes anti-tumor immunity in pancreatic cancer. *Cancer Cell* (2022) 40(7):720–37.E5. doi: 10.1016/j.ccell.2022.05.006

161. Hetze S, Barthel L, Lückemann L, Günther HS, Wülfing C, Salem Y, et al. Taste-immune associative learning amplifies immunopharmacological effects and attenuates disease progression in a rat glioblastoma model. *Brain Behavior Immunity.* (2022) 106:270–9. doi: 10.1016/j.bbi.2022.09.006

162. Ghanta VK, Hiramoto NS, Solvason HB, Soong S-J, Hiramoto RN. Conditioning: a new approach to immunotherapy. *Cancer Res* (1990) 50 (14):4295–9.

163. Ghanta VK, Miura T, Hiramoto NS, Hiramoto RN. Augmentation of natural immunity and regulation of tumor growth by conditioning. *Ann New York Acad Sci* (1988) 521:29–42. doi: 10.1111/j.1749-6632.1988.tb35263.x

164. Ghanta VK, Hiramoto RN, Solvason HB, Spector NH. Influence of conditioned natural immunity on tumor growth. *Ann New York Acad Sci* (1987) 496(1):637–46. doi: 10.1111/j.1749-6632.1987.tb35824.x

165. Ghanta VK, Hiramoto NS, Soong S-J, Hiramoto RN. Conditioning of the secondary cytotoxic T-lymphocyte response to YC8 tumor. *Pharmacol Biochem Behavior.* (1995) 50(3):399–403. doi: 10.1016/0091-3057(94)00286-R

166. Ghanta VK, Hiramoto NS, Soong S-J, Miller DM, Hiramoto RN. A multiple modality approach combining the effect of conditioning with adoptive chemoimmunotherapy. *Int J Neurosci* (1993) 71(1-4):251–65. doi: 10.3109/00207459309000608

167. Gorczynski RM, Kennedy M, Ciampi A. Cimetidine reverses tumor growth enhancement of plasmacytoma tumors in mice demonstrating conditioned immunosuppression. *J Immunol* (1985) 134(6):4261–6.

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CTLA4 mRNA is downregulated by miR-155 in regulatory T cells, and reduced blood *CTLA4* levels are associated with poor prognosis in metastatic melanoma patients

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Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) is an immune checkpoint expressed in regulatory T (Treg) cells and activated T lymphocytes. Despite its potential as a treatment strategy for melanoma, CTLA-4 inhibition has limited efficacy. Using data from The Cancer Genome Atlas (TCGA) melanoma database and another dataset, we found that decreased *CTLA4* mRNA was associated with a poorer prognosis in metastatic melanoma. To investigate further, we measured blood *CTLA4* mRNA in 273 whole-blood samples from an Australian cohort and found that it was lower in metastatic melanoma than in healthy controls and associated with worse patient survival. We confirmed these findings using Cox proportional hazards model analysis and another cohort from the US. Fractionated blood analysis revealed that Treg cells were responsible for the downregulated *CTLA4* in metastatic melanoma patients, which was confirmed by further analysis of published data showing downregulated CTLA-4 surface protein expression in Treg cells of metastatic melanoma compared to healthy donors. Mechanistically, we found that secretomes from human metastatic melanoma cells downregulate *CTLA4* mRNA at the post-transcriptional level through miR-155 while upregulating *FOXP3* expression in human Treg cells. Functionally, we demonstrated that *CTLA4* expression inhibits the proliferation and suppressive function of human Treg cells. Finally, miR-155 was found to be upregulated in Treg cells from metastatic melanoma patients compared to healthy donors. Our study provides new insights into the underlying

mechanisms of reduced *CTLA4* expression observed in melanoma patients, demonstrating that post-transcriptional silencing of *CTLA4* by miRNA-155 in Treg cells may play a critical role. Since CTLA-4 expression is downregulated in non-responder melanoma patients to anti-PD-1 immunotherapy, targeting miRNA-155 or other factors involved in regulating *CTLA4* expression in Treg cells without affecting T cells could be a potential strategy to improve the efficacy of immunotherapy in melanoma. Further research is needed to understand the molecular mechanisms regulating *CTLA4* expression in Treg cells and identify potential therapeutic targets for enhancing immune-based therapies.

KEYWORDS

melanoma, CTLA-4, biomarker, regulatory T cells, miRNA-155

1 Introduction

Melanoma is one of the aggressive forms of skin cancer. Its incidence increases by over 3% annually and continues to rise in Caucasians (1). Until recently, the survival rate for advanced melanoma patients was around 10% (2). However, recent advances in our understanding of tumor immunology and cancer biology have led to promising developments of immune checkpoint inhibitors and targeted therapies, resulting in significant improvements in long-term survival, with a substantial subset of melanoma patients experiencing durable responses (3). Despite these advances, some patients do not respond to immunotherapy or experience limited benefits, and approximately two-thirds of patients treated with immune checkpoint monotherapies eventually progress (4), underscoring the importance of understanding the mechanisms of immune evasion, drug resistance, and poor prognosis in these cancer patients.

Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) is a major immune checkpoint that negatively regulates T-cell activation by suppressing T-cell signaling through engagement by B7 (CD80/CD86) ligands (5). As such, CTLA-4 inhibition leads to increased T-cell activation and a reduction in mouse tumor size (6). In contrast to activated T cells expressing CTLA-4 after activation, regulatory T (Treg) cells constitutively express CTLA-4 and play a significant role in tumor tolerance through surface/intracellular CTLA-4 and its soluble counterpart (7). Therefore, targeting CTLA-4 affects both activated T and Treg cells. While anti-CTLA-4 therapy with the monoclonal antibody ipilimumab has improved the overall survival of metastatic melanoma patients (8) compared to anti-PD-1 therapy, a smaller percentage of patients benefited from anti-CTLA-4 treatment, and more immunotherapy-related adverse events were reported (9–11). To better understand the challenges of targeting CTLA-4 and close the fundamental knowledge gap, researchers have attempted to modify anti-CTLA-4 therapy and investigate TME (12).

Immune-related factors, including tumor-infiltrating lymphocytes and immune-related gene signatures, have been utilized to help determine prognosis and response to immunotherapy in melanoma patients (13). However, unlike PD-1 and PD-L1 expression, the expression of CTLA-4 in the TME has rarely been studied. Recent studies have shown that pre-treatment levels of *CTLA4* expression in tumor samples are associated with clinical benefits from anti-CTLA-4 immunotherapy in metastatic melanoma patients (14). Another study found that pre-treatment levels of *CTLA4* promoter methylation (*mCTLA4*) in the tumors inversely correlate with *CTLA4* mRNA expression and that low *mCTLA4* levels are associated with response to anti-PD-1/CTLA-4 therapy (15, 16), suggesting that tumors with higher *CTLA4* expression are associated with clinical benefits, while tumors with lower *CTLA4* expression are not. Interestingly, the study also found that *CTLA4* mRNA expression correlates with patient prognosis even without immunotherapy. These findings suggest that tumors with low *CTLA4* mRNA expression have a poor prognosis and resistance to immunotherapy. Since CTLA-4 expression is dynamically regulated in the TME, these results suggest that tumors with low *CTLA4* mRNA expression may have fewer CTLA-4-positive cells (activated T, Treg, and tumor cells) or a lower amount of *CTLA4* expression per cell. As CTLA-4 is an immune checkpoint that negatively regulates T-cell activation, we aim to investigate the significance of lower CTLA-4 expression in melanoma patients.

The T-cell-intrinsic function of CTLA-4 upregulation has been extensively investigated. On the other hand, Treg cells constitutively express CTLA-4 in physiological conditions; thus, its contribution to Treg cell function has been less studied, particularly in the presence of tumors. Germline depletion of *Ctla4* (17, 18) and Treg-specific deletion of *Ctla4* (19) resulted in severe autoimmunity with lethality, demonstrating the critical role of CTLA-4 in Treg cell function. Based on these findings, the tumors with downregulated CTLA-4 may have enhanced anti-tumor immunity, better prognosis, and greater response to immunotherapy. However, conditional ablation of *Ctla4* in adult mice was reported to confer protection from autoimmune and anti-tumor responses (20), suggesting the Treg-cell-intrinsic function of CTLA-4 to limit their activation and expansion. Therefore, it is also possible that the tumors with downregulated CTLA-4 have reduced anti-tumor immunity, poor prognosis, and resistance to immunotherapy.

In this study, we analyzed the expression of *CTLA4* mRNA in tumor and blood samples from melanoma patients and found a correlation between decreased tumor and blood *CTLA4* and a poorer prognosis in patients with metastatic melanoma. *CTLA4* was downregulated by approximately 70% in Treg cells, resulting in levels similar to those in non-Treg T cells. Mechanistically, we provide evidence that the metastatic melanoma secretome induces post-transcriptional *CTLA4* mRNA instability through the induction of miR-155. Consistent with this, we observed upregulation of miR-155 in Treg cells of metastatic melanoma patients. Functionally, we demonstrated that *CTLA4* expression inhibits the proliferation and suppressive function of human Treg cells. Our findings shed light on the mechanisms underlying the downregulation of *CTLA4* in Treg cells in patients with metastatic melanoma.

2 Materials and methods

2.1 TCGA and Swedish melanoma tumor datasets

Clinical information on 329 TCGA (TCGA-SKCM cohort) melanoma tumors was obtained from the cBioPortal website (21). Among them, 286 patients had complete pathological information, including 42 cases of primary melanoma and 244 cases of metastatic melanoma. Normalized RNA-seq gene expression profiles (Level 3, RSEM value) of these patients were downloaded from the cBioPortal website (22).

Swedish dataset containing 210 metastatic melanoma tumors was downloaded from the Gene Expression Omnibus (GEO) database with GSE65904, incorporating patient outcomes and relapse-free survival time. A natural log transformation was applied to the processed gene expression data before survival analysis (23).

2.2 Characteristics of Australian and US melanoma cohorts for blood analyses

For the AUS cohort, approval for the blood sample study was obtained from the Human Research Ethics Committee of Edith Cowan University (No. 2932) and Sir Charles Gardner Hospital (No. 2007-123). Eligible subjects included 103 healthy donors and 170 melanoma patients recruited in Australia between 2008-2011. All patients were from melanoma clinics in Perth, Western Australia (Medical Oncology Department of Sir Charles Gairdner Hospital and Perth Melanoma Clinic at Hollywood Hospital). Patients comprised 66 women and 104 men, IQR 57-78 years (median age of 66 years), while the aged-matched, healthy cohort from the general population comprised 63 women and 40 men, IQR 32-61.5 years (median age of 45 years). AJCC clinical staging of melanoma patients categorized 71.8% as stages 0-II and 28.2% as stages III-IV.

The US cohort was approved by the Institutional Review Boards of the University of Colorado (COMIRB#05-0309). A total of 263 eligible melanoma patients were recruited from the Cutaneous Oncology Department at the University of Colorado Cancer Center, Aurora, CO. This cohort consisted of 121 women and 142 men, IQR 41-63 years (median age of 53 years). AJCC clinical staging categorized 62.7% as stages 0-II and 37.3% as stages II-IV.

The demographic information of these two cohorts is summarized in Table 1. The medical records for each patient were reviewed retrospectively for pertinent past medical history and significant events such as disease progression and death. Local death records were reviewed if survival/mortality information was not determined by chart review. If no proof of death was obtained, the patient was presumed alive. The end of follow-up for AUS and US cohorts was April 14, 2020 and June 19, 2020, respectively. Subjects with non-melanoma-related death, lack of death information, and/or non-cutaneous primary melanoma were excluded from data analysis. Patients with metastatic melanoma of unknown primary origin were included.

2.3 Blood collection and RNA extraction

PAXgene RNA stabilization tubes (2.5 ml; PreAnalytiX, Hombrechtikon, CH) were used to collect, stabilize, and transport whole blood specimens in a closed evacuated system (24). Following the manufacturer's protocol, RNA was extracted from the samples using a PAXgene Blood RNA Kit (PreAnalytiX). The quality of RNA was verified on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and the quantity of RNA was determined by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) before being used in reverse transcription reactions with MMLV reverse transcriptase (Promega, Madison, WI, USA).

2.4 Fractionation of blood cell subtypes and RNA extraction

Blood samples to be fractionated were collected from metastatic melanoma patients at the Cutaneous Oncology Department at the University of Colorado Cancer Center, as mentioned previously (24, 25). The desired human immune cells ($CD3^+$, $CD8^+$, $CD14^+$, $CD15^+$, $CD19^+$, $CD45^+$, and $CD56^+$) were fractionated from collected blood samples using autoMACS^{TM} separator (Miltenyi Biotec, Auburn, CA, USA). Further isolation of T cell subsets was done using flow cytometric sorting of $CD4^+CD25^-CD127^{hi}$ (conventional T cells), $CD4^+CD25^+CD127^{dim}$ (Treg cells) and $CD8^+$ T cells from blood samples. RNA was immediately extracted from fractionated cells using a Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA).

2.5 Quantitative reverse transcription -PCR

qRT-PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on the MX3000P PCR system (Applied Biosystems). The gene expression was normalized

TABLE 1 Overview of patient demographic information of AUS and US melanoma patient cohorts.

		AUS melanoma	US Melanoma
Number of Patients		N = 170	N = 263
Sample taken post non-surgical treatment (%)	No	147 (86.5)	217 (82.5)
	Yes	23 (13.5)	46 (17.5)
Sex (%)	Female	66 (38.8)	121 (46.0)
	Male	104 (61.2)	142 (54.0)
Ulceration (%)	No	125 (84.5)	105 (76.1)
	Yes	23 (15.5)	33 (23.9)
Lymph Nodes (%)	No	124 (72.9)	190 (72.2)
	Yes	46 (27.1)	73 (27.8)
Locoregional recurrence (%)	No	157 (92.4)	247 (93.9)
	Yes	13 (7.6)	16 (6.1)
Progression prior to blood draw (%)	No	133 (81.1)	222 (84.4)
	Yes	31 (18.9)	41 (15.6)
Stage at blood draw (%)	0-II	122 (71.8)	165 (62.7)
	III+	48 (28.2)	98 (37.3)
Death (%)	No	136 (80.0)	198 (75.3)
	Yes	34 (20.0)	65 (24.7)
CTLA4 (median [IQR])		0.00 [0.00, 0.01]	0.01 [0.01, 0.02]
Log *CTLA4* (median [IQR])		-5.63 [-6.00, -5.29]	-4.64 [-5.01, -4.20]
Breslow Thickness (median [IQR])		0.75 [0.29, 2.28]	1.00 [0.52, 2.22]
Years Between Dx and Blood Draw (median [IQR])		1.61 [0.24, 4.41]	0.13 [0.04, 2.19]
Age at Blood Draw (median [IQR])		66.00 [57.00, 78.00]	53.00 [41.00, 63.00]
Years Since Dx (median [IQR])		8.5 [6.38, 11.47]	10.94 [9.2, 11.52]
Years Since Blood Draw (median [IQR])		6.98 [5.46, 7.55]	10.60 [7.68, 11.15]

relative to the housekeeping gene *GAPDH*. Primer sequences are listed in Supplementary Table S1.

2.6 Cell culture of melanoma cell lines

Human primary melanoma cell lines (WM35, WM115, and WM793) and metastatic melanoma cell lines (A375, 1205Lu, and HS294T) were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 (Thermo Scientific, Rockford, IL, USA) supplemented with 10% fetal bovine serum (Gemini Bioproducts, West Sacramento, CA, USA), 100 IU/ml penicillin-100 µg/ml streptomycin (Mediatech, Manassas, VA, USA) at 37°C and 5% CO_2 in the incubator. Melanoma-conditioned media (MCM) was obtained from culture supernatants of human melanoma cells after 24 h of cultivation in OptiMEM (Life Technologies, Grand Island, NY, USA) and centrifuged at 210 × g for 5 min (26). These cell lines have been authenticated using the short tandem repeat (STR) fingerprinting by the Barbara Davis Center Bioresource Core at the University of Colorado Anschutz Medical Campus. Cells were regularly monitored for mycoplasma contamination using PCR.

2.7 Isolation of human PBMCs for cell culture and *CTLA4* quantification

Blood from healthy donors was collected at the Children's Hospital Blood Donor Centre in Aurora, CO, USA, approved under COMIRB#17–0110. We isolated human peripheral blood mononuclear cells (PBMCs) using the density gradient separation media-Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA) and evaluated the effect of melanoma secretome on *CTLA4* expression. The isolated PBMCs were cultured with 50% culture media (RPMI 1640 + 10% fetal bovine serum) and 50% MCM from human primary melanoma cell lines (WM115, WM35, and WM793) and metastatic melanoma cell lines (1205Lu, A375, and HS294T) for 24 h. *CTLA4* expression in control media- and MCM-treated PBMCs was quantified by qRT-PCR as mentioned above. Primer sequences are listed in Supplementary Table S1.

2.8 Flow cytometry analysis

Treg cells ($CD4^+CD25^+CD127^{dim}$) were isolated from human healthy donor PBMCs using a $CD4^+CD25^+CD127^{dim}$ Treg cell Isolation kit (Miltenyi Biotech, San Diego, CA, USA) according to the manufacturer's instructions. Isolated human Treg cells were cultured in 50% lymphocyte cell culture media (RPMI 1640 supplemented with 10% fetal bovine serum, 0.1% β-mercaptoethanol (#21985023; Thermo Scientific, Rockford, IL, USA), 1% non-essential amino acids (#25025; Mediatech, Manassas, VA, USA), and 2 mM glutamine (#35050061; Thermo Scientific, Rockford, IL, USA)) with 50% MCM from 1205Lu cells. *CTLA4* and *FOXP3* expression in control media- and MCM-treated Treg cells were quantified by qRT-PCR. Primer sequences are listed in Supplementary Table S1.

Similarly, to evaluate the effect of melanoma TME on CTLA-4 and FOXP3 expression in Treg cells at the protein level, isolated Treg cells were cultured with and without 50% MCM for 48 h. Cultured Treg cells were stained for cell surface CTLA-4 and intracellular FOXP3 using the respective flow antibodies (BioLegend, San Diego, CA, USA) and FOXP3 Cytoperm/Cytofix staining kit (BD Pharmingen, San Diego, CA, USA) and analyzed using flowcytometry-Gallios 561 (Beckman Coulter, Indianapolis, IN, USA).

Next, to evaluate the effect of MCM or CTLA-4 downregulation in Treg cells on their proliferation and function, we assessed the proliferation of isolated Treg cells treated with 50% MCM or siRNA transfected or control Treg cells using an XTT assay. Briefly, Treg cells transfected with *CTLA4*-siRNA or control-siRNA were seeded into 96-well plates and cultured for 72 h. In another setting, Treg cells were seeded into 96-well plates and treated with or without 50% MCM in lymphocyte cell culture media for 72 h. Cell proliferation was assayed daily by adding XTT compound (2, 3-bis (2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide) (#X6493; Thermo Scientific, Rockford, IL, USA) followed by measuring absorbance at 450 nm in a microplate reader (Bio Tek, Winooski, VT, USA).

Likewise, T cell suppression assay was conducted using the CFSE- (#C34554; Thermo Scientific, Rockford, IL, USA) labeled T conventional (Tconv) cells ($CD4^+CD25^-CD127^{hi}$) in the presence of Treg cells subjected to various treatments. Isolated Treg cells were cultured with 50% MCM + 50% lymphocyte culture media (MCM-treated Treg cells) or 100% lymphocyte culture media (control Treg cells) for 48 h. CFSE-labeled Tconv cells were co-cultured with MCM-treated Treg cells or control Treg cells at ratios (1:1, 1:5, and 1:10; Treg: Tconv) in lymphocyte culture media with CD3/CD28 beads (1:2 beads: Tconv) (#130-095-345; Miltenyi Biotech, San Diego, CA, USA) and recombinant human IL-2 (100 IU/ml) (#202-IL-010/CF; R&D Systems, Minneapolis, MN, USA) for 72 h. In another setting, CFSE-labeled Tconv cells were co-cultured with siRNA-transfected Treg cells (*CTLA4*-siRNA or control-siRNA) for 72 h, as mentioned above. The dilution of CFSE in CFSE-labeled Tconv cells was analyzed using the flowcytometry-Gallios 561 (Beckman Coulter, Indianapolis, IN, USA).

2.9 miRNA bioinformatics tools

Three miRNA prediction bioinformatics tools, TargetScan (27) (https://www.targetscan.org/vert_80/), miRanda (28) (http://www.microrna.org/), and PicTar (29)(http://pictar.bio.nyu.edu/) were used to predict the potential miRNAs targeting the *CTLA4* mRNA. TargetScan was used to obtain the predicted complementary 3'UTR sequence of *CTLA4* to the seed sequence of miR-155 using (27).

2.10 miRNA quantification

miRNAs were extracted from the cells using the mirVana miRNA isolation kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. RT-PCR and qPCR were performed using miRNA-specific TaqMan™ MicroRNA assay kits for miRs-9, -34c, -142, -145, -155, -324, and -449 (Thermo Fisher Scientific, Waltham, MA, USA). U6 snRNA was used as an internal control to normalize miRNA expression (30).

2.11 Transfection of siRNAs, miR-155 mimics, and inhibitors

Human Treg cells were isolated from healthy donor PBMCs and transfected with 50 nM siRNA (a mixture of two preselected siRNAs; Horizon, Boulder, CO, USA) targeting argonaute-2 (*AGO2*), *CTLA4*, 50 nM miRNA mimics (Horizon), miRNA inhibitors (Horizon) targeting miR-155, or their corresponding non-target control using the P3 transfection buffer (Lonza, Hayward, CA, USA) with the protocol EO-104 on the Lonza 4D instrument (Lonza, Hayward, CA, USA). Transfected Treg cells were cultured for 24 h in the lymphocyte culture media with or without 50% MCM from 1205Lu cells. Then, RNA and miRNAs were isolated from cultured Treg cells and quantified for the expression of *AGO2*, *CTLA4*, *FOXP3*, miR-155, U6 snRNA, and *GAPDH*, as mentioned (31). The primers are listed in Supplementary Table S1.

2.12 Analysis of CTLA-4 protein expression in immune cell subtypes

We used the publicly available Cytometry by Time of Flight (CyTOF) data to evaluate the protein expression of CTLA-4 in blood samples of healthy donors (n = 5) and metastatic melanoma patients (n = 10). The clinical information of the cohort (n = 15) (#2 dataset) has been described previously (32). We accessed the normalized CyTOF data from Flow Repository using repository ID: FR-FCM-ZY34 (33) and analyzed CTLA-4 expression in $CD45^+$, $CD4^+$, $CD8^+$, and $CD4^+CD25^+CD127^-$ immune cell subtypes using the FlowJo software (BD Company, Ashland, OR, USA).

2.13 Statistical analysis

Statistical univariable analysis of melanoma-specific survival was determined using R 4.2.0 using Package 'survival' version 3.5-0. An optimal *CTLA4* expression level cut-point associated with overall survival (OS) was identified by incrementing over the range of expression values and dichotomizing patients into low and high expressers of natural log transformed *CTLA4* values that were higher or lower than the cut-point, with the cut-point that provided the strongest separation in OS was selected based on the log-rank test. Cut-points were assessed in increments of 0.01, and any that resulted in a large group imbalance (< 25% of patients in one group) were not evaluated.

The overall survival of melanoma patients with low and high expressers of *CTLA4* was calculated using the log-rank test and Kaplan-Meier (K-M) plot in the total, primary (stages 0 - II), and malignant (stages III – IV+) population of TCGA, Swedish AUS, and USA melanoma cohorts. For survival analyses, death was considered the endpoint, and overall survival was defined as the interval from primary diagnosis to death.

Since sex and age are known to dictate melanoma patient prognosis (34), Cox proportional hazards (Cox PH) regression was used for multivariable analysis. Cox PH analyses were performed with dichotomized (based on optimal cut-point) patient population and continuous *mRNA* expression data (natural log-transformed) after adjusting for age and sex, and disease stage. Two-sided P values < 0.05 were considered statistically significant, and we made no adjustments for multiple comparisons.

