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Chemo-immunotherapy combination after PD-1 inhibitor failure improves clinical outcomes in metastatic melanoma patients

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Management of PD-1 blockade resistance in metastatic melanoma (MM) remains challenging. Immunotherapy or chemotherapy alone provides limited benefit in this setting. Chemo-immunotherapy (CIT) has demonstrated favorable efficacy and safety profiles in lung cancer. Our pre-clinical study showed that in MM patients who have failed PD-1 blockade, the addition of chemotherapy increases CX3CR1+ therapy-responsive CD8+ T-cells with enhanced anti-tumor activity, resulting in improved clinical response. Here, we examined the clinical outcomes of CIT in MM patients after PD-1 blockade failure and the treatment-related changes in CX3CR1+ therapy-responsive CD8+ T-cells. We reviewed MM patients seen between January 2012 and June 2018 who failed anti-PD-1-based therapy and received subsequent CIT, immune checkpoint inhibitors (ICI) or chemotherapy alone. Overall survival (OS), objective response rate (ORR), event-free survival (EFS), and toxicities were assessed. Among 60 patients, 33 received CIT upon disease progression on PD-1 blockade. At a median follow-up of 3.9 years, the CIT group had a median OS of 3.5 years [95% confidence interval (CI) 1.7–NR] vs. 1.8 years (95% CI 0.9–2; $P = 0.002$) for those who received subsequent ICI (n = 9) or chemotherapy alone (n = 18), with ORR of 59% vs. 15% ($P = 0.0003$), respectively. The median EFS was 7.6 months (95% CI 6–10) following CIT vs. 3.4 months (95% CI 2.8–4.1; $P = 0.0005$) following ICI or chemotherapy alone. Therapy-responsive CX3CR1+CD8+ T-cells showed dynamic increase with successful CIT. CIT showed favorable clinical outcomes and acceptable safety profile in PD-1 blockade-resistant patients. CX3CR1+CD8+ therapy-responsive T-cells can be potentially used for monitoring disease response to CIT. *Melanoma Res* 30: 364–375 Copyright © 2020 The Author(s). Published by Wolters Kluwer Health, Inc.

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Background

Immune checkpoint inhibitors (ICI), especially PD-1 blockade, have changed the landscape of metastatic melanoma (MM) treatment, providing durable clinical response in a subset of patients. The majority of MM patients, however, either fail to respond or experience secondary resistance with later disease progressions after initial responses [1]. The clinical management following anti-PD-1 therapy failure remains challenging, and a standard of care is lacking. Current guidelines recommend immunotherapy, cytotoxic chemotherapy, targeted therapy (if BRAF V600 mutated), or a clinical trial, per clinicians' discretion, although evidence supporting their clinical benefits in patients who have failed previous anti-PD 1 therapy is limited [2]. One of the barriers in developing efficacious salvage therapeutic regimens is the lack of deep understanding of the cellular and molecular mechanisms that lead to PD-1 blockade resistance. In addition, mechanisms of overcoming the resistance and markers for monitoring anti-tumor response and therapeutic outcomes after PD-1 blockade failure are yet to be defined.

Chemotherapy drugs execute anti-tumor activity partly due to their immunomodulatory effects, including disruption of immune-suppressive pathways and increase of tumor immunogenicity [3–5], leading to synergistic

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anti-tumor activities when used in combination with anti-PD-1 agents [6]. In patients with non-small cell lung cancer (NSCLC), cytotoxic chemotherapy after anti-PD-1 therapy showed improved response rates, demonstrating anti-tumor activity in this setting, and we have reported similar findings in MM [7,8]. The combination of cytotoxic chemotherapy drugs