Other experimental results are derived from at least 3 independent experiments. The numerical data are expressed as mean ± SEM. Prism (version 7.0d) software (GraphPad Software, Inc, La Jolla, CA, USA) calculated the differences between the groups by one-way ANOVA with Bonferroni's or Dunnett's post-tests. A value $p < 0.05$ was considered significant.

3 Results

3.1 Correlation between lower tumor *CTLA4* mRNA levels and worse prognosis in metastatic melanoma patients across two cohorts

Since the association of tumor *CTLA4* expression with OS in melanoma patients has not been well characterized, we first analyzed this relationship in TCGA melanoma patients. Patients were divided into low (n = 110) and high (n = 176) *CTLA4* expressers based on the optimal cut-point (log –3.7) of *CTLA4* mRNA expression. The K-M survival curve analysis showed that patients with low *CTLA4* expression were associated with worse OS than patients with high *CTLA4* expression ($p = 0.0001$) (Figure 1A), similar to the findings from Goltz et al. (15). Further analysis showed that among these patients, not those with primary melanoma (n = 42) (Figure 1B) but those with metastatic melanoma (n = 244) had a significant association between low *CTLA4* expression and worse OS ($p = 0.0001$) (Figure 1C).

To verify this new finding, we performed a survival analysis with the log-rank test on another publicly available dataset of metastatic melanoma tumors from the Swedish cohort (23). Patients were segregated as low (n = 71) and high (n = 139) *CTLA4* expressers using the optimized cut-point (log –5.17). The results showed that downregulated *CTLA4* expression was associated with worse OS in the Swedish dataset of metastatic melanoma patients ($p = 0.0046$) (Figure 1D), consistent with the observations in the TCGA dataset. These findings collectively indicate that different from previously reported data, *CTLA4* is not upregulated in metastatic melanoma, and downregulated *CLTA4* correlates with poor prognosis in those metastatic melanoma patients.

3.2 Correlation between lower blood *CTLA4* mRNA levels and worse prognosis in metastatic melanoma patients across two cohorts

Although tumor *CTLA4* levels are associated with prognosis in patients with metastatic melanoma, tumor *CTLA4* expression levels are dynamically regulated by various factors and are technically challenging to test. Because CTLA-4 is expressed in immune cells such as Treg cells and activated T cells, we wondered whether we could observe similar or different trends in blood samples of melanoma patients. We collected blood samples from the AUS and US cohorts and analyzed them for *CTLA4* expression. Figure 2A explains the flow chart of blood sample collection from the AUS cohort. An overview of *CTLA4* expression data in the AUS cohort is shown in Supplementary Table S2. The AUS cohort comprised healthy donors (n = 103) and melanoma patients (n = 209, including 158 primary and 51 metastatic cases). Among the 209 patients, 36 primary and 3 metastatic patients were excluded due to non-melanoma-related death or lack of data on patient death, and the remaining 170 patients and 103 healthy donors were included for data analysis. The results showed that the expression levels of blood *CTLA4* in melanoma patients were significantly lower than those in healthy donors ($p = 0.037$) (Figure 2B). This significant difference was due to the significant downregulation of *CTLA4* in metastatic melanoma patients (stages III-IV, n = 48) ($p = 0.0001$) but not in primary melanoma patients (stages 0-II, n = 122) (Figure 2C). As expected, K-M plot analysis showed that OS worsened in melanoma patients as the disease progressed (Supplementary Figure 1), suggesting that blood *CTLA4* levels decline as melanoma progresses and the declined *CTLA4* could correlate with poor prognosis of patients.

Therefore, we categorized 170 melanoma patients as high and low expressers of *CTLA4* based on the optimized cut-point for survival analysis. As shown in Figure 2D, survival curve analysis with an optimal cut point (log –5.91) showed that patients with low blood *CTLA4* expression levels (n = 55) were significantly associated with worse OS compared to those patients with high blood *CTLA4* expression levels (n = 115) ($p = 0.0055$). Further

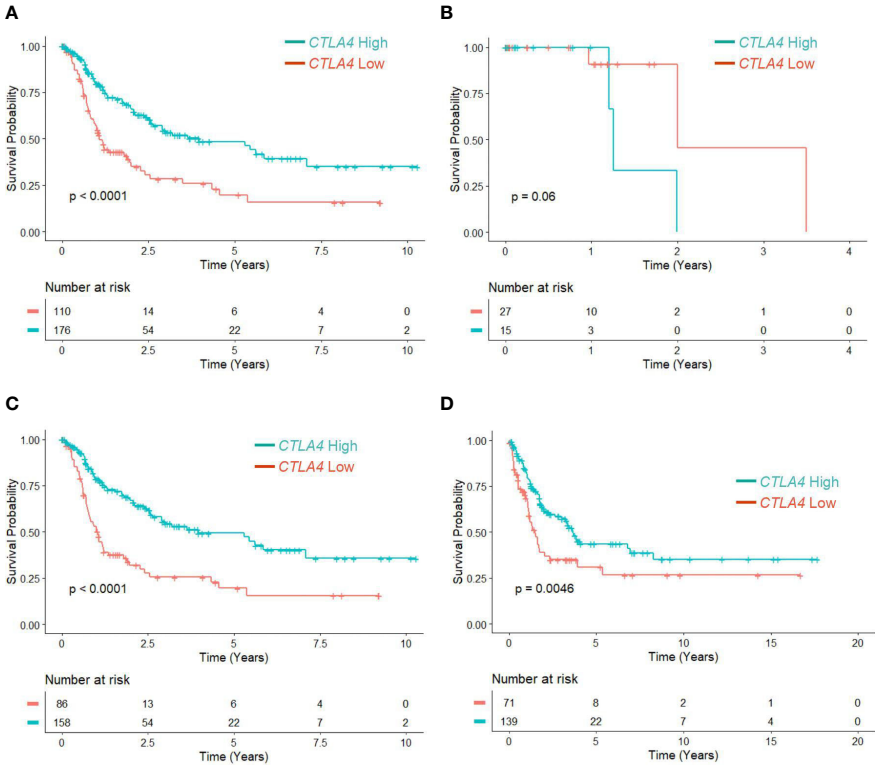

FIGURE 1
The prognostic value of *CTLA4* expression in tumor samples of melanoma patients from TCGA and Swedish cohorts. **(A-C)** Kaplan-Meier survival analyses of TCGA melanoma patients stratified as low and high based on the optimal cut-point of tumor *CTLA4* expression levels in whole melanoma patient population (n = 286) **(A)**, primary melanoma population (n = 42) **(B)**, and metastatic melanoma population (n = 244) **(C)**. **(D)** Kaplan-Meier survival analyses of the Swedish cohort of metastatic melanoma (n = 210). The significance of overall survival between low and high *CTLA4*-expressing patients was calculated by log-rank test.

analysis showed that among these melanoma patients, not those with primary melanoma (stages 0-II, n = 122, $p = 0.14$) (Figure 2E), but those with metastatic melanoma (stages III-IV, n = 48) had a significant association between low blood *CTLA4* expression and worse OS based on the optimal cut point (log –5.93) ($p = 0.017$) (Figure 2F). These data indicate that low tumor and low blood *CTLA4* expression levels are associated with worse OS in metastatic melanoma patients.

As sex and age are well-known determinants of melanoma patient prognosis (34), a multivariable Cox PH analysis was applied, including the confounding effects of sex, age, and stage to compare the cumulative mortality in two log-*CTLA4* level subgroups based on the cut-point in the respective AUS cohorts (Table 2). AUS patients with high (≥–5.91) *CTLA4* levels experienced a 47% lower risk of death (HR = 0.53 [95% CI: 0.27 – 1.06], $p = 0.074$) than patients with lower (<–5.91) *CTLA4*. Specifically, in AUS metastatic melanoma cohort, patients with high (≥–5.93) *CTLA4* experienced a better prognosis and a 55% lower risk of death (HR = 0.45 [95% CI: 0.2 – 1.02], $p = 0.057$) than those with lower (<–5.93) *CTLA4*. In contrast, in the primary melanoma cohort, no assessment was made as the hazard ratio (HR) was not supported by broader CI. These statistical analyses demonstrate that low blood *CTLA4* levels are associated with worse melanoma patient prognosis irrespective of age and sex in this cohort.

To verify our new finding in blood *CTLA4* levels and their prognostic values, we performed another study using the US melanoma cohort, shown in the flow chart (Figure 2G). Similar to the AUS cohort survival analysis, US cohort melanoma patients (n = 263) were segregated as *CTLA4* high and low expressers based on the optimized cut point (log –4.21) for survival analysis. Similar to the AUS cohort, univariable survival curve analysis showed that patients with low blood *CTLA4* expression (n = 196) had a worse prognosis compared to those with high blood *CTLA4* levels (n = 67) ($p = 0.072$) (Figure 2H). Similarly, further analysis showed that not those with primary melanoma (stages 0-II, n = 165, $p = 0.23$) (Figure 2I) but those with metastatic melanoma (stages III-IV, n = 98) had an association between low blood *CTLA4* expression and worse OS based on the optimal cut point (log –4.73); however, it was not statistically significant ($p = 0.053$) (Figure 2J).

Cox PH analysis was also applied to compare the cumulative death rates in two log-*CTLA4* level subgroups based on the cut-point in the respective US melanoma cohort (Table 3). US melanoma patients with higher (≥–4.21) *CTLA4* experienced a 35% lower risk of death (HR = 0.65 [95% CI: 0.34 - 1.26], $p = 0.202$) than those with lower (<–4.21) *CTLA4*. Likewise, in the US metastatic melanoma cohort, patients with higher (≥–4.73) *CTLA4* experienced better prognosis and 24% lower risk of death (HR = 0.76 [95% CI: 0.43 - 1.35], p = 0.353) than those with lower (<–4.73)

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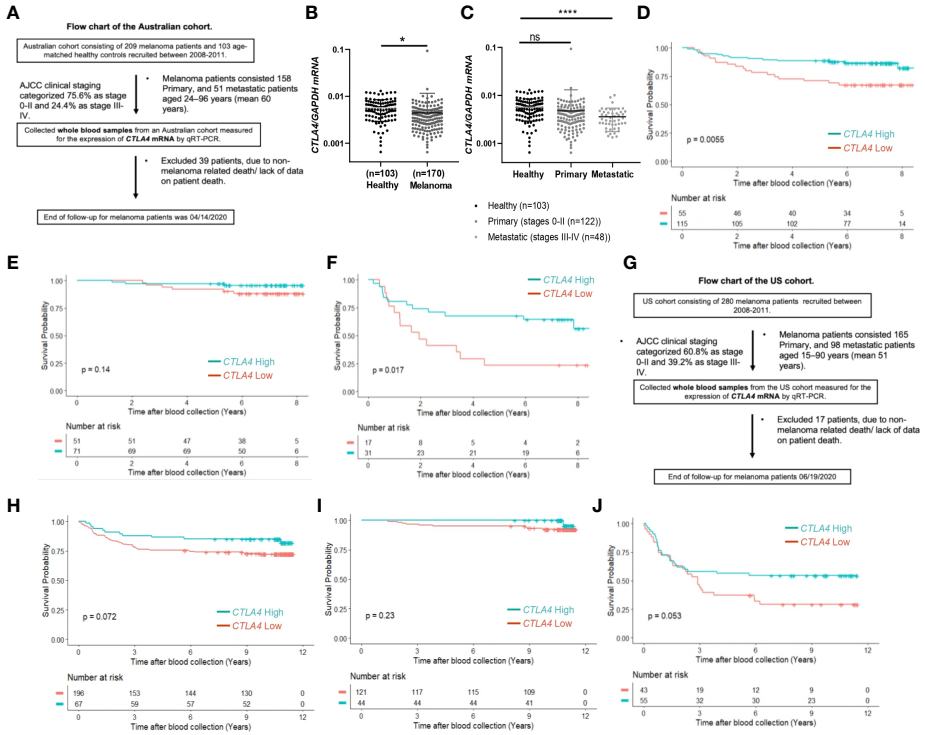

FIGURE 2
The prognostic value of *CTLA4* expression in blood samples of melanoma patients from AUS and US cohorts. **(A-F)** AUS cohort data. **(A)** The flow chart of the AUS cohort. **(B, C)** qRT-PCR analysis of blood *CTLA4* mRNA expression in healthy donors (n = 103) and melanoma patients (n = 170) (**B**), and healthy donors (n = 103), primary melanoma (n = 122), and metastatic melanoma (n = 48) **(C)**. *CTLA4* mRNA expression levels were normalized to *GAPDH* expression. Data are expressed as the mean ± SEM, ns: not significant, $*p < 0.05$, and $****p < 0.0001$. **(D–F)** Kaplan-Meier survival analyses of AUS melanoma patients stratified according to the optimal cut-point of *CTLA4* mRNA expression in blood samples of all melanoma patients (n = 210) **(D)**, primary (stages 0-II) melanoma patients (n = 122) **(E)**, and metastatic (stages III-IV+) melanoma patient (n = 48) **(F)**. **(G-J)** US cohort data. **(G)** The flow chart of the US cohort. **(H-J)** Kaplan-Meier survival analyses of the US cohort with all melanoma patients (n = 263) **(H)**, primary (stages 0-II) melanoma patients (n = 165) **(I)**, and metastatic (stages III-IV+) melanoma patients (n = 98) **(J)**. The significance of overall survival between low and high *CTLA4*-expressing patient groups was calculated by log-rank test.

CTLA4. However, in the primary melanoma cohort, no assessment was made as broader CI did not support HR.

The results from the AUS cohort confirm that lower blood *CTLA4* levels are associated with worse prognosis in metastatic melanoma patients. Although the results from the US cohort did not reach statistical significance, similar trends were observed in patients. Furthermore, the data from the AUS cohort indicate that blood *CTLA4* levels determine the melanoma patient prognosis independent of age and sex.

3.3 Treg cells contribute to reduced blood *CTLA4* mRNA levels in metastatic melanoma patients

After finding that blood *CTLA4* expression was associated with melanoma patients' OS, we aimed to investigate which specific blood cell subtypes were responsible for the downregulation of *CTLA4* expression. To test this, we analyzed blood samples from a different cohort of healthy donors and metastatic melanoma

TABLE 2 Association between survival, log *CTLA4* cut-point, melanoma stage, age, and sex by multivariable Cox PH model for AUS patients (n = 170, 34 events), primary melanoma (n = 122, 9 events), and metastatic melanoma (n = 48, 25 events).

Log(*CTLA4*)	AUS melanoma population			AUS primary melanoma			AUS metastatic melanoma		
	<-5.91 vs ≥-5.91			<-5.76 vs ≥-5.76			<-5.93 vs ≥-5.93		
	HR	95% CI	*p*	HR	95% CI	*p*	HR	95% CI	*p*
Stage (III+ vs. 0-II)	7.94	3.54 - 17.81	<0.001	–	–	–	–	–	–
Log(*CTLA4*)	0.53	0.27 - 1.06	0.074	0.29	0.07 - 1.17	0.082	0.45	0.2 - 1.02	0.057
Sex (males vs. females)	2.44	0.99 - 6.05	0.053	5.32	0.98 - 28.88	0.053	2.12	0.71 - 6.28	0.177
Age (years)	0.99	0.97 - 1.02	0.566	0.92	0.87 - 0.98	0.012	1.00	0.98 - 1.03	0.867

TABLE 3 Association between survival, log CTLA4 cut-point, melanoma stage, age, and sex by multivariable Cox PH model for US patients (n = 263, 65 events), primary melanoma (n = 165, 10 events), and metastatic melanoma (n = 98, 55 events).

Log(*CTLA4*)	US melanoma population			US primary melanoma			US metastatic melanoma		
	<-4.21 vs ≥-4.21			<-4.24 vs ≥-4.24			<-4.73 vs ≥-4.73		
	HR	95% CI	*p*	HR	95% CI	*p*	HR	95% CI	*p*
Stage (III+ vs. 0-II)	12.8	6.48 - 25.29	<0.001	–	–	–	–	–	–
Log(*CTLA4*)	0.65	0.34 - 1.26	0.202	0.37	0.05 - 2.98	0.353	0.76	0.43 - 1.35	0.353
Sex (males vs. females)	1.45	0.83 - 2.53	0.197	2.16	0.42 - 11.01	0.354	1.29	0.71 - 2.35	0.401
Age (years)	1.03	1.01 - 1.05	0.001	1.07	1.02 - 1.13	0.010	1.02	1 - 1.05	0.040

patients in the US (Figure 3). We first confirmed that levels of *CTLA4* were downregulated in whole blood samples from melanoma patients compared to healthy donors (Figure 3A). Along with immune cells, tumor cells also express *CTLA4*, induced by tumor cell-intrinsic b-catenin signaling (35). Because whole blood samples contain circulating tumor cells, we first fractionated the samples into $CD45^+$ (leukocytes) and $CD45^-$ (erythrocytes, platelets, and non-immune cells) fractions (Figure 3B). *CTLA4* was almost exclusively expressed in $CD45^+$ cells but rarely detected in $CD45^-$ cells in healthy donors. This trend was similar in metastatic melanoma patients, while *CTLA4* levels in $CD45^-$ cells were almost doubled compared to healthy donors. Similar to whole blood samples, *CTLA4* levels were downregulated in $CD45^+$ cells from melanoma patients compared to healthy donors.

We then compared *CTLA4* expression in various circulating immune cell fractions, including $CD3^+$ (T cells), $CD14^+$ (monocytes), $CD15^+$ (granulocytes), $CD19^+$ (B cells), and $CD56^+$ (neutrophils), from the two groups. While *CTLA4* expression was not significantly different in $CD14^+$, $CD15^+$, and $CD56^+$ cells between the two groups (Figure 3B), the levels were significantly downregulated by approximately 30% in both $CD3^+$ and $CD19^+$ cells from metastatic melanoma patients compared to healthy donors (Figure 3C).

Since *CTLA4* expression levels were much higher in $CD3^+$ cells than in $CD19^+$ cells, we further fractionated $CD3^+$ cells and analyzed *CTLA4* expression in Tconv cells ($CD4^+CD25^-CD127^{hi}$), Treg cells ($CD4^+$ $CD25^+$ $CD127^{dim}$), and $CD8^+$ T cell subsets (Figure 3D). As expected, *CTLA4* was highly expressed in the Treg cell subset but barely expressed in the CD8+ T cell subset in healthy donors. However, the expected *CTLA4* upregulation was not observed in the Treg cell subset of metastatic melanoma patients. In fact, *CTLA4* expression was downregulated by approximately 70% in Treg cell fractions of metastatic melanoma patients' blood compared to healthy donors. The *CTLA4* expression levels in the Treg cell subset of metastatic melanoma patients were similar to those in the Tconv cell subset of metastatic melanoma patients and were even lower than those in the Tconv cells of healthy donors. These results demonstrate that *CTLA4* expression levels in Treg cells from metastatic melanoma patients are severely downregulated or almost abolished, suggesting an active transcriptional or post-transcriptional inhibition of *CTLA4* mRNA induction in human Treg cells.

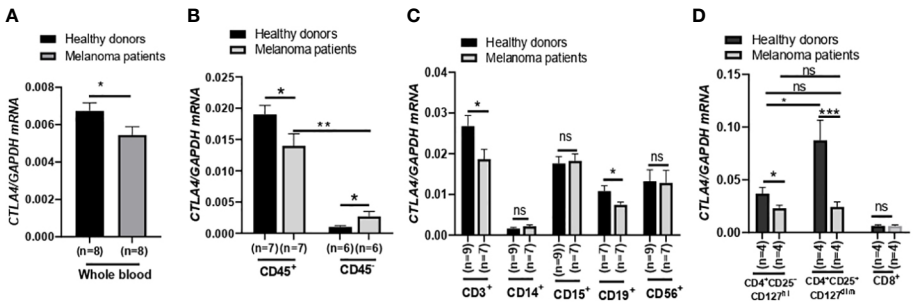

FIGURE 3
Expression of *CTLA4* mRNA and CTLA-4 protein in blood samples of healthy donors and metastatic melanoma patients. **(A)** qRT-PCR analysis of *CTLA4* expression in whole blood of healthy donors and metastatic melanoma patients (n = 8 each). **(B–D)** qRT-PCR analysis of *CTLA4* expression in fractionated $CD45^+$ and $CD45^-$ cells from healthy donors and metastatic melanoma patients (n = 8 each) **(B)**, T cells ($CD3^+$), monocytes/macrophages ($CD14^+$), granulocytes ($CD15^+$), B cells ($CD19^+$), and natural killer cells ($CD56^+$) from healthy donors (n = 9) and metastatic melanoma patients (n = 7) **(C)** and $CD4^+CD25^-CD127^{hi}$ (conventional T cells: Tconv cells), $CD4^+CD25^+CD127^{dim}$ (Treg cells), and $CD8^+$ T cells from healthy donors and metastatic melanoma patients (n = 4 each) **(D)**. *CTLA4* expression was normalized using *GAPDH* as an internal control. Representative data are shown and expressed as the mean ± SEM, ns, not significant, *$p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3.4 Human metastatic melanoma secretome upregulates *FOXP3* but downregulates *CTLA4* in human treg cells and enhances their proliferation and suppressive function

We aimed to understand the mechanisms underlying the downregulation of *CTLA4* expression in human Treg cells. As T-cell function is influenced by receptor signaling through T-cell receptors and cytokines, we hypothesized that the tumor cell secretome downregulates *CTLA4* expression in human Treg cells. To test this hypothesis, we analyzed the effects of human melanoma cell secretomes on *CTLA4* expression in human PBMCs by culturing them with MCM obtained from various human melanoma cell lines. qRT-PCR analysis showed that the expression of *CTLA4* was significantly downregulated in PBMCs treated with MCM from metastatic melanoma cell lines (1205Lu, A375, and HS294T) (Figure 4A). In contrast, MCM from 2 out of three primary melanoma cell lines tested (WM115 and WM35) did not inhibit the expression of *CTLA4* in PBMCs. These data suggest that tumor cell secretome from metastatic melanoma cells mediates the downregulation of *CTLA4* in immune cells.

FOXP3 is a known transcriptional activator of *CTLA4* expression in Treg cells (19). Therefore, we tested whether melanoma cell secretome-mediated *CTLA4* downregulation is regulated at the transcriptional level through FOXP3 in Treg cells. Isolated human Treg cells were cultured with or without 50% 1205Lu-MCM for 24 h. qRT-PCR and flow cytometry analyses confirmed that CTLA-4 expression was downregulated at mRNA (Figure 4B) and protein levels (Figure 4C). However, the mRNA and protein expression of FOXP3 was significantly upregulated in human Treg cells treated with MCM for 24 or 48 h (Figures 4D, E, respectively), suggesting that downregulation of *CTLA4* mRNA in Treg cells occurs at the post-transcriptional level. The time-course experiments showed that the exposure to MCM led to *CTLA4* downregulation in Treg cells by 12 h, and this effect persisted at least 94 h (Supplementary Figure 2).

Since MCM induces downregulation of CTLA-4 and upregulation of FOXP3 in Treg cells, we further assessed the effect of MCM on Treg cell proliferation and suppressive function. Human Treg cells treated with 50% MCM showed enhanced proliferation (Figure 5A) and increased suppressive function compared to control Treg cells without MCM treatment (Figure 5B; Supplementary Figure 3A). These results suggest that MCM augments Treg cell suppressive function by inducing Treg cell proliferation (as shown in Figure 5A) or promoting *FOXP3* expression and functionality in Treg cells (as shown in Figures 4D, E). As MCM upregulates *FOXP3* while downregulating *CTLA4*, we assessed the effect of downregulated *CTLA4* in Treg cells on their proliferation and suppressive function. We generated *CTLA4*-silenced Treg cells using siRNA (Supplementary Figure 3). As shown in Figure 5C, *CTLA4* knockdown did not affect Treg cell proliferation. However, Treg cells with *CTLA4* knockdown significantly suppressed CFSE-stained Tconv cell proliferation compared to control Treg cells (Figure 5D; Supplementary Figure 4B). These data suggest that *CTLA4* plays a role in decreasing Treg cell suppressive function.

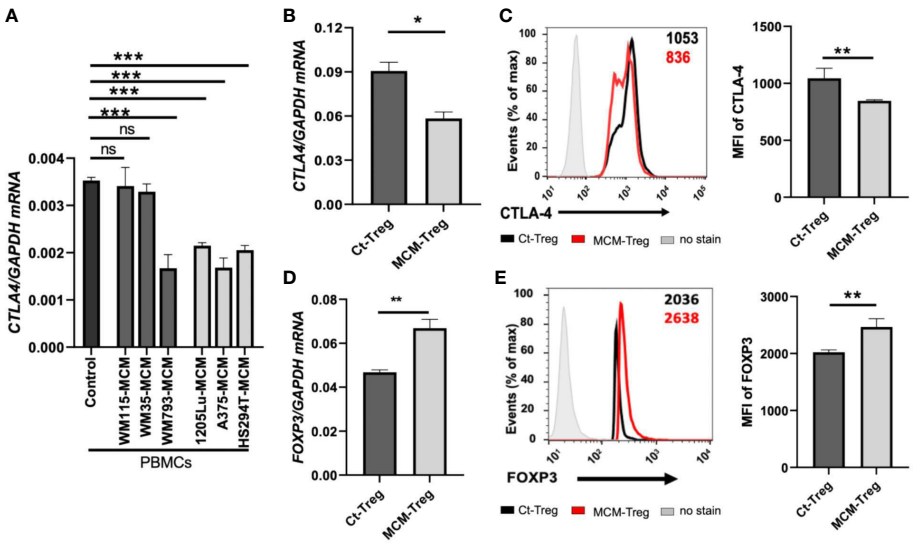

FIGURE 4
The effects of MCM on CTLA-4 and FOXP3 expression in PBMCs and human Treg cells. **(A)** qRT-PCR analysis of *CTLA4* in PBMCs cultured for 24 h in control media or 50% control media + 50% MCM from human primary melanoma cell lines (WM35, WM115, and WM793) or metastatic melanoma cell lines (A375, 1205Lu, and HS294T). **(B)** The qRT-PCR quantification of *CTLA4* mRNA expression in human Treg cells cultured in control media (Ct-Treg) or 50% control media + 50% 1205Lu-MCM (MCM-Treg) for 24 h. The expression of *CTLA4* was normalized using *GAPDH* as an internal control. **(C)** The flow cytometry analysis of CTLA-4 surface protein in Treg cells cultured for 48 h. Treg cells not stained with the antibodies are used as a control ("No stain"). Representative histogram with mean fluorescent intensity (MFI) in black (Ct-Treg) and red (MCM-Treg) (left panel) and the MFI analysis (right panel). **(D)** The qRT-PCR quantification of *FOXP3* mRNA expression in Ct-Treg and MCM-Treg cultured for 24 h. **(E)** The flow cytometry analysis of FOXP3 protein expression in Treg cells cultured for 48 h. Representative histogram with MFI in black (Ct-Treg) and red (MCM-Treg) (left panel) and the MFI analysis (right panel). Representative data are shown and expressed as the mean ± SEM (n = 3). ns: not significant, *$p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

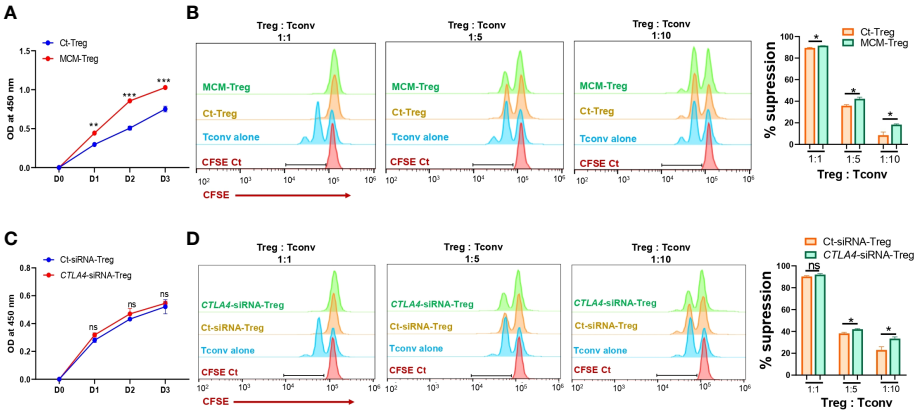

FIGURE 5
The effects of MCM and CTLA-4 on human Treg cell proliferation and functionality. **(A)** XTT colorimetric assessment of the proliferation of human Treg cells cultured in control media (Ct-Treg, blue) or 50% control media + 50% 1205Lu-MCM (MCM-Treg, red) for 72 h. **(B)** The flow cytometry analysis of CFSE dilution in CFSE-labeled Tconv cells co-cultured with control Tregs cells or MCM-treated Treg cells at different ratios (1:1, 1:5, and 1:10, Treg: Tconv) or CFSE-labelled Tconv cells alone in lymphocyte culture media with CD3/CD28 beads and rhIL-2 for 72 h. The representative histogram in orange (Ct-Treg), green (MCM-Treg), blue (Tconv alone), and red (CFSE-labeled Tconv on Day 0) (left panel) and percent suppression (right panel). **(C)** XTT colorimetric assessment of the proliferation of human Treg cells transfected with control siRNA (blue) or *CTLA4* siRNA (red) for 72 h. **(D)** The flow cytometry analysis of CFSE dilution in CFSE-labeled Tconv cells co-cultured with control-siRNA transfected Tregs cells or *CTLA4*-siRNA transfected Treg cells at different ratios (1:1, 1:5, and 1:10, Treg: Tconv) or CFSE-labeled Tconv cells alone in lymphocyte culture media with CD3/CD28 beads and rhIL-2 for 72 h. The representative histogram in orange (Ct-siRNA-Treg), green (*CTLA4*-siRNA-Treg), blue (Tconv alone), and red (CFSE-labeled Tconv on Day 0) (left panel) and percent suppression (right panel). Data are expressed as the mean ± SEM (n = 3). ns, not significant, *$p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3.5 *CTLA4* in human treg cells is post-transcriptionally downregulated by miRNAs induced by melanoma secretome

We investigated the mechanisms of *CTLA4* downregulation by melanoma cell secretome in human Treg cells. Given the opposing effects of the secretome on *CTLA4* and *FOXP3* expression, we hypothesized that the secretome-mediated downregulation of *CTLA4* occurs downstream of gene transcription. To test this, we knocked down *AGO2*, a major component of the miRNA-induced silencing complex, and examined its effect on *CTLA4* gene expression. AGO-2 typically binds to the 3′UTR of cytosolic mRNA targets, resulting in mRNA degradation (36). Human Treg cells were transfected with *AGO2* siRNA and cultured in 50% 1205Lu-MCM for 24 h. As shown in Figure 6A, silencing *AGO2* increased *CTLA4* expression in Treg cells, and this increase was significant in the presence of MCM, suggesting that RNA interference is likely involved in the instability of *CTLA4* mRNA in secretome-treated Treg cells. Therefore, the *CTLA4* downregulation observed in melanoma cell secretome-treated Treg cells was achieved through miRNA-mediated gene silencing at the post-transcriptional level.

To identify miRNAs targeting the *CTLA4* 3′UTR, we first performed an in silico screening of putative miRNA using three bioinformatics tools (PicTar, TargetScan, and miRanda). We selected several potential miRNAs, including miRs-9, -34c, -449, -324-5p, -145, -142, and -155, based on their binding sites spanning the *CTLA4* 3′UTR (Figure 6B). We observed that miR-155 was the only miRNA predicted to target the *CTLA4* 3′UTR by all three prediction tools. To test the involvement of miRNAs in melanoma secretome-mediated *CTLA4* mRNA instability, we cultured human Treg cells in the absence or presence of 50% 1205Lu-MCM for 24 h and analyzed mature miRNA expression by RT-PCR. We found that miR-155 was highly expressed in untreated Treg cells and was further upregulated in Treg cells treated with MCM was highly expressed among these putative miRNAs in untreated Treg cells, whereas other putative miRNAs were downregulated or upregulated to some extent without statistical significance in Treg cells treated with MCM (Figure 6C–I). The complementary alignment of the miR-155 seed sequence with the human *CTLA4* 3′UTR sequence with a 7mer site (Supplementary Figure 5) supports that miR-155 targets the 3′UTR of *CTLA4* mRNA. These data suggest that melanoma cell secretome affects blood *CTLA4* expression through miRNA-mediated gene-silencing, specifically miR-155.

3.6 Upregulation of miR-155 in treg cells of metastatic melanoma patients results in *CTLA4* degradation

To investigate the role of miR-155 in regulating *CTLA4* mRNA expression, we transfected human Treg cells with miR-155 inhibitors or miR-155 mimics and cultured them in the presence of 50% 1205Lu-MCM for 24 h. Gene expression analysis of *CTLA4* and *FOXP3* in Treg cells showed that transfection of human Treg cells with miR-155 inhibitor rescued downregulated *CTLA4* expression without affecting the expression of *FOXP3* (Figures 7A, B), supporting the idea that miR-155 targets *CTLA4* but not *FOXP3*. On the other hand, transfection of human Treg cells with miR-155 mimics in the presence of 50% 1205Lu-MCM did not affect *CTLA4* expression (Figure 7C) but significantly upregulated

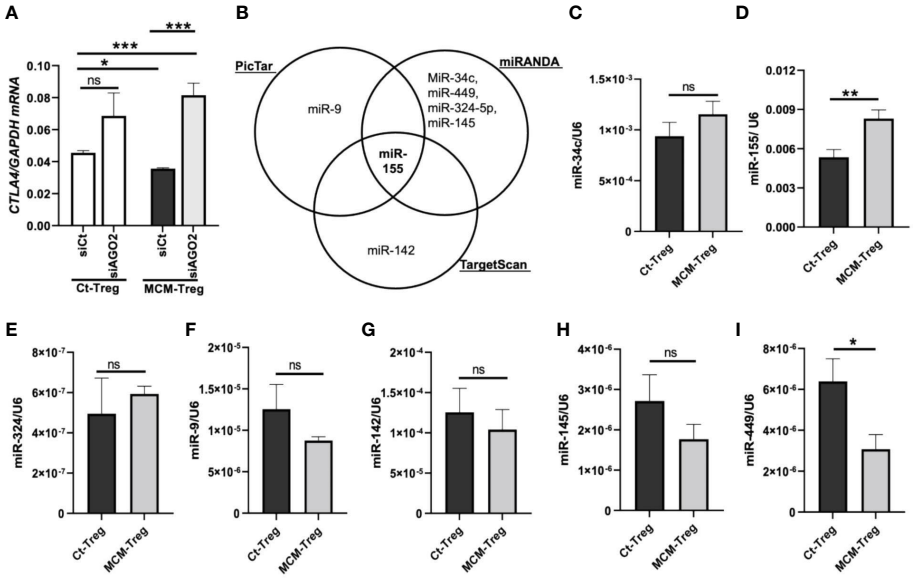

FIGURE 6
MicroRNAs regulation of *CTLA4* expression in human Treg cells. **(A)** qRT-PCR analysis of *CTLA4* expression in human Treg cells transfected with 50 nM control siRNA (siCt) or AGO2 siRNA (siAGO2) and subsequently cultured for 24 h in control media (Ct-Treg) or 50% control media + 50% 1205Lu-MCM (MCM-Treg). The expression of *CTLA4* was normalized using *GAPDH* as an internal control. **(B)** Venn diagram of predicted miRNAs targeting the 3'UTR of *CTLA4* mRNA from three bioinformatics tools (PicTar, miRanda, and TargetScan). **(C-I)** Quantitative analysis of mature miR-34c **(C)**, miR-155 **(D)**, miR-324 **(E)**, miR-9 **(F)**, miR-142 **(G)**, miR-145 **(H)**, and miR-449 **(I)** expression in Ct-Treg and MCM-Treg cells for 24 h. The expression of miRNAs were normalized using U6 snRNA as an internal control. Representative data are shown and expressed as the mean ± SEM (n = 3). ns, not significant, *$p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

FOXP3 expression (Figure 7D), suggesting that the effect of miR-155 on *CTLA4* is limited when the gene expression is already downregulated or miR-155 requires additional factors to be effective. To elucidate the former possibility, we transfected miR-155 mimics into control Treg cells that did not have downregulated *CTLA4*. The transfection of miR-155 mimics did not significantly affect the expression of *CTLA4* in these control Treg cells (Supplementary Figure 6). These findings suggest that miR-155 plays a critical role in downregulating *CTLA4* mRNA in MCM-treated Treg cells but additional factors may be necessary for miR-155 to downregulate *CTLA4* in Treg cells from melanoma.

Furthermore, we analyzed miR-155 expression levels in peripheral $CD3^+$ cells, $CD4^+$ cells, and $CD4^+$ $CD25^+$ cells from melanoma patients and compared them with healthy donors. The results showed that miR-155 expression was upregulated in these T cells in melanoma patients (Figures 7E–G).

4 Discussion

In the current study, we investigated the levels of *CTLA4* mRNA expression and found that low levels of *CTLA4* expression in both tumor tissue and blood cells are associated with poorer overall survival in patients with metastatic melanoma. The study also uncovered that reduced *CTLA4* expression in the blood cells of these patients is due to Treg cells, whose *CTLA4* was silenced at the post-transcriptional level by miR-155. Functionally, we demonstrated that *CTLA4* expression inhibits the proliferation and suppressive function of human Treg cells. These findings shed new light on the pathophysiology of metastatic melanoma and could potentially inform the development of innovative immune-based therapies.

We observed a significant decrease in the *CTLA4* levels in Treg cells from melanoma patients, which were almost equivalent to the levels observed in Tconv cells. CTLA-4 is a major immune checkpoint expressed in activated T cells and constitutively in Treg cells (7), but its role in Treg cells has been debated. Germline deletion of *Ctla4* resulted in severe autoimmunity with lethality (17, 18), which prompted researchers to delineate the effects of *Ctla4* on T cells and Treg cells separately. Tang et al. showed normal development, homeostasis, and uncompromised suppressive activity in *Ctla4*-deficient Treg cells from germline-depleted mice (37). Similarly, Schmidt et al. showed an increased peripheral Treg cell population in germline-depleted mice; however, this phenotype was not observed in thymic Treg cells (38). These data suggest that CTLA-4 negatively regulates the peripheral Treg cells' expansion, and that downregulated CTLA-4 may drive the proliferation of circulating Treg cells without decreasing their suppressive function. The conditional ablation of *Ctla4* in adult Treg cells protected mice from autoimmunity (20), supporting the notion that CTLA-4 is a negative regulator of Treg cell expansion. Our data demonstrated that *CTLA4* expression inhibits the proliferation and suppressive function of human Treg cells, suggesting that CTLA-4 is a negative regulator of Treg cell function.

Using the AUS cohort, we observed a decrease in *CTLA4* expression in the blood of melanoma patients, and this reduction was associated with a poorer prognosis for patients with metastatic melanoma. Similar results were observed from the US cohort, but

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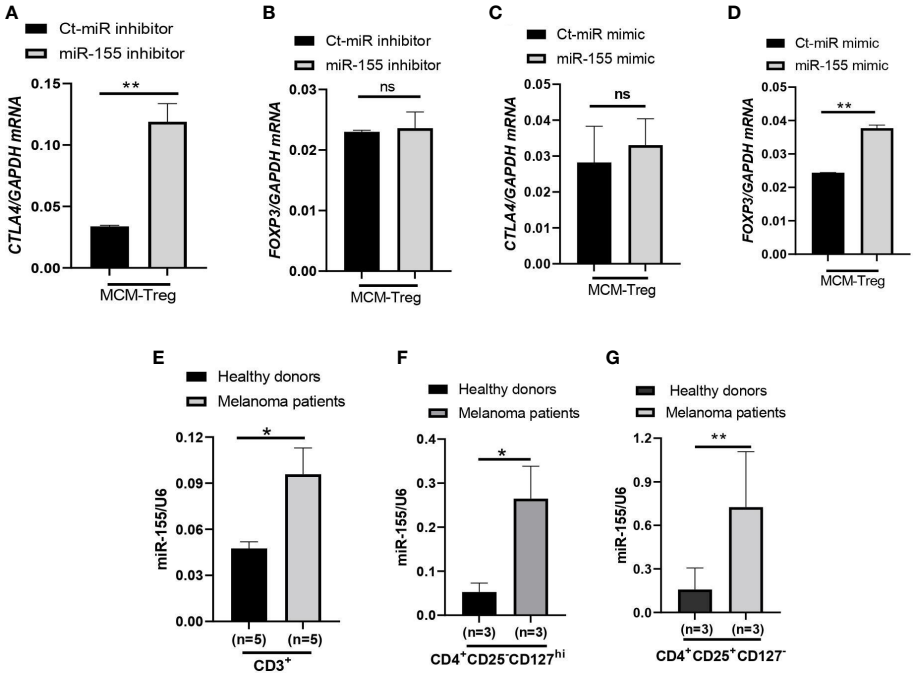

FIGURE 7
miR-155 regulation of *CTLA4* mRNA stability in human Treg cells cultured in MCM and its expression in immune cells of metastatic melanoma patients. **(A, B)** qRT-PCR analysis of *CTLA4* **(A)** and *FOXP3* **(B)** expression in human Treg cells transfected with 50 nM control (Ct-MiR) or miR-155 inhibitors and subsequently cultured in 50% control media + 50% 1205Lu-MCM (MCM-Treg) for 24 h. **(C, D)** qRT-PCR analysis of *CTLA4* **(C)** and *FOXP3* **(D)** expression in human Treg cells transfected with 50 nM control or miR-155 mimics and subsequently cultured in 50% control media + 50% 1205Lu-MCM (MCM-Treg) for 24 h. *CTLA4* and *FOXP3* mRNA levels were normalized with internal control *GAPDH* expression. **(E–G)** Expression of miR-155 in auto-MACS-fractionated $CD3^+$ T cells **(E)**, $CD4^+$ T cells **(F)**, and $CD4^+CD25^+$ T cells **(G)** from healthy donors (n = 3) and metastatic melanoma patients (n = 5 and 3 in E and F-G, respectively). The expression of miR-155 was normalized using U6 snRNA as an internal control. Representative data are shown and expressed as the mean ± SEM (n = 3). ns, not significant, *$p < 0.05$, and ** $p < 0.01$.

they were not supported by the statistical analysis. Further analysis of cohort characteristics indicated that the US cohort was skewed slightly younger and had higher levels of log *CTLA4* compared to the AUS cohort (data not shown), which might have led to marginal disconcordance between two cohorts. Furthermore, the data from the AUS cohort indicate that blood *CTLA4* levels determine the melanoma patient prognosis independent of age and sex, specifically in the AUS cohort. To confirm these results, we analyzed a publicly available CyTOF dataset (32) and found that CTLA-4 protein levels were reduced in circulating immune cells ($CD45^+$), $CD4^+$ T cells, and Treg cells ($CD4^+CD25^+CD127^-$) in metastatic melanoma patients compared to healthy donors (Figures 8A–C). The same paper also reported that higher CTLA-4 protein levels in peripheral $CD4^+$ and $CD8^+$ T cells were associated with a better response to anti-PD1 therapy in metastatic melanoma (32). Additionally, another study reported that *CTLA4* methylation in tumors, which leads to reduced *CTLA4* mRNA, is associated with resistance to anti-PD-1 and anti-CTLA-4 immunotherapy in melanoma patients (15). Therefore, the lack of upregulation of CTLA-4 in either peripheral blood or tumors might make inhibition of not only this molecule but also other checkpoints such as PD-1 ineffective and decrease their efficacy as monotherapy. The mechanism of reducing *CTLA4* mRNA expression in either peripheral blood or tumors may be related to resistance to immunotherapy.

Here, we provide mechanistic insights into the downregulation of *CTLA4* expression in Treg cells from melanoma through miR-155. Various mechanisms upregulate *CTLA4* expression. TCR triggering and CD28 co-stimulation upregulate *CTLA4* in T cells (39), and tumor cell-intrinsic *CTLA4* upregulation can be induced by β-catenin signaling (35). However, Treg cells constitutively express *CTLA4* due to the transcription factor FOXP3 (19). In contrast to the upregulation of *CTLA4*, downregulation of *CTLA4* expression can occur not only due to epigenetic changes, such as promoter methylation (15), but also through RNA interference, which affects the stability of expressed *CTLA4* mRNA, as seen in our current study. Recent studies have highlighted the role of miRNAs in regulating immune checkpoint (PD-1, PD-L1, and CTLA-4) gene expression and their importance as regulators of T-cells and tumor cells (40). While some miRNAs, such as miRs-9, -105, -155, and -487a-3p, directly modulate *CTLA4* expression (40, 41), others like miRs-24 and -210 indirectly downregulate *CTLA4* through direct downregulation of FOXP3 (42). Moreover, miR-155 has been shown to directly target the 3'UTR of *CTLA4* in $CD8^+CD25^+$ Treg cells (43) and helper T-cells, increasing their proliferative response by downregulation of *CTLA4* (44). Consistent with this, our study confirmed that miR-155 downregulates *CTLA4* at the mRNA level without interfering with *FOXP3* expression in circulating Treg cells from melanoma patients. miRNAs regulate target genes through the miRNA-induced silencing complex

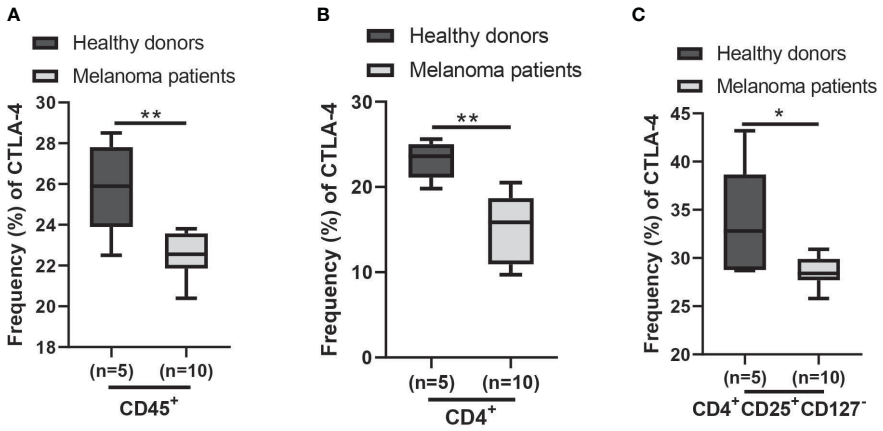

FIGURE 8
Expression of CTLA-4 protein in blood samples of healthy donors and metastatic melanoma patients. Analysis of blood CTLA-4 expression in immune cell subsets of metastatic melanoma patients using the publicly available CyTOF data (dataset #2 results of (32)). The percent frequency of CTLA-4-expressing cells in $CD45^+$ cells **(A)**, $CD4^+$ T cells **(B)**, and $CD4^+CD25^+CD127^-$ T cells (Treg cells) **(C)** from healthy donors (n = 5) and metastatic melanoma patients (n = 10). Blood samples were obtained before the immunotherapy. Representative data are shown and expressed as the mean ± SEM, $*p < 0.05$ and $** p < 0.01$.

(miRSC), which interacts with various molecules, such as RNA-binding proteins, poly (A)-binding proteins, and GW182 (45). While miR-155 inhibitor rescued downregulated *CTLA4* in Treg cells treated with MCM, miR-155 mimic did not affect *CTLA4* expression even in control naïve Treg cells. These data suggest that miR-155 is one of the major factors regulating the stability of blood *CTLA4* mRNA in melanoma but additional factors may be necessary for miR-155 to play this critical role. Further identification of co-factors that regulate *CTLA4* mRNA stability in Treg cells from melanoma patients is needed to fully understand the mechanisms involved.

Furthermore, we show that melanoma secretome induces downregulation of *CTLA4* through miR-155 expression in Treg cells without decreasing their *FOXP3* expression. Similar to other miRNAs, the function of miR-155 is context-dependent (46). While miR-155 has been reported to suppress tumors by dampening anti-tumor immunity in T cells (47), it has also been shown to suppress T helper cell activation (48), enhance myeloid-derived suppressor cells (49), and expand Treg cells (50), indicating its oncogenic role. The induction of miR-155 is mediated by various signaling pathways, including transforming growth factor (TGF) β through Smad4 (51). TGFβ is a major component of the secretome from melanoma cells (25), and has been shown to induce miR-155 expression in various immune cells. Therefore, TGFβ in melanoma secretome may induce miR-155 intrinsically in Treg cells, leading to downregulating their *CTLA4* expression. This could explain why we observed higher upregulation of miR-155 in the T cells and Treg cells from melanoma patients than in healthy donors. Moreover, miR-155 is transcriptionally induced by FOXP3 and is expressed more highly in Treg cells than in other CD4 T cells (52). miR-155 promotes suppressive competence and proliferation by inhibiting the suppressors of cytokine signaling (SOCS)1 in Treg cells (53). Similar to our study, *CTLA4* is also shown to be a direct target of miR-155 (43). These studies suggest that downregulated *CTLA4 via* miR-155 predominantly contributes to the expansion of Treg cells without affecting their suppressive function. In support of this, Paterson and colleagues showed that Treg cells with conditional ablation of *Ctla4* in adult mice remained functionally suppressive and sufficient to protect mice from experimental autoimmune encephalomyelitis (20). CTLA-4 is a negative regulator of Treg cell homeostasis and their cell proliferation (54), and anti-CTLA-4 treatment in tumor models enhances the Treg cell population even though it yields tumor-specific immune responses (55), opposing their therapeutic outcomes in tumor studies. Our study reveals that the post-transcriptional silencing of *CTLA4* by miRNA-155 in Treg cells may contribute to reducing *CTLA4* mRNA expression observed in melanoma patients. These findings suggest that targeting miRNA-155 or other factors involved in regulating *CTLA4* expression in Treg cells without affecting T cells could be a potential approach for improving the efficacy of immunotherapy in melanoma.

5 Conclusions

Our study provides new insights into the underlying mechanisms of reduced *CTLA4* expression observed in melanoma patients, demonstrating that post-transcriptional silencing of *CTLA4* by miR-155 in Treg cells may play a critical role. Our findings suggest that targeting miR-155 or other factors involved in regulating *CTLA4* expression in Treg cells, without affecting T cells, could be a potential strategy to improve the efficacy of immunotherapy in melanoma. These results may have broader implications for other cancers where immune checkpoint inhibition has been shown to be less effective, as these phenotypes have also been observed in anti-PD-1 therapy. Further research is needed to

understand the molecular mechanisms that regulate *CTLA4* and miR-155 expression in Treg cells and identify potential therapeutic targets for enhancing immune-based therapies.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author/s.

Ethics statement

The studies involving human participants were reviewed and approved by Human Research Ethics Committee of Edith Cowan University (No. 2932) and Sir Charles Gardner Hospital (No. 2007-123) for AUS cohort. The US cohort was approved by the Institutional Review Boards of the University of Colorado (COMIRB#05-0309). Blood from healthy donors was collected at the Children's Hospital Blood Donor Centre in Aurora, CO, USA, approved under COMIRB#17–0110. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization, MF. Methodology, PV, DO, AN, DS. Formal analysis, PV, AN, DM, DG, ZZ. Data collection, DS, JM, AR, JD, GN, WR, MZ. Data curation, PV, AN, DG, ZZ, MF. Writing—original draft preparation, PV, AN, NW, JM, ZZ. Writing—review and editing, PV, DO, DM., DG, ZZ, MF. Supervision, MF. Funding acquisition, MZ, MF. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1173035/full#supplementary-material

References

1. Whiteman DC, Green AC, Olsen CM. The growing burden of invasive melanoma: projections of incidence rates and numbers of new cases in six susceptible populations through 2031. *J Invest Dermatol* (2016) 136(6):1161–71. doi: 10.1016/j.jid.2016.01.035

2. O'Neill CH, Scoggins CR. Melanoma. *J Surg Oncol* (2019) 120(5):873–81. doi: 10.1002/jso.25604

3. Siegel RL, Miller KD, Wagle NS, Jemal A. Cancer statistics, 2023. *CA Cancer J Clin* (2023) 73(1):17–48. doi: 10.3322/caac.21763

4. Carlino MS, Larkin J, Long GV. Immune checkpoint inhibitors in melanoma. *Lancet* (2021) 398(10304):1002–14. doi: 10.1016/S0140-6736(21)01206-X

5. Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 system of T cell costimulation. *Annu Rev Immunol* (1996) 14:233–58. doi: 10.1146/annurev.immunol.14.1.233

6. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. *Science* (1996) 271(5256):1734–6. doi: 10.1126/science.271.5256.1734

7. Chambers CA, Kuhns MS, Egen JG, Allison JP. CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. *Annu Rev Immunol* (2001) 19:565–94. doi: 10.1146/annurev.immunol.19.1.565

8. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* (2010) 363(8):711–23. doi: 10.1056/NEJMoa1003466

9. Lipson EJ, Drake CG. Ipilimumab: an anti-CTLA-4 antibody for metastatic melanoma. *Clin Cancer Res* (2011) 17(22):6958–62. doi: 10.1158/1078-0432.CCR-11-1595

10. Weber J, Mandala M, Del Vecchio M, Gogas HJ, Arance AM, Cowey CL, et al. Adjuvant nivolumab versus ipilimumab in resected stage III or IV melanoma. *N Engl J Med* (2017) 377(19):1824–35. doi: 10.1056/NEJMoa1709030

11. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Rutkowski P, Lao CD, et al. Five-year survival with combined nivolumab and ipilimumab in advanced melanoma. *N Engl J Med* (2019) 381(16):1535–46. doi: 10.1056/NEJMoa1910836

12. Liu Y, Zheng P. Preserving the CTLA-4 checkpoint for safer and more effective cancer immunotherapy. *Trends Pharmacol Sci* (2020) 41(1):4–12. doi: 10.1016/j.tips.2019.11.003

13. Maibach F, Sadozai H, Jafari SMS, Hunger RE, Schenk M. Tumor-infiltrating lymphocytes and their prognostic value in cutaneous melanoma. *Front Immunol* (2020) 11. doi: 10.3389/fimmu.2020.02105

14. Van Allen EM, Miao D, Schilling B, Shukla SA, Blank C, Zimmer L, et al. Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. *Science* (2015) 350(6257):207–11. doi: 10.1126/science.aad0095

15. Goltz D, Gevensleben H, Vogt TJ, Dietrich J, Golletz C, Bootz F, et al. CTLA4 methylation predicts response to anti-PD-1 and anti-CTLA-4 immunotherapy in melanoma patients. *JCI Insight* (2018) 3(13):e96793. doi: 10.1172/jci.insight.96793

16. Fietz S, Zarbl R, Niebel D, Posch C, Brossart P, Gielen GH, et al. CTLA4 promoter methylation predicts response and progression-free survival in stage IV melanoma treated with anti-CTLA-4 immunotherapy (ipilimumab). *Cancer Immunol Immun* (2021) 70(6):1781–8. doi: 10.1007/s00262-020-02777-4

17. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, et al. Lymphoproliferative disorders with early lethality in mice deficient in ctla-4. *Science* (1995) 270(5238):985–8. doi: 10.1126/science.270.5238.985

18. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of ctla-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of ctla-4. *Immunity* (1995) 3(5):541–7. doi: 10.1016/1074-7613(95)90125-6

19. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4 control over Foxp3(+) regulatory T cell function. *Science* (2008) 322 (5899):271–5. doi: 10.1126/science.1160062

20. Paterson AM, Lovitch SB, Sage PT, Juneja VR, Lee Y, Trombley JD, et al. Deletion of CTLA-4 on regulatory T cells during adulthood leads to resistance to autoimmunity. *J Exp Med* (2015) 212(10):1603–21. doi: 10.1084/jem.20141030

21. Cancer Genome Atlas N. Genomic classification of cutaneous melanoma. *Cell* (2015) 161(7):1681–96. doi: 10.1016/j.cell.2015.05.044

22. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov* (2012) 2(5):401–4. doi: 10.1158/2159-8290.CD-12-0095

23. Cirenajwis H, Ekedahl H, Lauss M, Harbst K, Carneiro A, Enoksson J, et al. Molecular stratification of metastatic melanoma using gene expression profiling: prediction of survival outcome and benefit from molecular targeted therapy. *Oncotarget* (2015) 6(14):12297–309. doi: 10.18632/oncotarget.3655

24. Luo YC, Robinson S, Fujita J, Siconolfi L, Magidson J, Edwards CK, et al. Transcriptome profiling of whole blood cells identifies PLEK2 and C1QB in human melanoma. *PloS One* (2011) 6(6):e20971. doi: 10.1371/journal.pone.0020971

25. Osborne DG, Domenico J, Luo Y, Reid AL, Amato C, Zhai Z, et al. Interleukin-37 is highly expressed in regulatory T cells of melanoma patients and enhanced by melanoma cell secretome. *Mol Carcinog.* (2019) 58(9):1670–9. doi: 10.1002/mc.23044

26. Okamoto M, Liu W, Luo Y, Tanaka A, Cai X, Norris DA, et al. Constitutively active inflammasome in human melanoma cells mediating autoinflammation *via* caspase-1 processing and secretion of interleukin-1beta. *J Biol Chem* (2010) 285 (9):6477–88. doi: 10.1074/jbc.M109.064907

27. McGeary SE, Lin KS, Shi CY, Pham TM, Bisaria N, Kelley GM, et al. The biochemical basis of microRNA targeting efficacy. *Science* (2019) 366(6472):eaav1741. doi: 10.1126/science.aav1741

28. John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS. Human microRNA targets (vol 2, pg 1862, 2005). *PloS Biol* (2005) 3(7):1328. doi: 10.1371/journal.pbio.0020363

29. Chen K, Rajewsky N. Natural selection on human microRNA binding sites inferred from SNP data. *Nat Genet* (2006) 38(12):1452–6. doi: 10.1038/ng1910

30. Feng B, Cao Y, Chen S, Chu X, Chu Y, Chakrabarti S. miR-200b mediates endothelial-to-Mesenchymal transition in diabetic cardiomyopathy. *Diabetes* (2016) 65 (3):768–79. doi: 10.2337/db15-1033

31. Zhai ZL, Samson JM, Yamauchi T, Vaddi PK, Matsumoto Y, Dinarello CA, et al. Inflammasome sensor NLRP1 confers acquired drug resistance to temozolomide in human melanoma. *Cancers* (2020) 12(9):2518. doi: 10.3390/cancers12092518

32. Krieg C, Nowicka M, Guglietta S, Schindler S, Hartmann FJ, Weber LM, et al. High-dimensional single-cell analysis predicts response to anti-PD-1 immunotherapy. *Nat Med* (2018) 24(2):144–53. doi: 10.1038/nm.4466

33. Spidlen J, Brinkman RR. Use FlowRepository to share your clinical data upon study publication. *Cytom Part B-Clin Cy.* (2018) 94(1):196–8. doi: 10.1002/cyto.b.21393

34. Olsen CM, Thompson JF, Pandeya N, Whiteman DC. Evaluation of sex-specific incidence of melanoma. *JAMA Dermatol* (2020) 156(5):553–60. doi: 10.1001/jamadermatol.2020.0470

35. Spranger S, Bao R, Gajewski TF. Melanoma-intrinsic beta-catenin signalling prevents anti-tumour immunity. *Nature* (2015) 523(7559):231–5. doi: 10.1038/nature14404

36. Li XJ, Wang XY, Cheng ZN, Zhu QB. AGO2 and its partners: a silencing complex, a chromatin modulator, and new features. *Crit Rev Biochem Mol* (2020) 55 (1):33–53. doi: 10.1080/10409238.2020.1738331

37. Tang Q, Boden EK, Henriksen KJ, Bour-Jordan H, Bi M, Bluestone JA. Distinct roles of CTLA-4 and TGF-beta in CD4+CD25+ regulatory T cell function. *Eur J Immunol* (2004) 34(11):2996–3005. doi: 10.1002/eji.200425143

38. Schmidt EM, Wang CJ, Ryan GA, Clough LE, Qureshi OS, Goodall M, et al. CTLA-4 controls regulatory T cell peripheral homeostasis and is required for suppression of pancreatic islet autoimmunity. *J Immunol* (2009) 182(1):274–82. doi: 10.4049/jimmunol.182.1.274

39. Carreno BM, Collins M. The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. *Annu Rev Immunol* (2002) 20:29–53. doi: 10.1146/annurev.immunol.20.091101.091806

40. Kipkeeva F, Muzaffarova T, Korotaeva A, Mansorunov D, Apanovich P, Nikulin M, et al. The features of immune checkpoint gene regulation by microRNA in cancer. *Int J Mol Sci* (2022) 23(16):9324. doi: 10.3390/ijms23169324

41. Skafi N, Fayyad-Kazan M, Badran B. Immunomodulatory role for MicroRNAs: regulation of PD-1/PD-L1 and CTLA-4 immune checkpoints expression. *Gene* (2020) 754:144888. doi: 10.1016/j.gene.2020.144888

42. Fayyad-Kazan H, Rouas R, Fayyad-Kazan M, Badran R, El Zein N, Lewalle P, et al. MicroRNA profile of circulating CD4-positive regulatory T cells in human adults and impact of differentially expressed microRNAs on expression of two genes essential to their function. *J Biol Chem* (2012) 287(13):9910–22. doi: 10.1074/jbc.M111.337154

43. Jebbawi F, Fayyad-Kazan H, Merimi M, Lewalle P, Verougstraete JC, Leo O, et al. A microRNA profile of human CD8(+) regulatory T cells and characterization of the effects of microRNAs on treg cell-associated genes. *J Transl Med* (2014) 12:1-6. doi: 10.1186/s12967-014-0218-x

44. Sonkoly E, Janson P, Majuri ML, Savinko T, Fyhrquist N, Eidsmo L, et al. MiR-155 is overexpressed in patients with atopic dermatitis and modulates T-cell proliferative responses by targeting cytotoxic T lymphocyte-associated antigen 4. *J Allergy Clin Immun* (2010) 126(3):581–U306. doi: 10.1016/j.jaci.2010.05.045

45. Duchaine TF, Fabian MR. Mechanistic insights into MicroRNA-mediated gene silencing. *Csh Perspect Biol* (2019) 11(3):a032771. doi: 10.1101/cshperspect.a032771

46. Svoronos AA, Engelman DM, Slack FJ. OncomiR or tumor suppressor? the duplicity of MicroRNAs in cancer. *Cancer Res* (2016) 76(13):3666–70. doi: 10.1158/0008-5472.CAN-16-0359

47. Huffaker TB, Lee SH, Tang WW, Wallace JA, Alexander M, Runtsch MC, et al. Antitumor immunity is defective in T cell-specific microRNA-155-deficient mice and is rescued by immune checkpoint blockade. *J Biol Chem* (2017) 292(45):18530–41. doi: 10.1074/jbc.M117.808121

48. Goncalves-Alves E, Saferding V, Schliehe C, Benson R, Kurowska-Stolarska M, Brunner JS, et al. MicroRNA-155 controls T helper cell activation during viral infection. *Front Immunol* (2019) 10:1367. doi: 10.3389/fimmu.2019.01367

49. Chen SQ, Wang L, Fan J, Ye C, Dominguez D, Zhang Y, et al. Host miR155 promotes tumor growth through a myeloid-derived suppressor cell-dependent mechanism. *Cancer Res* (2015) 75(3):519–31. doi: 10.1158/0008-5472.CAN-14-2331

50. Schjenken JE, Moldenhauer LM, Zhang BH, Care AS, Groome HM, Chan HY, et al. MicroRNA miR-155 is required for expansion of regulatory T cells to mediate robust pregnancy tolerance in mice. *Mucosal Immunol* (2020) 13(4):609–25. doi: 10.1038/s41385-020-0255-0

51. Kong W, Yang H, He L, Zhao JJ, Coppola D, Dalton WS, et al. MicroRNA-155 is regulated by the transforming growth factor beta/Smad pathway and contributes to epithelial cell plasticity by targeting RhoA. *Mol Cell Biol* (2008) 28(22):6773–84. doi: 10.1128/MCB.00941-08

52. Kohlhaas S, Garden OA, Scudamore C, Turner M, Okkenhaug K, Vigorito E. Cutting edge: the Foxp3 target miR-155 contributes to the development of regulatory T cells. *J Immunol* (2009) 182(5):2578–82. doi: 10.4049/jimmunol.0803162

53. Lu LF, Thai TH, Calado DP, Chaudhry A, Kubo M, Tanaka K, et al. Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein. *Immunity* (2009) 30(1):80–91. doi: 10.1016/j.immuni.2008.11.010

54. Tang AL, Teijaro JR, Njau MN, Chandran SS, Azimzadeh A, Nadler SG, et al. CTLA4 expression is an indicator and regulator of steady-state CD4(+)FoxP3(+) T cell homeostasis. *J Immunol* (2008) 181(3):1806–13. doi: 10.4049/jimmunol.181.3.1806

55. Marangoni F, Zhakyp A, Corsini M, Geels SN, Carrizosa E, Thelen M, et al. Expansion of tumor-associated treg cells upon disruption of a CTLA-4-dependent feedback loop. *Cell* (2021) 184(15):3998. doi: 10.1016/j.cell.2021.05.027

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Tuberculosis infection following immune checkpoint inhibitor treatment for advanced cancer: a case report and literature review

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Objective: To investigate the clinical features of active tuberculosis (TB) infection due to immune checkpoint inhibitors (ICIs) treatment in patients with advanced cancer.

Methods: We report the diagnosis and treatment of a case of pulmonary malignancy (squamous cell carcinoma, cT4N3M0 IIIC), secondary to active TB infection following ICIs therapy. Moreover, we summarize and analyze other related cases collected from the China National Knowledge Infrastructure (CNKI), Wanfang Database, PubMed, the Web of Science, and EMBASE (up to October 2021).

Results: A total of 23 patients, including 20 males and 3 females who were aged 49-87 years with a median age of 65 years, were included in the study. Twenty-two patients were diagnosed by Mycobacterium tuberculosis culture or DNA polymerase chain reaction (PCR), while the remaining patient was diagnosed by tuberculin purified protein derivative and pleural biopsy. One case had an interferon-gamma release assay (IGRA) to rule out latent TB infection prior to the application of ICI. Fifteen patients received an anti-tuberculosis regimen. Among the 20 patients with a description of clinical regression, 13 improved and 7 died. Seven of the patients who improved were treated with ICI again and four of them did not experience a recurrence or worsening of TB. The case diagnosed in our hospital also improved after receiving anti-TB treatment after stopping ICI therapy, and continued chemotherapy on the basis of anti-TB treatment, and his condition is relatively stable at present.

Conclusion: Due to the lack of specificity of TB infection following ICIs therapy, patients should be followed for fever and respiratory symptoms for 6.3 months after drug administration. It is recommended that IGRA should be performed before ICIs therapy and the development of TB during immunotherapy in patients who are positive in IGRA should be closely monitored. The symptoms of TB in most patients can be improved with ICIs withdrawal and anti-TB treatment, but there is still a need to be alert to the potentially fatal risk of TB.

KEYWORDS

immune checkpoint inhibitors, tuberculosis, drug-related adverse reactions, interferon-γ release assay, tuberculosis screening

1 Introduction

Mycobacterium tuberculosis (MTB) poses a huge burden on the whole world, with 1.7 billion people worldwide estimated to be potentially infected with tuberculosis (TB) (1). Several studies have shown that patients with hemopathy and malignant solid tumors like head and neck cancers and lung cancers are at increased risk of developing TB (2, 3). Some guidelines, including that of the United States Preventive Services Task Force (USPSTF), suggest patients at higher risk of developing active TB should take latent TB screening (4).

Immunotherapy represents an important and growing area of clinical oncology. Immune checkpoint inhibitors (ICIs) exert their antitumor activity by inhibiting the suppression pathway of immune cells, which can enhance the activity of immune cells and reduce T cell depletion (5). Currently, approved checkpoint inhibitors include cytotoxic T lymphocyte-associated antigen-4 (CTLA4) inhibitors, programmed death receptor-1 (PD-1), and programmed death ligand-1 (PD-L1) inhibitors. Despite the significant effects of ICIs, immune-related adverse events usually occur during treatment, with increasing reports of MTB infections occurring during the treatment of ICIs (Table S1). Clinical studies have shown that immune checkpoint pathways, such as the PD-1 and PD-L1 axes, play an important role in immune homeostasis in TB, and that the deficiency of PD-1 leads to the worsening of TB in animal models (6). After being exposed to tuberculosis, PD-1-deficient mice developed necrotizing pulmonary lesions with abundant acid-fast bacilli, characterized by many granulomas, local necrosis, and neutrophil infiltration (7). Laboratory tests showed a greatly increased MTB-specific CD4 T cell immune response and high levels of inflammatory cytokines in the lung of PD-1 deficient mice (7). This suggests that inhibition of PD-1 may enhance CD4 T cell-mediated immunity, leading to excessive inflammatory response and tissue destruction in the context of TB infection, thereby reactivating tuberculosis (6, 8, 9). In addition, Langan and others (10) summarized all published cases of TB infection during ICIs immunotherapy and pointed out that patients with non-small cell lung cancer treated with ICIs are at increased risk because activation of TB may result from activation of specific immune cell subsets. This evidence suggests that ICIs treatment may directly or indirectly lead to TB infection, but the exact mechanism remains unclear.

In the present study, a case of active TB associated with the application of camrelizumab in 2022 was studied and a systematic review of the collected related literature was taken to further elucidate the potential impact of ICIs on the development of active TB.

Abbreviations: AFB, acid fast bacilli; ART, antiretroviral therapy; BAL, bronchoalveolar lavage fluid; CEA, carcinoembryonic antigen; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4; CNKI, China National Knowledge Infrastructure; HIV, human immunodeficiency virus; hrze, isoniazid, rifampicin, pyrazinamide, ethambutol; ICIs, immune checkpoint inhibitors; IGRA, interferon-gamma release assay; irAEs, immune-related adverse events; MTB, Mycobacterium tuberculosis; NSE, neuron-specific enolase; PCR, polymerase chain reaction; PD-1, programmed death receptor-1; PD-L1, programmed death ligand-1; TB, Tuberculosis; TST, tuberculin skin test

2 Subjects and methods

2.1 Subjects

One patient with active TB infection following camrelizumab treatment who was admitted to Shengli Oilfield Central Hospital and patients collected through literature search with TB associated with ICIs.

2.2 Methods

We first consulted and organized the electronic medical records of the patient admitted to Shengli Oilfield Central Hospital to form a case report. Moreover, we searched through the China National Knowledge Infrastructure (CNKI) and Wanfang Database with the terms 'ICIs' and 'TB' up to October 2021, but no similar reports were found in China. Then, the compound terms 'TB' and 'PD-1' or 'PD-L1' or 'CTLA-4' or 'pembrolizumab' or 'nivolumab' or 'caliplimab' or 'atezolizumab' or 'avelumab' or 'duvalumab' or 'ipilimumab' or 'temlimumab' were searched for on PubMed and the Web of Science. Furthermore, 'TB' and ('pembrolizumab', 'nivolumab', 'cymplimab', 'atezolizumab', 'avelumab', 'duvalumab', 'ipilimumab' or 'temlimumab') were searched for on EMBASE. Each of the obtained results was relevant to the topic of TB in ICIs therapy, and the data provided in the cases were relatively complete.

Figures 1A, B show the lung window scans of enhanced chest CTs, which describe the contents of the left upper lobe of the lung with bronchial truncation and inflammation of the left lower lobe of the lung, respectively. All scans were from different slices of the same patient on the same date. The following comparative CT slices were all obtained from the same machine, and each slice was corresponding to the previous one, so they can be directly compared.

In addition, an Excel data extraction sheet was applied to extract relevant data on patients from clinical records and literature, including general data (such as gender, age, region, and primary tumor), ICIs application, applications of other immunosuppressants, the development of TB (time lag from ICIs administration to TB occurrence and laboratory results of TB), treatment and regression of TB, and the reapplication of ICIs. Finally, we performed statistical analysis of the collected data.

3 Results

3.1 Case report

The patient, a 68-year-old male, was admitted to our hospital because of "cough and sputum with breathlessness for 3 months". The patient presented with cough and sputum 3 months ago. Specifically, the patient had a paroxysmal cough with white

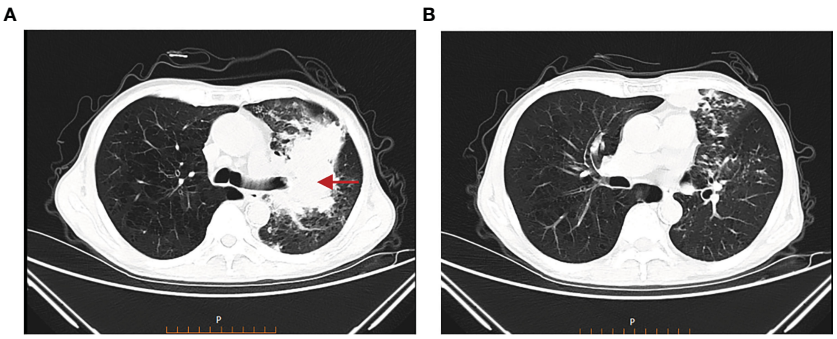

FIGURE 1
Chest CT on 05 March 2022. **(A)** Truncated bronchi in the upper lobe of the left lung, multiple small patchy and nodular high-density shadows in the surrounding lung. **(B)** Multiple small nodules and small patchy high-density shadows in the lower lobe of the left lung.

mucous sputum, accompanied by chest distress, shortness of breath, and numbness of both lower limbs. He had a previous history of smoking for 50 years (20 cigarettes/day) but had quit smoking for 5 months. The rest of his past medical history was negative.

Physical examination: general condition was acceptable, superficial lymph nodes were not palpably enlarged, and the breath sounds of both lungs were rough without rhonchus and moist rales. No abnormalities were found on cardiac or abdominal examination.

The examinations undertaken are listed in Table 1. All images were taken by contrast-enhanced CT.

1. Chest CT plain scan and enhanced scan: (Figures 1A, B, 05 March 2022): a mixed-density mass measuring approximately 12.6 × 7.6 cm was found on the left upper lobe of the lung, with truncated bronchi in the upper lobe of the left lung, localized thinning of the left pulmonary artery, and multiple small patchy and nodular high-density shadows in the surrounding lung; multiple small nodules and small patchy high-density shadows were found in the lower lobe of the left lung.

2. Cranial MRI plain scan and enhanced scan (06 March 2022): abnormal lesion signal shadow was found in the anterior horn of the left lateral ventricle, which was presumed to be a cavernous hemangioma.

3. Tracheoscopy (09 March 2022): cauliflower-like neoplasm was found to block the orifice in the upper lobe of the left lung, and the opening of the lower lobe was slightly compressed and narrowed; no abnormalities were found in the bronchial tubes of the bilateral lobes.

4. Tumor markers (03 March 2022): carcinoembryonic antigen (CEA) was 6mg/ml, CA125 was 88.18U/ml, section 19 of cytokeratin was 12.91ng/ml, and neuron-specific enolase (NSE) was 33.72ng/ml.

There were no abnormalities in the routine blood test, whole-body bone imaging, biochemistry analysis, blood clotting, echocardiography, and electrocardiogram.

Pathological findings: the moderately differentiated squamous cell carcinoma was revealed (through bronchoscopy biopsy of left upper lobe lung). Immunohistochemical findings: CK5/6 (+), CK7 (-), Ki-67 (+, 50%-60%), NapsinA (-), P40 (+), TTF-1 (-), and P63 (+). No abnormalities were found in etiological examinations for bacteria, fungus, *M. tuberculosis*, Pneumocystis carinii, *Actinomyces*, nocardiosis, and cytomegalovirus.

Final diagnosis: there was a malignant tumor (squamous cell carcinoma, cT4N3M0 IIIC) with obstructive pneumonia in the upper lobe of the left lung. Since the patient had no surgical indications, he was suggested for chemotherapy combined with radiotherapy and PD-L1 testing. Genetic testing showed negative results for EGFR, ALK, ROS1, and PD-L1 expression.

Treatment:

Treatment course: a brief description of the treatment course is shown in Figure 2. After excluding contraindications, the first cycle of DP chemotherapy (docetaxel 120mg d1 + cisplatin 40mg d1-d3) was administered on 15 March 2022. Here, the chemotherapy went smoothly and the patient did not have any complaints. Then, the second cycle of chemotherapy was administered on 05 April 2022 with the same regimen as the first cycle. During chemotherapy, the patient had a severe gastrointestinal reaction and bone marrow suppression, with white blood cells falling to 1.3×10^9/L and neutrophils dropping to 0.23×10^9/L. Also, the patient was given symptomatic treatment such as leuke-raising injections but refused to accept another cycle of chemotherapy. After excluding contraindications, he was treated with 200 mg of camrelizumab for immunotherapy on 25 April 2022. On 21 May 2022, the patient began to cough up blood in phlegm with a volume of about 20 ml and he was reviewed using a chest CT on 22 May 2022 (Figures 3A, B), which showed that the lung cancer in the upper lobe of the left lung was less extensive than before, while multiple small nodules and small patchy high-density shadows in both lungs were more extensive than before, which could not exclude pneumonia or infection associated with ICIs. The patient was diagnosed with a tracheoscopy and a 13-item nucleic acid test for respiratory pathogens in the lavage fluid, which was positive for MTB. Moreover, the acid-fast bacillus stain test was positive. Therefore, the patient was considered to have active TB. Multidisciplinary

TABLE 1 The examination results of the patient in our hospital.

Examination	Results		
	Indicators	Normal person	Patient
Chest CT plain scan + enhanced scan (Figure 1A, B, 05 March 2022)	Mass	–	~ 12.6 × 7.6 cm
	Truncated bronchi	–	+
	Pulmonary artery	Normal	Localized thinning
Cranial MRI plain scan + enhanced scan (06 March 2022)	Signal shadow	Normal	Abnormal
Tracheoscopy (09 March 2022)	Cauliflower-like neoplasm	–	
	Bronchial tubes of the bilateral lobes	Normal	Normal
Tumor markers (03 March 2022)	CEA	≤ 5 ng/ml	(+) 6 mg/ml
	CA125	0-35 U/ml	88.18 U/ml
	cytokeratin	0.10-4 ng/ml	12.91 ng/ml
	NSE	< 13 μg/ml	33.72 ng/ml
Blood routine	Normal		
Biochemistry analysis			
Coagulation function			
Echocardiography			
Electrocardiogram			
Whole-body bone scintigraphy			
Pathological findings	CK5/6	<10% (-)	+
	CK7	–	–
	Ki-67	–	+, (50%-60%)
	Napsin A	–	–
	P40	–	+
	TTF-1	–	–
	P63	–	+
	bacteria	Normal	
Chest CT (Figure 3A, B, 22 May 2022),	Cancer area	–	Less extensive
	Mass	–	More extensive
13-item nucleic acid test (lavage fluid)	MTB		+
Acid-fast bacillus stain (tracheoscopy lavage solution)	Acid-fast bacillus	–	+
CT enhanced scan (19 August 2022)	Mass	–	Less extensive

oncology consultation suggested that the patient should stop immunotherapy and be treated with a four-drug anti-TB regimen of isoniazide, rifampicin, ethambutol, and pyrazinamide. After 3 months of anti-TB treatment, the patient was reviewed using an enhanced CT scan on 19 August 2022, which showed that the primary focus of cancer in the right lung was stable and the nodular and small patchy high-density shadows in both lungs were less extensive than before (Figures 4A, B). Finally, the lung was stable, and no further ICIs were administered.The patient is currently in a relatively stable condition and is being followed up.

3.2 Patient characteristics

There were 23 patients, 20 men and three women aged 49-87 years, with a median age of 65 years. Among the 19 patients with a specified

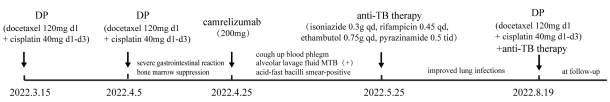

FIGURE 2
The timeline of treatment of the patient in our hospital.

race, there were six Caucasian, two Japanese, three Chinese, two Greeks, one Vietnamese, one Belgian, and four Korean. Of these patients, there were twelve cases of non-small cell lung cancer, one case of undifferentiated lung cancer, five cases of melanoma (including one case of ocular melanoma), two cases of oral squamous cell carcinoma, one case of nasopharyngeal carcinoma, one case of Hodgkin lymphoma, and one case of Merkel cell carcinoma.

3.3 Application of immunotherapy and other therapies

All patients were treated with PD-1 inhibitors. Only one patient (9) received the treatment of CTLA-4 inhibitor (Ipilimumab) before pabrolizumab. Thirteen patients were treated with nivolumab, eight patients were treated with pembrolizumab, one patient was treated with atezolizumab, and one patient was treated with camrelizumab. Thirteen of 18 patients with prior treatment data received chemotherapy. Among the 20 patients that developed active MTB infection, four patients (10, 11) required steroids or infliximab to treat immune-related adverse events (irAEs) and a further two patients (12, 13) had immune-related adverse events such as Sjogren's syndrome and adrenal insufficiency before the diagnosis and treatment of TB, but the specific treatment of immune-related adverse events for these patients was not documented. There were no reports about the application of immunosuppressive drugs in these cases except for the six patients mentioned above who might have had immunosuppressive drugs applied to treat immune-related adverse reactions.

3.4 TB screening and occurrence before the treatment

Only one patient (14) screened negative for TB with an IGRA before starting with immunization. Two other patients with TB were initially thought to have developed secondary lesions of primary cancer. One patient (13) with melanoma developed a right upper lobe pulmonary nodule during treatment with pabrolizumab. One patient (11) with lung adenocarcinoma presented with pericardial thickening, which was also initially thought to be related to cancer until it had not subsided with the treatment of nivolumab.

Two of the 23 patients were presumed to have latent TB before the application of ICIs. One of them (8) was from Vietnam, where TB is endemic, and the other (15) had contact with a patient with active TB 10 years ago. For three other patients (12, 16, 17) it was explicitly stated that TB represented reactivation, but the reasons for this were not given.

3.5 Diagnosis of TB

Twenty-two of the 23 cases were confirmed to have TB by the culture of Mycobacterium TB or by PCR analysis of TB DNA. The analytical specimens were mainly sputum or bronchoalveolar lavage fluid, but reactivation of TB in other organs (pericardium, bone, liver, and gastrointestinal tract; one case each) was also recorded. The remaining case (18) was diagnosed mainly by purified tuberculin skin test and pathological findings of caseous granuloma on pleural biopsy and symptomatic remission with four anti-TB drugs. The imaging mainly showed new or worsening nodules, whose pathological biopsies were all granulomatous inflammation. The time lag from initiation of ICIs therapy to the diagnosis of TB of these patients ranged from 1 month to 2 years, with a median of 6.3 months.

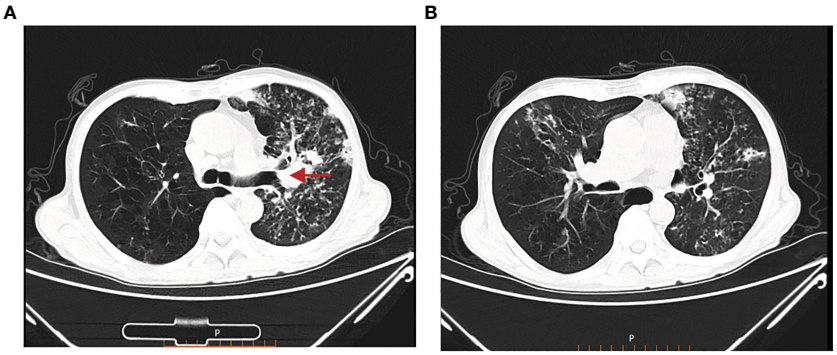

FIGURE 3
Chest CT on 22 May 2022. **(A)** The lung cancer in the upper lobe of the left lung was less extensive than before. **(B)** Multiple small nodules and small patchy high-density shadows in both lungs were more extensive than before.

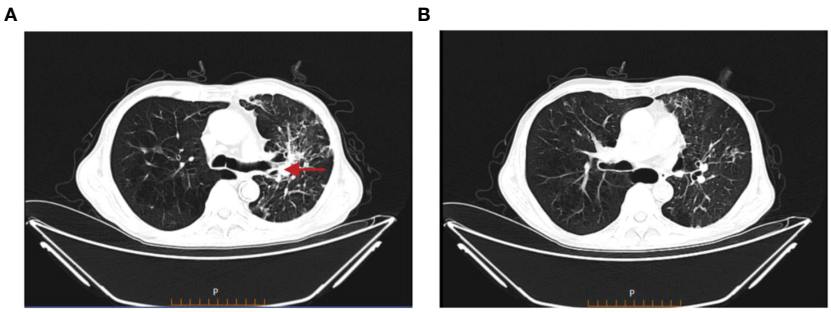

FIGURE 4
Chest CT on 19 August 2022. **(A)** The primary focus of cancer in the right lung was stable. **(B)** The nodular and small patchy high-density shadows in both lungs were less extensive than before.

3.6 Effectiveness of anti-TB and ICIs therapy

Immunotherapy was discontinued in all but five patients (10, 18, 19) (three (19) of these cases are unknown) after the diagnosis of TB. Seven patients restarted ICIs therapy, two of whom (11, 20) after finding resolution of TB symptoms and no recurrence one month after discontinuation of ICIs. Four other patients (12, 13, 21, 22) suspended immunotherapy after diagnosis of TB but restarted with immunotherapy before completion of anti-TB treatment. One patient (8) restarted with ICIs therapy at the time of progression of the primary disease, with the metastatic mass shrinking.

The specific anti-TB regimens of eight patients were not listed. Of the other 15 patients (including the patient in the study), 11 of them were initially treated with isoniazid, rifampicin, pyrazinamide, and ethambutol (8, 10, 13, 15, 19, 21, 22), one of them (16) was initially treated with rifampicin, isoniazid, and ethambutol, and two (18) of them were treated with four drugs not specified. Some patients changed their regimens due to adverse events during treatment.

Seven patients died during follow-up. The first (18) died intraoperatively while attempting to remove a tuberculoma that caused spinal cord compression. The second (8) died of intestinal perforation due to disseminated TB. The third (17) died of respiratory failure secondary to pneumonia, but it was unclear whether this was related to TB. The fourth (22) had apparently an improved TB condition and restarted with nivolumab, but eventually died of respiratory failure in the progression of lung adenocarcinoma. The fifth (10) died of acute respiratory failure after 3 days of anti-TB treatment. The sixth (19) died due to peritonitis, and the seventh (19) died of the progression of lung adenocarcinoma. Of these seven deaths, two were directly attributable to the TB, and the third might have been directly or indirectly related to the TB as he died of respiratory failure after developing pneumonia in the setting of active TB.

4 Discussion

Our results suggest that inhibition of the PD-1/PD-L1 pathway is associated with the development of TB, and the ICIs leading to TB was a PD-1 inhibitor in all cases. Moreover, there were no cases of TB disease found that occurred during treatment with CTLA-4 inhibitors. In the majority of cases, TB-related signs and symptoms improved with TB treatment, but two patients died of TB-related complications. Moreover, our findings also suggest that active TB can develop in a variety of organs other than the lungs.

It has been demonstrated that the PD-1/PD-L1 pathway is closely related to the pathophysiology of TB. PD-1 (CD279/PDCD1) is a cell surface inhibitory receptor that is expressed on activated T and B cells upon binding to the Ag receptor. PD-1 binds to either of its two ligands, PD-L1 (B7-H1/CD274) and PD-L2 (B7-DC/CD273), inhibiting T cell proliferation and cytokine secretion. ICIs can relieve the immunosuppression of T cell activation by tumor cells, and promote the activation and proliferation of T cells, thereby killing tumor cells. When the body is infected with TB, MTB is phagocytosed by macrophages and presented to T cells after processing by antigen-presenting cells, and then T lymphocytes secrete a variety of cytokines, such as γ interferon (IFN-γ) and tumor necrosis factor (TNF)-α, to activate the anti-TB activity of macrophages. When TB granulomas are formed, the body presents latent tuberculosis infection, at which the expression of PD-1 increases, inducing apoptosis of T cells. ICIs can block PD-1, restore the function of lymphocytes, release inflammatory cytokines such as IFN-γand TNF-αin T cells again, and excessive inflammatory cells and cytokines destroy the extracellular matrix, which is conducive to the growth of MTB, disrupts the homeostatic control of latent pulmonary tuberculosis infection, and active tuberculosis infection occurs (Figure 5). Studies have shown that the type I IFN signaling pathway is enhanced in patients receiving PD-1 blockade. TB activity represents an enhanced immune response to a pre-existing pathogen in the presence of PD-1 or PD-L1 inhibition, which is similar to the immune reconstitution inflammatory syndrome in HIV (human immunodeficiency virus) (14, 18). Furthermore, corticosteroids may have a role in the treatment of the immune reconstitution inflammatory syndrome as it is associated with an enhanced immune response. Therefore, the developing rate of immune reconstitution inflammatory syndrome associated with TB in HIV patients can be reduced with the combined application of prednisone and antiretroviral therapy (ART) (23). In this research, three (11, 21, 22) patients

received the combined treatment of corticosteroids and anti-TB at some point after the diagnosis of TB and all of them were considered to be in remission or showed improvement. The TB of five other patients had also been relieved without the application of steroids, which shows that whether steroids play a role in the treatment of patients who develop active TB during ICIs therapy needs to be further investigated, and the potential benefits of corticosteroids must be weighed against their attenuating effect on immunotherapy in the process of application (24, 25).

TB screening in high-risk patient groups, particularly those exposed to epidemiological risk factors for TB, should be applied to patients before the initiation of immunotherapy. In this study, 10 of the 19 patients were from East or South East Asia where TB is endemic. If patients receiving anti-PD1 antibodies in areas with high TB prevalence develop fever and respiratory symptoms, the reactivation of TB should be considered after respiratory infection and pneumonia has been excluded. Therefore, latent TB screening needs to be applied before starting ICIs therapy in TB-endemic countries. A previous study has shown that adequate treatment of latent TB infection significantly reduces the risk of active TB in these patients (26). Although immunosuppression is also a risk factor for TB, the extent to which immunosuppression promotes the development of active TB in cancer patients treated with ICIs is unknown. Most of the patients in the study received chemotherapy before ICIs therapy, which may have contributed to immunosuppression. Whether the concomitant use of immunosuppressive drugs such as glucocorticoids and ICIs therapy are associated with the development of active TB disease still needs to be confirmed in larger studies. It cannot be ruled out whether the development of tuberculosis is associated with high doses of steroid drugs. In the above cases, no concomitant use of immunosuppressive drugs was reported, except for the case of four patients who required steroids or infliximab to treat irAEs and two patients who might require immunosuppressive drugs such as glucocorticoids to treat irAEs. Therefore, cancer and ICIs immunotherapy should be considered as a possible basis for MTB susceptibility.

Although acute TB infection is diagnosed according to clinical symptoms, chest X-ray, and sputum culture or PCR, the initial symptoms and radiological findings may not distinguish between tumor progression, TB infection, or changes in immune-mediated imaging. Then, IGRA testing should be performed before the treatment of immune checkpoint inhibitors therapy to rule out the possibility of latent Mycobacterium TB infection. Only one of these patients took IGRA testing before ICIs administration. The IGRA result of the patient, which was negative at first, changed to positive after new abnormalities on imaging, which contributed to the diagnosis of TB. Therefore, it is necessary to take IGRA testing before the treatment of immune checkpoint inhibitors therapy.

It is an important clinical question whether ICIs therapy can be continued after the development of active TB. Given that continued treatment with ICIs may worsen TB, it is reasonable to discontinue ICIs after the diagnosis of active TB. However, the appropriate time to restart ICIs therapy is still being worked out. Although the four reported patients who restarted immunotherapy did not have a relapse or worsening of TB, these four cases are not sufficient to confirm the appropriateness of restarting ICIs therapy in the presence of TB. In clinical practice, the decision to continue, temporarily interrupt, or permanently suspend treatment with ICIs should take several aspects into account, including the

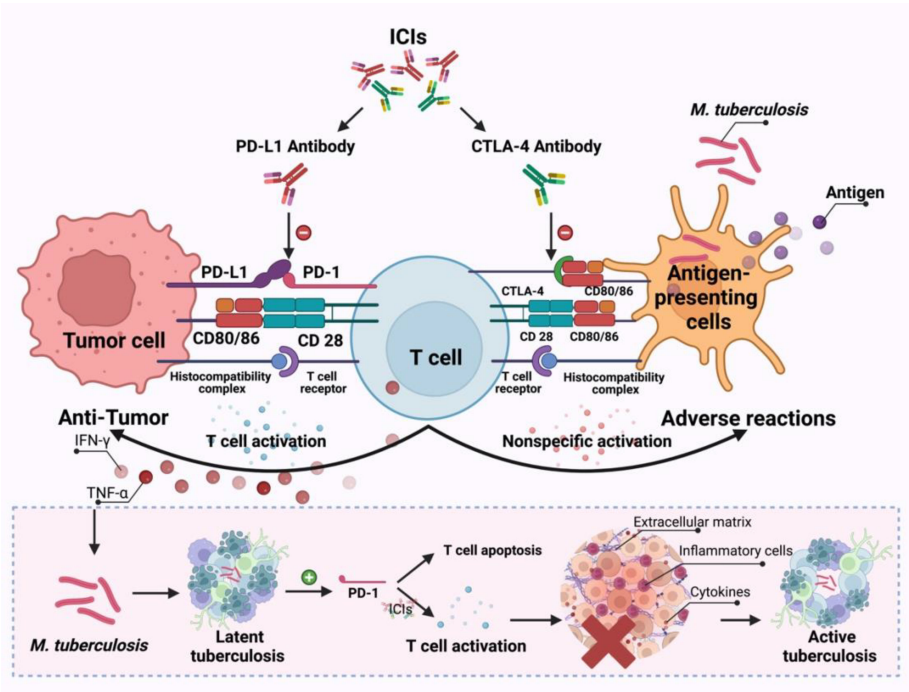

FIGURE 5
The possible mechanisms through which ICIs treatment promotes the development of TB infection.

severity of MTB infection, tumor control, and the severity of complications. Therefore, it is better to let multidisciplinary specialists in oncology discuss whether ICIs can be restarted or not.

Above all, ICIs have revolutionized cancer treatment, namely, greatly improving the overall survival of patients. However, potential MTB infection or reactivation of the disease during the treatment with ICIs still needs to be considered. It is recommended that IGRA testing be performed before ICIs therapy, and the development of TB during immunotherapy should be closely monitored for in patients who are positive in the results of IGRA testing to enable early diagnosis and timely treatment.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials further inquiries can be directed to the corresponding author/s.

Ethics statement

The studies involving human participants were reviewed and approved by Shengli Oilfield Central Hospital. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article. The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Institutional Review Board of Shengli Oilfield Central Hospital (No. 2022026). All the study subjects provided informed consent.

Author contributions

SL, CL, and GX designed the overall research strategy. CL collected the clinical information. CL and GX wrote the manuscript. CL, GX, TF, and SG participated in data discussion. All authors contributed to the article and approved the submitted version. CL and GX contributed equally to this work and share first authorship.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1162190/full#supplementary-material

References

1. Houben RMGJ, Dodd PJ. The global burden of latent tuberculosis infection: a re-estimation using mathematical modelling. *PloS Med* (2016) 13:e1002152. doi: 10.1371/journal.pmed.1002152

2. Cheng MP, Abou Chakra CN, Yansouni CP, Cnossen S, Shrier I, Menzies D, et al. Risk of active tuberculosis in patients with cancer: a systematic review and meta-analysis. *CLINID* (2016) 64(5):ciw838. doi: 10.1093/cid/ciw838

3. Dobler CC, Cheung K, Nguyen J, Martin A. Risk of tuberculosis in patients with solid cancers and haematological malignancies: a systematic review and meta-analysis. *Eur Respir J* (2017) 50:1700157. doi: 10.1183/13993003.00157-2017

4. Preventive Services Task Force US, Bibbins-Domingo K, Grossman DC, Curry SJ, Bauman L, Davidson KW, et al. Screening for latent tuberculosis infection in adults: US preventive services task force recommendation statement. *JAMA* (2016) 316:962. doi: 10.1001/jama.2016.11046

5. National Cancer Institute. Definition of immune checkpoint inhibi-tor-nci dictionary of cancer terms[EB/OL]. (2022).

6. Barber DL, Mayer-Barber KD, Feng CG, Sharpe AH, Sher A. CD4 T cells promote rather than control tuberculosis in the absence of PD-1–mediated inhibition. *J Immunol* (2011) 186:1598–607. doi: 10.4049/jimmunol.1003304

7. Inthasot V, Bruyneel M, Muylle I, Ninane V. Severe pulmonary infections complicating nivolumab treatment for lung cancer: a report of two cases. *Acta Clin Belg* (2020) 75:308–10. doi: 10.1080/17843286.2019.1629078

8. Barber DL, Sakai S, Kudchadkar RR, Fling SP, Day TA, Vergara JA. Tuberculosis following PD-1 blockade for cancer immunotherapy. *Sci Transl Med* (2019) 11: eaat2702. doi: 10.1126/scitranslmed.aat2702

9. Elkington PT, Bateman AC, Thomas GJ, Ottensmeier CH. Implications of tuberculosis reactivation after immune checkpoint inhibition. *Am J Respir Crit Care* (2018) 198:1451–3. doi: 10.1164/rccm.201807-1250LE

10. Anastasopoulou A, Ziogas DC, Samarkos M, Kirkwood JM, Gogas H. Reactivation of tuberculosis in cancer patients following administration ofimmune checkpoint inhibitors: Current evidence and clinical practicerecommendations []. *J Immunother Cancer* (2019) 7(1):239. doi: 10.1186/s40425-019-0717-7

11. Chu Y-C, Fang K-C, Chen H-C, Yeh Y-C, Tseng C-E, Chou T-Y, et al. Pericardial tamponade caused by a hypersensitivity response to tuberculosis reactivation after anti–PD-1 treatment in a patient with advanced pulmonary adenocarcinoma. *J Thorac Oncol* (2017) 12:e111–4. doi: 10.1016/j.jtho.2017.03.012

12. Tetikkurt S, Taş F, Emre F, Özsoy Ş, Bilece ZT. Significant neutrophilic emperipolesis in squamous cell carcinoma. *Case Rep Oncol Med* (2018) 2018:1–5. doi: 10.1155/2018/1301562

13. He W, Zhang X, Li W, Kong C, Wang Y, Zhu L, et al. Activated pulmonary tuberculosis in a patient with melanoma during PD-1 inhibition: a case report. *OTT* (2018) 11:7423–7. doi: 10.2147/OTT.S178246

14. Fujita K, Terashima T, Mio T. Anti-PD1 antibody treatment and the development of acute pulmonary tuberculosis. *J Thorac Oncol* (2016) 11:2238–40. doi: 10.1016/j.jtho.2016.07.006

15. Suliman AM, Bek SA, Elkhatim MS, Husain AA, Mismar AY, Eldean MZS, et al. Tuberculosis following programmed cell death receptor-1 (PD-1) inhibitor in a patient with non-small cell lung cancer. case report and literature review. *Cancer Immunol Immun* (2021) 70:935–44. doi: 10.1007/s00262-020-02726-1

16. Lee JJX, Chan A, Tang T. Tuberculosis reactivation in a patient receiving anti-programmed death-1 (PD-1) inhibitor for relapsed hodgkin's lymphoma. *Acta Oncol* (2016) 55:519–20. doi: 10.3109/0284186X.2015.1125017

17. Tsai C-C, Chen J-H, Wang Y-C, Chang F-Y. Re-activation of pulmonary tuberculosis during anti-programmed death-1 (PD-1) treatment. *QJM: Int J Med* (2019) 112:41–2. doi: 10.1093/qjmed/hcy243

18. Picchi H, Mateus C, Chouaid C, Besse B, Marabelle A, Michot JM, et al. Infectious complications associated with the use of immune checkpoint inhibitors in oncology: reactivation of tuberculosis after anti PD-1 treatment. *Clin Microbiol Infect* (2018) 24:216–8. doi: 10.1016/j.cmi.2017.12.003

19. Im J, Kim SJ, Koh WJ, Jhun BW, Lee SH. Development of tuberculosis in cancer patients receiving immune checkpoint inhibitors. *Respir Med* (2020) 161:105853. doi: 10.1016/j.rmed.2019.105853

20. Kim TH, Kim J. P3.CR-16 A Case of Toxic Hepatic Event Occurring in Combination Treatment with Nivolumab and Anti-Tuberculosis in Advanced Lung Cancer. *J Thorac Oncol* (2018) 13:S1034. doi: 10.1016/j.jtho.2018.08.1995

21. Takata S, Koh G, Han Y, Yoshida H, Shiroyama T, Takada H, et al. Paradoxical response in a patient with non-small cell lung cancer who received nivolumab followed by anti-mycobacterium tuberculosis agents. *J Infect Chemother* (2019) 25:54–8. doi: 10.1016/j.jiac.2018.06.016

22. van Eeden R, Rapoport BL, Smit T, Anderson R. Tuberculosis infection in a patient treated with nivolumab for non-small cell lung cancer: case report and literature review. *Front Oncol* (2019) 9:659. doi: 10.3389/fonc.2019.00659

23. Meintjes G, Stek C, Blumenthal L, Thienemann F, Schutz C, Buyze J, et al. Prednisone for the prevention of paradoxical tuberculosis-associated IRIS. *N Engl J Med* (2018) 379:1915–25. doi: 10.1056/NEJMoa1800762

24. Arbour KC, Mezquita L, Long N, Rizvi H, Auclin E, Ni A, et al. Impact of baseline steroids on efficacy of programmed cell death-1 and programmed death-ligand 1 blockade in patients with non–Small-Cell lung cancer. *JCO* (2018) 36:2872–8. doi: 10.1200/JCO.2018.79.0006

25. Scott SC, Pennell NA. Early use of systemic corticosteroids in patients with advanced NSCLC treated with nivolumab. *J Thorac Oncol* (2018) 13:1771–5. doi: 10.1016/j.jtho.2018.06.004

26. Lee J, Kim E, Jang EJ, Lee C-H, Lee EY, Im JP, et al. Efficacy of treatment for latent tuberculosis in patients undergoing treatment with a tumor necrosis factor antagonist. *Ann Am Thorac Soc* (2017) 14:690–7. doi: 10.1513/AnnalsATS.201608-647OC

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Bispecific antibodies targeting CTLA-4: game-changer troopers in cancer immunotherapy

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Antibody-based cancer immunotherapy has become a powerful asset in the arsenal against malignancies. In this regard, bispecific antibodies (BsAbs) are a ground-breaking novel approach in the therapy of cancers. Recently, BsAbs have represented a significant advancement in improving clinical outcomes. BsAbs are designed to target two different antigens specifically. Over a hundred various BsAb forms currently exist, and more are constantly being manufactured. An antagonistic regulator of T cell activation is cytotoxic T lymphocyte-associated protein 4 (CTLA-4) or CD152, a second counter-receptor for the B7 family of co-stimulatory molecules was introduced in 1996 by Professor James P. Allison and colleagues. Contrary to the explosive success of dual immune checkpoint blockade for treating cancers, a major hurdle still yet persist is that immune-related adverse events (irAEs) observed by combining immune checkpoint inhibitors (ICIs) or monoclonal antibodies such as ipilimumab (anti-CTLA-4) and nivolumab (anti-PD-1). A promising strategy to overcome this hurdle is using BsAbs. This article will summarize BsAbs targeting CTLA-4, their applications in cancer immunotherapy, and relevant clinical trial advances. We will also discuss the pre-clinical rationale for using these BsAbs, and provide the current landscape of the field.

KEYWORDS

CTLA-4 (cytotoxic T lymphocyte-associated protein 4), CD152, bispecific antibody (BsAbs), cancer immunotherapy, Bi-specific T cell engagers (BiTEs)

Abbreviations: BsAb, Bispecific antibody; CTLA-4, Cytotoxic T-lymphocyte-associated protein 4; irAE, Immune-related adverse event; TME, Tumor microenvironment; PD-1, Programmed cell death protein 1; PD-L1, Programmed death-ligand 1; Treg, Regulatory T cell; Teff, Effector T cell; ICI, Immune checkpoint inhibitor; NSCLC, non-small-cell lung cancer; mAb, Monoclonal antibody; ADCC, Antibody-dependent cellular cytotoxicity; NK, Natural killer cell; BiTE, Bi-specific T cell engager; TAA, Tumor-associated antigen; HER2, Human epidermal growth factor receptor 2.

1 Introduction

Numerous advancements in cancer immunotherapy during the last decade have included altering T cell-mediated immunity. Using three kinds of cancer immunotherapies, including immune checkpoint inhibitors (ICIs) or monoclonal antibodies (mAbs), genetically modified T cells expressing chimeric antigen receptors (CARs), and bispecific antibodies (BsAbs), are now licensed for clinical use (1, 2). mAb-based cancer immunotherapy has become a potent weapon in the arsenal against malignancies. Up to now, the FDA has approved several therapeutic mAbs for treating various malignancies and autoimmune disorders. Target cells may undergo cellular cytotoxicity when mAbs selectively attach to their antigens. After gaining crowning achievements of mAbs in treating cancer, another molecular platform of mABs was developed. Meanwhile, BsAbs, which target two antigens simultaneously, were introduced as therapeutic medications for various cancers (3).

During the 1960s, Nisonoff and colleagues played a significant role in popularizing the concept of BsAbs. The subsequent development of innovative antibody engineering methods produced numerous BsAb molecular platforms. BsAbs are superior to mAbs in several ways. Outstanding cytotoxicity effects of BsAbs in cancers are one of these benefits. BsAbs also exhibit a lower level of therapeutic resistance (4). Some BsAbs have a small size, typically consisting of two basic single-chain fragments with variable domain (5). As an example, BsAbs that are based on single-chain fragment variables (scFvs) have demonstrated a high degree of specificity for tumor cells and are able to penetrate tissues effectively (6). BsAbs may be an innovative new pillar in the fight against cancer. In terms of improving clinical treatment results, BsAbs represent a promising milestone. Many BsAbs have been developed within the context of tumor immunotherapy throughout the last few decades, and the first BsAb named blinatumomab was licensed in 2009 (7, 8). Lymphomas, in particular, appear to respond well to BsAbs, while myeloid neoplasias and solid tumors have shown more limited success (9). Although BsAbs have been shown to penetrate solid tumors effectively, there are concerns about their safety due to their short half-life, and this remains an area of active research (10).

An antagonistic regulator of T cell activation is cytotoxic T lymphocyte-associated protein 4 (CTLA-4) or CD152, a second counter-receptor for the B7 family of co-stimulatory molecules, which was introduced in 1996 by professor James Allison and colleagues (11). CTLA-4 is an inhibitory immune checkpoint, which inhibits immune responses through both an intrinsic mechanism that transmits a negative signal directly to effector T lymphocytes (T_{eff}) and an extrinsic mechanism that is primarily connected to regulatory T lymphocyte (T_{reg}) functions (12). B7-1 (CD80) and B7-2 (CD86) are the ligands shared by CD28 and CTLA-4, which have a greater affinity to bind CTLA-4. Consequently, CTLA-4 and CD28 are antagonistic concerning ligand binding (12, 13). The discovery that CTLA-4 can impede T cell activation provided the basis for the notion that interrupting its function might allow T cells to mount a therapeutic attack on cancer (14, 15). In mice, mAbs against CTLA-4 improved the immune system's ability to fight against colon cancer and fibrosarcoma (11). Moreover, in subsequent exposure to tumor cells, animals that had been given anti-CTLA-4 treatment were able to quickly eradicate the cancerous cells by means of the immune system. This suggests that suppressing CTLA-4 leads to sustained immunological memory (11, 16).

At present, numerous clinical trials are underway to investigate the effectiveness of BsAbs targeting CTLA-4 in treating various types of cancer. These trials have yielded promising results in certain types of tumor cells and have been associated with a prolonged anti-tumoral response (17–19). Although BsAbs targeting CTLA-4 have been a significant breakthrough in the field of cancer immunotherapy, there are still numerous aspects that require clarification and challenges that need to be addressed related to the safety, effectiveness, and range of tumors that could be treated. This review summarizes BsAbs targeting CTLA-4, their applications in cancer immunotherapy, relevant challenges, and clinical trial advances. We also discuss the pre-clinical rationale for using BsAbs targeting CTLA-4 and provide the current landscape of the field.

2 Bispecific antibodies mechanisms of action: a general viewpoint

BsAbs are designed to bind two distinct antigens concurrently. BsAbs exert their functions by redirecting and activating immune cells, inhibiting immune cell co-inhibitory receptors, activating co-stimulatory molecules, inhibiting signaling pathways, and combinatorial targeting of cancer antigens (Figure 1) (20).

Recruiting Immune Cells. In addition to stimulating T cells, BsAbs such as blinatumomab and catumaxomab that target CD3, CD19, and EpCAM also guide these cells toward cancer cells, where they can lead to lysis them (4). Besides, BsAbs such as AFM13 can crosslink the cancer antigen CD30 on tumor cells with CD16a on natural killer (NK) cells, rerouting NK cells to the tumor cells for lysing by antibody-dependent cellular cytotoxicity (ADCC) (3).

Blocking of Immune Checkpoints and Co-inhibitory Molecules. Programmed death-ligand 1 (PD-L1), programmed cell death protein 1 (PD-1), and CTLA-4 are well-known immune checkpoints that prevent immune cells from becoming activated. Thus, blocking immune checkpoints with antibodies can reinvigorate immune cells. BsAbs targeting immune checkpoints are mostly used for treating solid tumors, including AK104 (PD-1×CTLA-4) (17), MGD019 (PD-1×CTLA-4) (18), XmAb20717 (PD-1×CTLA-4) (21), MEDI5752 (PD-1×CTLA-4) (22), MGD013 (PD-1×Lymphocyte-activation gene 3 [LAG-3]) (23), and RO7121661 (PD-1×T-cell immunoglobulin and mucin domain 3 [TIM-3]) (NCT03708328), which are capable of targeting two immune checkpoints simultaneously. Dysregulation of lymphocyte functions co-expressed by PD-1 and LAG-3 is common, and combined therapy involving PD-1 and LAG-3 can effectively restore T cell function (24). Furthermore, BsAbs targeting immune checkpoints combined with tumor-associated antigens (TAAs) have been developed, including AK112 (PD-

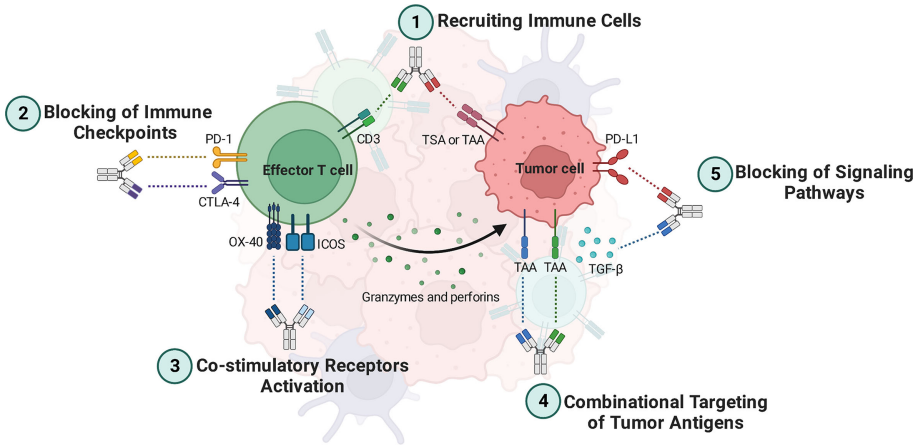

FIGURE 1
Bispecific antibodies (BsAbs) mechanisms of action. **1)** BsAbs can guide effector T cells toward cancer cells, where they can lead to lysis them. **2)** Simultaneous inhibition of two inhibitory immune checkpoints leads to more reinvigoration of effector T cells to eradicate the tumor cells. **3)** BsAbs can be deployed as agonistic agents activating co-stimulatory receptors such as ICOS and OX-40, leading to better activation of tumor-specific effector T cells. **4)** BsAbs targeting dual TAAs bind specifically to tumor cells that express both antigens. Thus, tumor cells can be targeted more specifically. **5)** BsAbs can suppress signaling pathways that are involved in angiogenesis and T cell exhaustion, such as those that inhibit TGF-β and PD-L1 signaling pathways. TAA, Tumor-associated antigen; TSA, Tumor-specific antigen; ICOS, Inducible T cell co-stimulator; TGF-β, Transforming growth factor-beta; PD-1, Programmed cell death protein 1; PD-L1, Programmed death-ligand 1; CTLA-4, Cytotoxic T-lymphocyte associated protein 4.

1×Vascular endothelial growth factor [VEGF]) and IBI315 (PD-1×Human epidermal growth factor receptor 2 [HER2]) (4, 25).

Co-stimulatory Receptors Activation. To maintain a stable anti-cancer immune response against advanced malignancies, one strategy might be proposed that co-stimulatory receptors can be targeted along with the blockage of immunological checkpoints. Numerous BsAbs have been emerging for binding co-stimulatory receptors, such as inducible T cell co-stimulator (ICOS) or CD278, OX40, and 4–1BB (3, 20). OX40, like CTLA-4, is significantly up-regulated on activated T cells in the tumor microenvironment (TME), particularly on Tregs (26, 27). Targeting two overexpressed receptors, for instance CTLA-4 and OX40, in the tumor has the potential to increase the localization of BsAbs to the tumor site compared to monospecific antibodies, which may reduce the risk of systemic T cell activation and improve efficacy. Additionally, it has been suggested that combining a checkpoint inhibitor with a T cell co-stimulatory agonistic antibody may convert a "cold" tumor into a "hot" tumor by enhancing T cell expansion and effector functions while controlling the suppressive function of Tregs (28, 29).

Combinational Targeting of Tumor Antigens. "On-target off-tumor" toxicity is a significant issue that needs to be considered in antibody-based therapeutic modalities. This toxicity refers to a side effect of cancer immunotherapy where the immune system attacks not only cancer cells but also healthy cells expressing the same target antigen, leading to unintended damage to healthy tissues (30). In line with this issue, both healthy and malignant cells express TAAs. One of the BsAb's advantages is that it targets tumor cells more precisely, which directs its power to only tumor cells in circulation, TME, and metastatic sites and not healthy cells. Thus, healthy cells are unharmed as a result of this. In other words, BsAbs targeting dual TAAs bind specifically to cancer cells that express both antigens, while avoiding healthy cells that express only one antigen (31).

The fact that many cancer antigens are cleaved by enzymes, releasing soluble extracellular domains, presents another obstacle. Indeed, a major challenge in developing immunotherapies for hematologic malignancies, for instance, acute lymphocytic leukemia (ALL), is that cancer cells can lose the CD19 antigen while retaining CD22 after CD19 shedding (32). Due to this mechanism, tumor antigen escape happens, but BsAbs can offset this mechanism *via* combinational targeting. As an illustration, a BsAb called DT2219ARL has been developed to overcome antigen loss-mediated relapse in ALL. DT2219ARL targets CD19 and CD22, which are two antigens commonly expressed in ALL cells (20, 33, 34).

Blocking of Signaling Pathways. BsAbs can reduce the progression of tumors by targeting the molecules of signaling pathways involved in angiogenesis, metastasis, and proliferation. In this regard, there are several BsAbs such as YM101 (35–37), MCLA-128 (zenocutuzumab) (38, 39), BI 836880 (40, 41), vanucizumab (42), ABT-165 (43), OMP-305B83 (navixizumab) (44, 45), TR009 (ABL001) (46), and EMB01 (47, 48). For instance, YM101 has the ability to specifically bind to transforming growth factor-beta (TGF-β) and PD-L1. Results from *in vitro* experiments demonstrated that YM101 effectively inhibited the biological impacts of TGF-β and the PD-1/PD-L1 pathways, which includes the activation of Smad signaling, induction of epithelial-mesenchymal transition, and immunosuppressive activities (35). Furthermore, MCLA-128 targets VEGF and angiopoietin-2 (4).

3 Mechanism of action of bispecific anti-CTLA-4 antibodies

BsAbs that target CTLA-4 are a novel class of immunotherapies that are being developed to improve the effectiveness and safety of immune checkpoint blockade in cancer treatment. The mechanism of action of bispecific anti-CTLA-4 antibodies involves, for instance, the simultaneous binding of CTLA-4 on T_{regs} and co-stimulatory receptor such as OX-40 on T_{eff} (49). The dual engagement of these two molecules helps to modulate the balance between immune activation and suppression, which is important for achieving an optimal anti-tumor response. For instance, by binding to CTLA-4 on T_{regs}, ATOR-1015 can selectively deplete or down-regulate these cells (49), which are known to play a role in suppressing immune responses and promoting tumor growth. At the same time, by binding to OX-40 on T_{eff}, ATOR-1015 can enhance T cell activation and proliferation, leading to increased colon, pancreatic, and bladder tumor cell killing (49, 50).

BsAbs targeting both CTLA-4 and another molecule, such as PD-1 (17, 51) or PD-L1 (52), enhance the immune response against cancer by blocking two separate immune checkpoints that can inhibit anti-tumor immunity. By targeting both CTLA-4 and PD-1, these bispecific antibodies can simultaneously promote activation and proliferation of T cells, reduce regulatory T cell function, and enhance the killing of tumor cells by cytotoxic T lymphocytes. The combination of these effects allows for a more robust and sustained anti-tumor response (4, 20).

4 Anti-CTLA-4 antibody and combinational therapies

There is a compelling strategy regarding combinational using the anti-CTLA-4 mAbs with other therapies against poorly immunogenic tumors (53). Wei et al. (54) demonstrated that contrary to either monotherapy, CTLA-4/PD-1 dual immune checkpoint blockade is sufficient to stimulate specific cellular responses. Anti-CTLA-4 and anti-PD-1 inhibition considerably improves responses in pre-clinical tumor models, leading to much higher T_{eff}-to-suppressor cells (myeloid-derived suppressor cell and T_{reg}) ratios and the generation of pro-inflammatory cytokines including interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) (55). Combinational therapy ipilimumab plus nivolumab led to enhance the effectiveness of treatment in melanoma (56), renal cell carcinoma (57), colorectal cancer (58) compared with monotherapy. In melanoma, the combination of PD-1 and CTLA-4 blockade was linked to specific cellular immune responses, including a dramatic increase in cytokine production, an increase in T cell frequency, and a decrease in circulating B cells (59, 60).

5 Pre-clinical rationale for using bispecific antibodies targeting CTLA-4

The currently known BsAbs are fragment-based, symmetric, asymmetric or Bi-specific T cell engagers (BiTEs). These forms determine their half-life, immunogenicity, target selectivity, and production complexity (61). Contrary to the explosive success of dual immune checkpoint blockade for treating cancers, two major hurdles persist. First, immune-related adverse events (irAEs) were observed by combination ICIs such as ipilimumab and nivolumab (62). Second, compared to BsAbs, there is an MHC restriction of the TCR when using ICIs, leading to immune escape (63). A promising strategy to overcome these hurdles is simultaneously co-blockade of two cancer antigens, for example, CTLA-4 and PD-1, preferentially in the TME by BsAbs, restricting immune responses specific to the tumor site. Thus, it can be expected that immune-mediated toxicity toward normal tissues is reduced (12).

Immunotherapies for cancer, such as the blockade of CTLA-4 and PD-1, often result in severe autoimmunity as a side effect (64, 65). Overall, there is a tendency for the efficacy of anti-tumor responses to be associated with the occurrence of autoimmune diseases, particularly when a systemic approach is taken to deplete T_{regs} (66). A promising strategy to elicit potent anti-tumor immune responses while minimizing the risk of inducing detrimental autoimmune reactions might be to selectively engage effector T_{regs} in the TME using BsAbs. This approach has the potential to preserve the pool of naive T_{regs} in non-tumor tissues, which are essential for maintaining immune tolerance and preventing autoimmunity. In other words, it is desirable to avoid T_{reg} depletion in normal tissues while targeting immune checkpoint pathways in the TME. One potential advantage of using BsAbs is that they can be designed to selectively bind to cells expressing both PD-1 and CTLA-4, which are mainly tumor-infiltrating lymphocytes (TILs). Thus, by selectively targeting TILs, BsAbs may spare T_{regs} in normal tissues and avoid the potential adverse effects associated with their systemic depletion.

On the one hand, the possibility that anti-tumor reactive T cells will be inadequate in quantity or malfunctioning and anergic are two key issues with chimeric antigen receptor (CAR) T cell treatment that are addressed by BsAbs. On the other hand, increased tumor-reactive T cell frequency is the ultimate goal of BsAbs. In order to accomplish this, T cells and tumor cells are connected effectively *via* BsAbs in an intercellular space name immunological synapse. Their activation occurs without the need for co-stimulation, peptide antigen presentation, and major histocompatibility complex (MHC) class 1/2 (61, 67). All in all, the aforementioned basic concepts provide a rationale for using BsAbs.

There are some BsAbs, which have been described in pre-clinical (Table 1) and clinical investigation (Table 2). These include XmAb20717 (Vudalimab), MEDI5752, AK104, MGD019, KN046, and ATOR-1015. According to preliminary results, XmAb20717, which targets CTLA-4 and PD-1 simultaneously, was well-tolerated in patients with advanced cancer and had complete and partial responses in various tumor types. T cell population changes in the tumor and surrounding tissues were consistent with effective dual checkpoint inhibition (21). In pre-clinical studies, using another BsAb targeting CTLA-4 and PD-1 named MEDI5752 was associated with lower cytotoxicity and equivalent activity to anti-PD-1 and anti-CTLA-4 antibodies, providing an improved therapeutic index (22). In the following, we will elaborate on the clinical trials of BsAbs targeting CTLA-4 (Table 2).

TABLE 1 Pre-clinical status of BsAbs targeting CTLA-4.

Name	Targets	Main findings	Reference
MEDI5752	CTLA-4 + PD-1	1. High saturation of CTLA-4 on PD-1$^+$ tumor cells by MEDI5752. 2. Inhibition of CTLA-4 on TILs while sparing peripheral T cell populations and reducing toxicity. 3. MEDI5752 induces internalisation and subsequent degradation of PD-1 by tethering CTLA-4 to PD-1.	(22)
MGD019	CTLA-4 + PD-1	1. Combinatorial blockade of PD-1 and CTLA-4 *via* single molecule. 2. MGD019 is well-tolerated in non-human primates. 3. Increasing in the count of Ki67$^+$CD8$^+$ and ICOS$^+$CD4$^+$ T cells upon MGD019 administration.	(18)
ATOR-1015	CTLA-4 + OX40	1. ATOR-1015 activates T cells and reduces Tregs *in vitro*. 2. In various preclinical models, ATOR-1015 reduces tumor growth and improves survival, including in bladder, colon, and pancreas cancer models. 3. ATOR-1015 generates long-term tumor-specific immunological memory and enhances response to PD-1 inhibition. 4. ATOR-1015 targets the tumor area, where it boosts the number and activation of CD8$^+$ T-cells and decreases Tregs.	(49)

CTLA-4, Cytotoxic T-lymphocyte-associated protein 4; PD-1, Programmed cell death protein 1; Treg, Regulatory T cell; TIL, Tumor-infiltrating lymphocyte; ICOS, Inducible T-cell costimulator.

TABLE 2 BsAbs targeting CTLA-4 in clinical development.

Name	Type and Structure	Targets	Indication	Treatment regimen	Status/ Phase	Participants	Developed by	NCT identifier
XmAb20717 (Vudalimab)	Humanized BsAb/XmAb technology	CTLA-4 + PD-1	-Melanoma -Breast carcinoma -HCC -Renal cell carcinoma -Colorectal carcinoma -non-small-cell lung carcinoma - Cervical cancer -Mesothelioma	MT	Active, not recruiting/I	154	Xencor	NCT03517488
MEDI5752	Fully human BsAb (a monovalent bispecific human IgG_1 mAb with an engineered Fc domain)	CTLA-4 + PD-1	Advanced solid tumors	Combinational therapy: Pemetrexed, Carboplatin, Pembrolizumab, Paclitaxel	Recruiting/ I	366	MedImmune	NCT03530397
			Renal cell carcinoma	Combinational therapy: Axitinib, Lenvatinib	Recruiting/ I	70	MedImmune	NCT04522323
			Metastatic NSCLC	Combinational therapy: Durvalumab, Danvatirsen, Oleclumab, Pemetrexed, Carboplatin, Gemcitabine, Cisplatin, Nab-paclitaxel, AZD2936	Active, not recruiting/I	258	AstraZeneca	NCT03819465
AK104 (Cadonilimab)	IgG_1 scaffold Fc-engineered humanized antibody	CTLA-4 + PD-1	Cervical cancer	MT	Completed/ II	30	Akeso	NCT04380805
			Recurrent or metastatic cervical cancer	Combinational therapy: Bevacizumab, Paclitaxel, Cisplatin or Carboplatin	Active, not recruiting/ II	50	Akeso	NCT04868708
			Nasopharyngeal carcinoma	MT	Completed/ II	34	Akeso	NCT04220307
			Advanced MSI-H/ dMMR gastric	MT		29	Peking University	NCT04556253

(Continued)

TABLE 2 Continued

Name	Type and Structure	Targets	Indication	Treatment regimen	Status/ Phase	Participants	Developed by	NCT identifier
			carcinoma and colorectal cancer		Not yet recruiting/ II			
			-Gastric adenocarcinoma -Advanced solid tumors -Gastroesophageal Junction Adenocarcinoma	Combinational therapy: Oxaliplatin, Capecitabine	Active, not recruiting/ I,II	338	Akeso	NCT03852251
			HCC	Combinational therapy: Lenvatinib	Active, not recruiting/ II	32	Akeso	NCT04728321
			Liver cancer	Combinational therapy: Lenvatinib	Recruiting/ I,II	30	Akeso	NCT04444167
			NSCLC	Combinational therapy: Anlotinib	Active, not recruiting/ I,II	114	Akeso	NCT04646330
			NSCLC	Combinational therapy: Anlotinib	Not yet recruiting/ II	30	Chinese PLA General Hospital	NCT04544644
			Advanced or metastatic solid tumors	Combinational therapy: AK119 (anti-CD73)	Recruiting/ I	195	Akeso	NCT04572152
MGD019	a tetravalent bispecific (2 × 2) Fc-bearing based on DART platform	CTLA-4 + PD-1	-NSCLC -Prostate cancer metastatic -Cutaneous melanoma -Colorectal cancer	Combinational therapy: Lorigerlimab	Active, not recruiting/I	287	MacroGenics	NCT03761017
KN046	Humanized bispecific single domain Fc fusion protein antibody	CTLA-4 + PD-L1	NSCLC	Combinational therapy: Paclitaxel, Pemetrexed, Carboplatin	Unknown/ II	50	Jiangsu Alphamab Biopharmaceuticals	NCT04054531
			Stage IV NSCLC	MT	Unknown/ II	149	Jiangsu Alphamab Biopharmaceuticals	NCT03838848
			HER2 positive solid tumor	Combinational therapy: KN026 (anti-HER2)	Recruiting/ I	24	Peking University	NCT04040699
			TNBC	Combinational therapy: Nab-paclitaxel	Active, not recruiting/ I,II	52	Jiangsu Alphamab Biopharmaceuticals	NCT03872791
			Esophageal squamous cell carcinoma	MT	Completed/ II	45	Jiangsu Alphamab Biopharmaceuticals	NCT03925870
			Advanced gastrointestinal tumors	Combinational therapy: Donafenib Tosilate	Recruiting/ I, II	42	Suzhou Zelgen Biopharmaceuticals	NCT04612712
			HCC	Combinational therapy: Lenvatinib	Recruiting/ II	55	Peking University Cancer Hospital & Institute	NCT04542837
			Locally advanced and metastatic pancreatic cancer	Combinational therapy: Gemcitabine, Albumin-Paclitaxel,	Recruiting/ I,II	60	Changhai Hospital	NCT04324307

(Continued)

TABLE 2 Continued

Name	Type and Structure	Targets	Indication	Treatment regimen	Status/ Phase	Participants	Developed by	NCT identifier
				Oxaliplatin, Irinotecan, Leucovorin, Fluorouracil				
			Squamous NSCLC	MT	Active, not recruiting/ III	482	Jiangsu Alphamab Biopharmaceuticals	NCT04474119
			Thymic carcinoma	MT	Recruiting/ II	29	Weill Medical College of Cornell University	NCT04925947
			Thymic carcinoma	MT	Recruiting/ II	66	Jiangsu Alphamab Biopharmaceuticals	NCT04469725
ATOR-1015	Human IgG_1 BsAb	CTLA-4 + OX40	-Solid Tumor -Neoplasms	MT	Completed/ I	33	Alligator Bioscience AB	NCT03782467

mAb, Monoclonal antibody; BsAb, Bispecific antibody; Fc, Fragment crystallizable region; CTLA-4, T-lymphocyte-associated protein 4; PD-1, Programmed cell death protein 1; NSCLC, Non-small cell lung cancer; HCC, Hepatocellular carcinoma; TNBC, Triple-negative breast cancer; MT, Monotherapy; DART, Dual affinity retargeting.

6 Current landscape and clinical trials

The landscape of clinical anti-CTLA-4 BsAbs immunotherapy continues to progress promptly. Recently, there have been numerous illustrations of these BsAbs in clinical and pre-clinical phases. Rudimentary findings revealed positive clinical outcomes, which can be classified into three categories, including i) Anti-tumor immune responses, ii) Immune-related toxicities, and iii) TME (Figure 2). The following sections summarize the current landscape and the clinical trials.

6.1 CTLA-4×PD-1 BsAbs

XmAb20717. XmAb20717 is a heterodimeric antibody composed of two different protein subunits, designed with a modified Fc domain that prevents interactions with Fc-gamma receptor (FcγR). Additionally, the incorporation of Xtend™ technology enhances its pharmacokinetic properties to promote a longer half-life (21). A human clinical trial is investigating the effects of XmAb20717 (NCT03517488). Patients with advanced solid tumors, including melanoma, renal cell carcinoma, non-

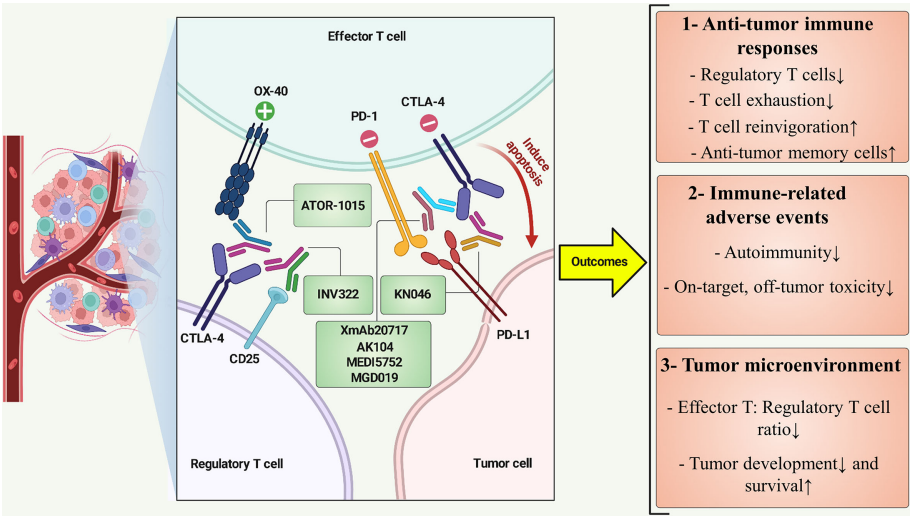

FIGURE 2
BsAbs targeting CTLA-4 at a view of cross-talk between immune and cancer cells. ATOR-1015 can simultaneously target CTLA-4 and OX-40 on regulatory T cell and effector T cell, respectively, resulting in effector T-cell stimulation and improved cancer immunity. INV322 binds to CD25 and CTLA-4, which restricts the function of regulatory T cells in the tumor microenvironment. CTLA-4 and PD-L1 are recognized by KN046, eventually preventing T-cell exhaustion and inducing apoptosis in tumor cells. BsAbs such as XmAb20717, AK104, MEDI5752, and MGD019 inhibit PD-1 and CTLA-4, revitalizing tumor-specific cytotoxic T lymphocytes. Positive and negative symbols indicate stimulatory and inhibitory role of markers, respectively. ↑, Increase; ↓, Decrease.

small cell lung cancer (NSCLC), castration-resistant prostate cancer, are enrolled in phase 1 multiple doses, ascending dose escalation clinical trial to determine the pharmacokinetic (PK), maximum-tolerated dose, safety, and tolerability.

MEDI5752. MEDI5752 is a fully human monovalent bispecific human IgG1 mAb with an engineered Fc domain designed to suppress the PD-1 pathway and provide regulated CTLA-4 inhibition, favoring greater blocking of PD-1$^+$ activated T cells. The variable domains of an anti-PD-1 mAb and tremelimumab (an anti-CTLA-4 mAb) were combined to generate this BsAb. The engineering of the IgG1 constant heavy chains also diminished the Fc-mediated immune effector functions. The higher anti-tumor activity was stimulated by MEDI5752, which was found to accelerate PD-1 internalization and degradation and to accumulate preferentially in tumors. MEDI5752 could fully saturate CTLA-4 on cells expressing PD-1 and CTLA-4 at lower doses than those needed to fully saturate CTLA-4 on cells that did not express PD-1 (51). A phase 1, first-time-in-human, multicenter, open-label, dose-escalation and dose-expansion clinical trial was designed to evaluate the safety, tolerability, efficacy, PK, and immunogenicity of MEDI5752 in participants with advanced solid tumors, when administered as a single agent or combined with chemotherapeutic drugs (NCT03530397).

AK104. PD-1 and CTLA-4 are concurrently targeted by the humanized IgG1 tetrameric BsAb known as AK104. Indeed, AK104 is an IgG_1 scaffold Fc-engineered humanized antibody. Early research revealed that AK104 has promising anti-tumor effectiveness in liver cancer and a better safety profile than co-administering anti-PD-1 and anti-CTLA-4 mAbs (17). A phase 2, global, multicenter, open-label, single-arm study evaluated the efficacy, safety, tolerability, PK, and immunogenicity of AK104 monotherapy in adult subjects with previously treated recurrent or metastatic cervical carcinoma (NCT04380805).

MGD019. Berezhnoy et al. (18) have developed a BsAb in the IgG4 isotype known as MGD019 using a dual-affinity re-targeting antibody (DART) platform. This BsAb consists of an engineered tetravalent CTLA-4/PD-1 molecule. Two variable fragments with their variable heavy chain components switch to form a DART molecule (68). The PD-1 and CTLA-4 binding domains are derived from retifanlimab and human mAb 4B6, respectively (18). Compared to bi-single domain antibodies, this novel structure enables greater conformational flexibility during antigen-antibody recognition (18). To prevent the potential depletion of T_{eff} and T_{regs}, MGD019 carries Fc mutations, which cause the lack of Fc-mediated effector function and limit the ability to trigger ADCC. Innovative approaches include modulating the Fc effector domain or directing the immune response preferentially toward TME, which may improve therapy efficacy and minimize immune-mediated damage (27, 69). Despite increasing human T cell activation *in vitro*, MGD019 did not exhibit any Fc-mediated effector activity. MGD019 showed promising therapeutic efficacy with acceptable safety in patients with advanced solid tumor cancer, including ovarian, breast, lung, colon cancer, who had had much pre-treatment and was well-tolerated in cynomolgus monkeys. Notably, in animals treated with MGD019, no alterations were observed in either the tissue-resident or circulating T_{reg} populations (18).

6.2 CTLA-4×PD-L1 BsAb

KN046. KN046 (also known as alphamab), a unique humanized bispecific single domain Fc fusion protein antibody, can simultaneously inhibit the PD-1/PD-L1 pathway and the CTLA-4 pathway. Thus, it reinvigorates the immunological responses of exhausted T lymphocytes to the tumor. The findings of both pre-clinical and clinical testing with KN046 have shown good effectiveness, accompanied by decreased levels of systemic toxicity. KN046 is now being evaluated in several clinical studies in phase 1 and 2 as either a single agent or as part of combination regimens in tumor types and stages (52, 70). In patients with HER2-positive gastrointestinal tumors, preliminary efficacy and safety results of KN026 (a BsAb targeting two distinct HER2 epitopes) and KN046 have been reported. In both treatment-naive and extensively pretreated HER2-positive gastrointestinal cancers, KN026 combined with KN046 demonstrated the potential to improve clinical benefit to the current standard of care (71). A phase 2 clinical trial of KN046 was investigated in patients with metastatic NSCLC who failed first-line treatment. Results showed that KN046 was a successful second-line therapy for advanced NSCLC and was well-tolerated. In both squamous and non-squamous NSCLC, KN046 demonstrated a potential overall survival improvement (19). KN046 and Nab-paclitaxel showed safety, tolerability, and efficacy results in patients with metastatic triple-negative breast cancer (72).

6.3 CTLA-4×OX40 BsAb

ATOR-1015. An improved form of the Ig-like V-type domain of human CD86 was linked to an agonistic OX40 antibody, and eventually, a BsAb in the IgG1 form known as ATOR-1015 was generated. This BsAb simultaneously targets CTLA-4 and OX40. OX40, or CD134, is a powerful immunological co-stimulatory molecule that can be induced on activated CD4$^+$ and CD8$^+$ T cells. *In vitro*, ATOR-1015 stimulated T lymphocyte activation and T_{reg} reduction. Many syngeneic tumor models, such as those of bladder, colon, and pancreatic cancer, responded well to treatment with ATOR-1015, which slowed tumor development and increased survival. It is further shown that ATOR-1015 increases the response to PD-1 blockade and generates tumor-specific and long-term immunological memory. Additionally, ATOR-1015 is localized to the tumor site, enhancing the frequency and activation of CD8$^+$ T lymphocytes while decreasing the frequency of T_{regs} (49). A phase 1 clinical trial investigated the safety and tolerability of ATOR-1015 when administered as repeated intravenous infusions to patients with advanced and refractory solid malignancies (NCT03782467).

6.4 CTLA-4×GITR BsAb

A receptor called glucocorticoid-induced TNFR family-related gene (GITR) promotes T lymphocyte activation against tumor cells. A patent known as WO2018091739 employs a BsAb targeting

CTLA-4 and GITR to treat colon carcinoma. This BsAb has a basic structure that includes two binding sites for GITR, which are formed by the antigen-binding sites of both the heavy and light chains. In addition, there are two binding sites for CTLA-4, which are created by a CTLA-4 binding domain that is connected to the kappa constant region of the light chain through a linker at the carboxyl end (73). BsAb triggered T_{eff} and tumor inhibition in colon carcinoma-bearing mice (73). However, no clinical studies still demonstrate using this BsAb to treat cancer patients.

6.5 CTLA-4×CD25 BsAb

In the clinic, targeting T_{regs} has shown promise, but current strategies are constrained by on-target, off-tumor stimulation of autoimmune-related toxicities linked to global T_{reg} blocking. To limit these toxicities, a BsAb known as INV322 was engineered to engage the Tregs of TME preferentially. It is created using Invenra's B-Body® platform, which is fully human in origin, and has a wild-type IgG_1 structure that enables Fc-gamma-mediated effector function (74). INV322 targets CD25 and CTLA-4 on T_{regs} with lower-affinity monovalent interactions, supporting the selective blockade of tumor-restricted T_{regs} function and depletion by Fc-mediated clearance. *In vivo*, the development of anti-tumor memory cells, tumor-protective activity, T_{reg} depletion, and a rise in the T_{eff}: T_{reg} ratio inside the TME were associated with INV323 treatment (74).

7 Conclusion, challenges, and future directions

Over the past three decades, there has been a significant evolution from the simple development and modification of mAbs without any further engineering to more complex antibody derivatives in a wide range of shapes and sizes, particularly BsAbs (75). Researchers' interest in BsAb technology has grown significantly over time due to its outstanding potential for clinical applications. This development has established a strong basis for BsAb-based cancer immunotherapy. BsAbs are a novel approach to the fight against cancer. Overall, BsAbs have represented a significant advancement in improving clinical results. BsAbs might play a fascinating function in treating cancer. IrAEs caused by combining ICIs like ipilimumab and nivolumab remain a significant obstacle despite the tremendous success of treating cancers. BsAbs are a viable strategy to overcome this obstacle because the simultaneous co-blockade of two cancer antigens, such as CTLA-4 and PD-1, primarily in the TME by BsAbs may restrict immune responses, particularly to the tumor site. Thus, healthy cells and tissues are not damaged.

Although BsAbs are well positioned as a safe immunotherapy, outstanding questions remain open. Elucidating the significant parameters that determine anti-CTLA-4 BsAbs potency and persistence will be essential as the field progresses to evolving strategies to address obstacles specific to each type of cancer. Remarkably, as the field of cancer immunotherapy, particularly BsAbs, continues to innovate rapidly, it is important to consider potential safety risks linked to BsAbs in humans, as concerns associated with possible off-target effects might be very relevant. Establishing multidisciplinary approaches to sketch a holistic path to the clinical translation of anti-CTLA-4 BsAbs will also be crucial.

Nevertheless, despite the advantages discussed above, there are still several challenges and obstacles, such as immunogenicity and chain mispairing issues in the commercial production and development of anti-CTLA-4 BsAbs. More specifically, it takes time and funds to manufacture BsAbs. Obtaining the necessary products necessitates using suitable, secure, and economical cell line manufacturing processes and analytical and purifying techniques (76). Furthermore, before patients may benefit from anti-CTLA-4 BsAbs, some post-antibody production problems, such as degradation, aggregation, denaturation, fragmentation, and oxidation of BsAbs, must be addressed (76). Further clinical trials are necessary to investigate the most effective dose and mode of administration that can result in controlled release formulations, lower systemic adverse effects, and greater concentrations in target tissues. Besides, the unavoidable negative consequences on healthy organs such as neurotoxicity or other intricate aspects, such as an immunotolerant cancer stroma, disrupted neovasculature, and insufficient penetration of BsAbs, make BsAbs targeting solid tumors deserving of additional study (77, 78). Notably, the suppressive TME, which restricts T cell activation and causes immunological insufficiency (79), is a significant barrier to anti-CTLA-4 BsAbs in advanced solid tumors. Thus, there is a genuine eagerness for the continuing investigations of anti-CTLA-4 BsAbs in solid tumors, which are predicted to provide positive outcomes soon, even if developing these BsAbs from bench to bedside may take a long time and involve a huge endeavor.

Author contributions

PF and A-AD: conceptualization. PF and SM: writing the original draft. A-AD and MA: editing. PF: visualization. A-AD and MA: supervision. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Blanco B, Domínguez-Alonso C, Alvarez-Vallina L. Bispecific immunomodulatory antibodies for cancer immunotherapy. *Clin Cancer Res* (2021) 27:5457–64. doi: 10.1158/1078-0432.CCR-20-3770

2. Farhangnia P, Akbarpour M, Yazdanifar M, Aref AR, Delbandi A-A, Rezaei N. Advances in therapeutic targeting of immune checkpoints receptors within the CD96-TIGIT axis: clinical implications and future perspectives. *Expert Rev Clin Immunol* (2022):1217–37. doi: 10.1080/1744666X.2022.2128107

3. Jin S, Sun Y, Liang X, Gu X, Ning J, Xu Y, et al. Emerging new therapeutic antibody derivatives for cancer treatment. *Signal Transduct Target Ther* (2022) 7:39. doi: 10.1038/s41392-021-00868-x

4. Ma J, Mo Y, Tang M, Shen J, Qi Y, Zhao W, et al. Bispecific antibodies: from research to clinical application. *Front Immunol* (2021) 12:626616. doi: 10.3389/fimmu.2021.626616

5. Wang S, Chen K, Lei Q, Ma P, Yuan AQ, Zhao Y, et al. The state of the art of bispecific antibodies for treating human malignancies. *EMBO Mol Med* (2021) 13: e14291. doi: 10.15252/emmm.202114291

6. Fan G, Wang Z, Hao M, Li J. Bispecific antibodies and their applications. *J Hematol Oncol* (2015) 8:130. doi: 10.1186/s13045-015-0227-0

7. Kontermann RE, Brinkmann U. Bispecific antibodies. *Drug Discovery Today* (2015) 20:838–47. doi: 10.1016/j.drudis.2015.02.008

8. Viardot A, Bargou R. Bispecific antibodies in haematological malignancies. *Cancer Treat Rev* (2018) 65:87–95. doi: 10.1016/j.ctrv.2018.04.002

9. Duell J, Lammers PE, Djuretic I, Chunyk AG, Alekar S, Jacobs I, et al. Bispecific antibodies in the treatment of hematologic malignancies. *Clin Pharmacol Ther* (2019) 106:781–91. doi: 10.1002/cpt.1396

10. Wu Y, Yi M, Zhu S, Wang H, Wu K. Recent advances and challenges of bispecific antibodies in solid tumors. *Exp Hematol Oncol* (2021) 10:56. doi: 10.1186/s40164-021-00250-1

11. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. *Science* (1996) 271:1734–6. doi: 10.1126/science.271.5256.1734

12. Lisi L, Lacal PM, Martire M, Navarra P, Graziani G. Clinical experience with CTLA-4 blockade for cancer immunotherapy: from the monospecific monoclonal antibody ipilimumab to probodies and bispecific molecules targeting the tumor microenvironment. *Pharmacol Res* (2022) 175:105997. doi: 10.1016/j.phrs.2021.105997

13. Alegre M-L, Frauwirth KA, Thompson CB. T-Cell regulation by CD28 and CTLA-4. *Nat Rev Immunol* (2001) 1:220–8. doi: 10.1038/35105024

14. Waldman AD, Fritz JM, Lenardo MJ. A guide to cancer immunotherapy: from T cell basic science to clinical practice. *Nat Rev Immunol* (2020) 20:651–68. doi: 10.1038/s41577-020-0306-5

15. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* (2012) 12:252–64. doi: 10.1038/nrc3239

16. Kwon ED, Hurwitz AA, Foster BA, Madias C, Feldhaus AL, Greenberg NM, et al. Manipulation of T cell costimulatory and inhibitory signals for immunotherapy of prostate cancer. *Proc Natl Acad Sci U.S.A.* (1997) 94:8099–103. doi: 10.1073/pnas.94.15.8099

17. Bai L, Sun M, Xu A, Bai Y, Wu J, Shao G, et al. Phase 2 study of AK104 (PD-1/CTLA-4 bispecific antibody) plus lenvatinib as first-line treatment of unresectable hepatocellular carcinoma. *J Clin Oncol* (2021) 39:4101. doi: 10.1200/JCO.2021.39.15_suppl.4101

18. Berezhnoy A, Sumrow BJ, Stahl K, Shah K, Liu D, Li J, et al. Development and preliminary clinical activity of PD-1-Guided CTLA-4 blocking bispecific DART molecule. *Cell Rep Med* (2020) 1(9):100163. doi: 10.1016/j.xcrm.2020.100163

19. Zhou C, Xiong A, Fang J, Li X, Fan Y, Zhuang W, et al. 1022P a phase II study of KN046 (a bispecific anti-PD-L1/CTLA-4) in patients with metastatic non-small cell lung cancer (NSCLC) who failed first line treatment. *Ann Oncol* (2022) 33:S1022. doi: 10.1016/j.annonc.2022.07.1148

20. Farhangnia P, Delbandi A-A, Sadri M, Akbarpour M. Bispecific antibodies in targeted cancer immunotherapy BT - handbook of cancer and immunology. In: Rezaei N, editor. *Handbook of cancer and immunology*. Cham: Springer International Publishing (2023). p. 1–46. doi: 10.1007/978-3-030-80962-1_189-1

21. Shum E, Reilley M, Najjar Y, Daud A, Thompson J, Baranda J, et al. 523 preliminary clinical experience with XmAb20717, a PD-1 x CTLA-4 bispecific antibody, in patients with advanced solid tumors. *J Immunother Cancer* (2021) 9: A553 LP–A553. doi: 10.1136/jitc-2021-SITC2021.523

22. Dovedi SJ, Mazor Y, Elder M, Hasani S, Wang B, Mosely S, et al. Abstract 2776: MEDI5752: a novel bispecific antibody that preferentially targets CTLA-4 on PD-1 expressing T-cells. *Cancer Res* (2018) 78:2776. doi: 10.1158/1538-7445.AM2018-2776

23. Wang J, Asch AS, Hamad N, Weickhardt A, Tomaszewska-Kiecana M, Dlugosz-Danecka M, et al. A phase 1, open-label study of MGD013, a bispecific DART® molecule binding PD-1 and LAG-3 in patients with relapsed or refractory diffuse Large b-cell lymphoma. *Blood* (2020) 136:21–2. doi: 10.1182/blood-2020-139868

24. Woo S-R, Turnis ME, Goldberg MV, Bankoti J, Selby M, Nirschl CJ, et al. Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. *Cancer Res* (2012) 72:917–27. doi: 10.1158/0008-5472.CAN-11-1620

25. Zhou C, Ren S, Luo Y, Wang L, Xiong A, Su C, et al. A phase Ib/II study of AK112, a PD-1/VEGF bispecific antibody, as first- or second-line therapy for advanced non–small cell lung cancer (NSCLC). *J Clin Oncol* (2022) 40:9040. doi: 10.1200/JCO.2022.40.16_suppl.9040

26. Montler R, Bell RB, Thalhofer C, Leidner R, Feng Z, Fox BA, et al. OX40, PD-1 and CTLA-4 are selectively expressed on tumor-infiltrating T cells in head and neck cancer. *Clin Transl Immunol* (2016) 5:e70. doi: 10.1038/cti.2016.16

27. Arce Vargas F, Furness AJS, Litchfield K, Joshi K, Rosenthal R, Ghorani E, et al. Fc effector function contributes to the activity of human anti-CTLA-4 antibodies. *Cancer Cell* (2018) 33:649–663.e4. doi: 10.1016/j.ccell.2018.02.010

28. Galon J, Bruni D. Approaches to treat immune hot, altered and cold tumours with combination immunotherapies. *Nat Rev Drug Discovery* (2019) 18:197–218. doi: 10.1038/s41573-018-0007-y

29. Redmond WL, Linch SN, Kasiewicz MJ. Combined targeting of costimulatory (OX40) and coinhibitory (CTLA-4) pathways elicits potent effector T cells capable of driving robust antitumor immunity. *Cancer Immunol Res* (2014) 2:142–53. doi: 10.1158/2326-6066.CIR-13-0031-T

30. Castellarin M, Sands C, Da T, Scholler J, Graham K, Buza E, et al. A rational mouse model to detect on-target, off-tumor CAR T cell toxicity. *JCI Insight* (2020) 5. doi: 10.1172/jci.insight.136012

31. Huang S, van Duijnhoven SMJ, Sijts AJAM, van Elsas A. Bispecific antibodies targeting dual tumor-associated antigens in cancer therapy. *J Cancer Res Clin Oncol* (2020) 146:3111–22. doi: 10.1007/s00432-020-03404-6

32. Fry TJ, Shah NN, Orentas RJ, Stetler-Stevenson M, Yuan CM, Ramakrishna S, et al. CD22-targeted CAR T cells induce remission in b-ALL that is naive or resistant to CD19-targeted CAR immunotherapy. *Nat Med* (2018) 24:20–8. doi: 10.1038/nm.4441

33. Vallera DA, Todhunter DA, Kuroki DW, Shu Y, Sicheneder A, Chen H. A bispecific recombinant immunotoxin, DT2219, targeting human CD19 and CD22 receptors in a mouse xenograft model of b-cell Leukemia/Lymphoma. *Clin Cancer Res* (2005) 11:3879–88. doi: 10.1158/1078-0432.CCR-04-2290

34. Bachanova V, Frankel AE, Cao Q, Lewis D, Grzywacz B, Verneris MR, et al. Phase I study of a bispecific ligand-directed toxin targeting CD22 and CD19 (DT2219) for refractory b-cell malignancies. *Clin Cancer Res* (2015) 21:1267–72. doi: 10.1158/1078-0432.CCR-14-2877

35. Yi M, Zhang J, Li A, Niu M, Yan Y, Jiao Y, et al. The construction, expression, and enhanced anti-tumor activity of YM101: a bispecific antibody simultaneously targeting TGF-β and PD-L1. *J Hematol Oncol* (2021) 14:27. doi: 10.1186/s13045-021-01045-x

36. Yi M, Niu M, Zhang J, Li S, Zhu S, Yan Y, et al. Combine and conquer: manganese synergizing anti-TGF-β/PD-L1 bispecific antibody YM101 to overcome immunotherapy resistance in non-inflamed cancers. *J Hematol Oncol* (2021) 14:146. doi: 10.1186/s13045-021-01155-6

37. Yi M, Wu Y, Niu M, Zhu S, Zhang J, Yan Y, et al. Anti-TGF-β/PD-L1 bispecific antibody promotes T cell infiltration and exhibits enhanced antitumor activity in triple-negative breast cancer. *J Immunother Cancer* (2022) 10. doi: 10.1136/jitc-2022-005543

38. de Vries Schultink AHM, Bol K, Doornbos RP, Murat A, Wasserman E, Dorlo TPC, et al. Population pharmacokinetics of MCLA-128, a HER2/HER3 bispecific monoclonal antibody, in patients with solid tumors. *Clin Pharmacokinet* (2020) 59:875–84. doi: 10.1007/s40262-020-00858-2

39. Schram AM, Odintsov I, Espinosa-Cotton M, Khodos I, Sisso WJ, Mattar MS, et al. Zenocutuzumab, a HER2xHER3 bispecific antibody, is effective therapy for tumors driven by NRG1 gene rearrangements. *Cancer Discovery* (2022) 12:1233–47. doi: 10.1158/2159-8290.CD-21-1119

40. Le Tourneau C, Becker H, Claus R, Elez E, Ricci F, Fritsch R, et al. Two phase I studies of BI 836880, a vascular endothelial growth factor/angiopoietin-2 inhibitor, administered once every 3 weeks or once weekly in patients with advanced solid tumors. *ESMO Open* (2022) 7:100576. doi: 10.1016/j.esmoop.2022.100576

41. Girard N, Wermke M, Barlesi F, Kim D-W, Ghiringhelli F, Bennouna J, et al. Phase ib study of BI 836880 (VEGF/Ang2 nanobody) plus ezabenlimab (BI 754091; anti-PD-1 antibody) in patients (pts) with solid tumors. *J Clin Oncol* (2021) 39:2579. doi: 10.1200/JCO.2021.39.15_suppl.2579

42. Hidalgo M, Martinez-Garcia M, Le Tourneau C, Massard C, Garralda E, Boni V, et al. First-in-Human phase I study of single-agent vanucizumab, a first-in-Class bispecific anti-Angiopoietin-2/Anti-VEGF-A antibody, in adult patients with advanced solid tumors. *Clin Cancer Res* (2018) 24:1536–45. doi: 10.1158/1078-0432.CCR-17-1588

43. Li Y, Hickson JA, Ambrosi DJ, Haasch DL, Foster-Duke KD, Eaton LJ, et al. ABT-165, a dual variable domain immunoglobulin (DVD-ig) targeting DLL4 and VEGF, demonstrates superior efficacy and favorable safety profiles in preclinical models. *Mol Cancer Ther* (2018) 17:1039–50. doi: 10.1158/1535-7163.MCT-17-0800

44. Jimeno A, Moore KN, Gordon M, Chugh R, Diamond JR, Aljumaily R, et al. A first-in-human phase 1a study of the bispecific anti-DLL4/anti-VEGF antibody

navicixizumab (OMP-305B83) in patients with previously treated solid tumors. *Invest New Drugs* (2019) 37:461–72. doi: 10.1007/s10637-018-0665-y

45. Fu S, Corr BR, Culm-Merdek K, Mockbee C, Youssoufian H, Stagg R, et al. Phase ib study of navicixizumab plus paclitaxel in patients with platinum-resistant ovarian, primary peritoneal, or fallopian tube cancer. *J Clin Oncol* (2022) 40:2568–77. doi: 10.1200/JCO.21.01801

46. Yeom D-H, Lee Y-S, Ryu I, Lee S, Sung B, Lee H-B, et al. ABL001, a bispecific antibody targeting VEGF and DLL4, with chemotherapy, synergistically inhibits tumor progression in xenograft models. *Int J Mol Sci* (2021) 22. doi: 10.3390/ijms22010241

47. Qing Z, Gabrail N, Uprety D, Rotow J, Han B, Jänne PA, et al. 22P EMB-01: an EGFR-cMET bispecific antibody, in advanced/metastatic solid tumors phase I results. *Ann Oncol* (2022) 33:S39–40. doi: 10.1016/j.annonc.2022.02.031

48. Ren F, Wu X, Yang D, Wu D, Gong S, Zhang Y, et al. Abstract 528: EMB-01: an innovative bispecific antibody targeting EGFR and cMet on tumor cells mediates a novel mechanism to improve anti-tumor efficacy. *Cancer Res* (2020) 80:528. doi: 10.1158/1538-7445.AM2020-528

49. Kvarnhammar AM, Veitonmäki N, Hägerbrand K, Dahlman A, Smith KE, Fritzell S, et al. The CTLA-4 x OX40 bispecific antibody ATOR-1015 induces anti-tumor effects through tumor-directed immune activation. *J Immunother Cancer* (2019) 7:103. doi: 10.1186/s40425-019-0570-8

50. Perez-Santos M, Anaya-Ruiz M, Herrera-Camacho I, Millán-Pérez Peña L, Rosas-Murrieta NH. Bispecific anti-OX40/CTLA-4 antibodies for advanced solid tumors: a patent evaluation of WO2018202649. *Expert Opin Ther Pat* (2019) 29:921–4. doi: 10.1080/13543776.2019.1681400

51. Dovedi SJ, Elder MJ, Yang C, Sitnikova SI, Irving L, Hansen A, et al. Design and efficacy of a monovalent bispecific PD-1/CTLA4 antibody that enhances CTLA4 blockade on PD-1(+) activated T cells. *Cancer Discovery* (2021) 11:1100–17. doi: 10.1158/2159-8290.CD-20-1445

52. Xing B. 938P KN046 (an anti-PD-L1/CTLA-4 bispecific antibody) in combination with lenvatinib in the treatment for advanced unresectable or metastatic hepatocellular carcinoma (HCC): preliminary efficacy and safety results of a prospective phase II trial. *Ann Oncol* (2021) 32:S822. doi: 10.1016/j.annonc.2021.08.158

53. Egen JG, Kuhns MS, Allison JP. CTLA-4: new insights into its biological function and use in tumor immunotherapy. *Nat Immunol* (2002) 3:611–8. doi: 10.1038/ni0702-611

54. Wei SC, Anang N-AAS, Sharma R, Andrews MC, Reuben A, Levine JH, et al. Combination anti-CTLA-4 plus anti-PD-1 checkpoint blockade utilizes cellular mechanisms partially distinct from monotherapies. *Proc Natl Acad Sci U.S.A.* (2019) 116:22699–709. doi: 10.1073/pnas.1821218116

55. Curran M, Montalvo-Ortiz W, Yagita H, Allison J. PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. *Proc Natl Acad Sci U.S.A.* (2010) 107:4275–80. doi: 10.1073/pnas.0915174107

56. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, et al. Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. *N Engl J Med* (2015) 373:23–34. doi: 10.1056/NEJMoa1504030

57. Motzer RJ, Tannir NM, McDermott DF, Arén Frontera O, Melichar B, Choueiri TK, et al. Nivolumab plus ipilimumab versus sunitinib in advanced renal-cell carcinoma. *N Engl J Med* (2018) 378:1277–90. doi: 10.1056/NEJMoa1712126

58. Overman MJ, Lonardi S, Wong KYM, Lenz H-J, Gelsomino F, Aglietta M, et al. Durable clinical benefit with nivolumab plus ipilimumab in DNA mismatch repair-Deficient/Microsatellite instability-high metastatic colorectal cancer. *J Clin Oncol* (2018) 36:773–9. doi: 10.1200/JCO.2017.76.9901

59. Das R, Verma R, Sznol M, Boddupalli CS, Gettinger SN, Kluger H, et al. Combination therapy with anti-CTLA-4 and anti-PD-1 leads to distinct immunologic changes. *vivo. J Immunol* (2015) 194:950–9. doi: 10.4049/jimmunol.1401686

60. Das R, Bar N, Ferreira M, Newman AM, Zhang L, Bailur JK, et al. Early b cell changes predict autoimmunity following combination immune checkpoint blockade. *J Clin Invest* (2018) 128:715–20. doi: 10.1172/JCI96798

61. Zhou X, Einsele H, Danhof S. Bispecific antibodies: a new era of treatment for multiple myeloma. *J Clin Med* (2020) 9. doi: 10.3390/jcm9072166

62. Almutairi AR, McBride A, Slack M, Erstad BL, Abraham I. Potential immune-related adverse events associated with monotherapy and combination therapy of ipilimumab, nivolumab, and pembrolizumab for advanced melanoma: a systematic review and meta-analysis. *Front Oncol* (2020) 10:91. doi: 10.3389/fonc.2020.00091

63. Brischwein K, Parr L, Pflanz S, Volkland J, Lumsden J, Klinger M, et al. Strictly target cell-dependent activation of T cells by bispecific single-chain antibody constructs of the BiTE class. *J Immunother* (2007) 30:798–807. doi: 10.1097/CJI.0b013e318156750c

64. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* (2012) 366:2443–54. doi: 10.1056/NEJMoa1200690

65. Postow MA, Sidlow R, Hellmann MD. Immune-related adverse events associated with immune checkpoint blockade. *N Engl J Med* (2018) 378:158–68. doi: 10.1056/NEJMra1703481

66. Tanaka A, Sakaguchi S. Regulatory T cells in cancer immunotherapy. *Cell Res* (2017) 27:109–18. doi: 10.1038/cr.2016.151

67. Stamova S, Koristka S, Keil J, Arndt C, Feldmann A, Michalk I, et al. Cancer immunotherapy by retargeting of immune effector cells *via* recombinant bispecific antibody constructs. *Antibodies* (2012) 1:172–98. doi: 10.3390/antib1020172

68. Huang L, Shah K, Barat B, Lam C-YK, Gorlatov S, Ciccarone V, et al. Multispecific, multivalent antibody-based molecules engineered on the DART® and TRIDENT(TM) platforms. *Curr Protoc Immunol* (2020) 129:e95. doi: 10.1002/cpim.95

69. Ha D, Tanaka A, Kibayashi T, Tanemura A, Sugiyama D, Wing JB, et al. Differential control of human treg and effector T cells in tumor immunity by fc-engineered anti-CTLA-4 antibody. *Proc Natl Acad Sci U.S.A.* (2019) 116:609–18. doi: 10.1073/pnas.1812186116

70. Jin G, Guo S, Zhang Y, Ma Y, Guo X, Zhou X, et al. Efficacy and safety of KN046 plus nab-paclitaxel/gemcitabine as first-line treatment for unresectable locally advanced or metastatic pancreatic ductal adenocarcinoma (PDAC). *J Clin Oncol* (2021) 39:4138. doi: 10.1200/JCO.2021.39.15_suppl.4138

71. Gong J, Shen L, Luo S, Dong Z, Liu D, An S, et al. 1377P preliminary efficacy and safety results of KN026 (a HER2-targeted bispecific antibody) in combination with KN046 (an anti-PD-L1/CTLA-4 bispecific antibody) in patients (pts) with HER2-positive gastrointestinal tumors. *Ann Oncol* (2021) 32:S1042. doi: 10.1016/j.annonc.2021.08.1486

72. Xu B, Li Q, Zhang Q, Zhang Y, Ouyang Q, Zhang Y, et al. Abstract 1660: preliminary safety tolerability & efficacy results of KN046 (an anti-PD-L1/CTLA-4 bispecific antibody) in combination with nab-paclitaxel in patients with metastatic triple-negative breast cancer (mTNBC). *Cancer Res* (2021) 81:1660. doi: 10.1158/1538-7445.AM2021-1660

73. Millán-Pérez Peña L, Martin P-S, Herrera-Camacho I, Bandala C, Anaya-Ruiz M. Colon carcinoma treatment using bispecific anti-GITR/CTLA-4 antibodies: a patent evaluation of WO2018091739. *Expert Opin Ther Pat* (2020) 30:307–11. doi: 10.1080/13543776.2020.1732352

74. Ritter A, Marshal N, Pulukkunat D, Pereira N, Rakhmilevich A, Gawdzik J, et al. 500 INV322, a TME selective CD25 x CTLA4 bispecific antibody approach for depletion of tumor restricted tregs. *J Immunother Cancer* (2022) 10:A521 LP–A521. doi: 10.1136/jitc-2022-SITC2022.0500

75. Carter PJ, Lazar GA. Next generation antibody drugs: pursuit of the "high-hanging fruit". *Nat Rev Drug Discovery* (2018) 17:197–223. doi: 10.1038/nrd.2017.227

76. Elgundi Z, Reslan M, Cruz E, Sifniotis V, Kayser V. The state-of-play and future of antibody therapeutics. *Adv Drug Delivery Rev* (2017) 122:2–19. doi: 10.1016/j.addr.2016.11.004

77. Labrijn AF, Janmaat ML, Reichert JM, Parren PWHI. Bispecific antibodies: a mechanistic review of the pipeline. *Nat Rev Drug Discovery* (2019) 18:585–608. doi: 10.1038/s41573-019-0028-1

78. Li H, Er Saw P, Song E. Challenges and strategies for next-generation bispecific antibody-based antitumor therapeutics. *Cell Mol Immunol* (2020) 17:451–61. doi: 10.1038/s41423-020-0417-8

79. Anderson K, Stromnes I, Greenberg P. Obstacles posed by the tumor microenvironment to T cell activity: a case for synergistic therapies. *Cancer Cell* (2017) 31:311–25. doi: 10.1016/j.ccell.2017.02.008

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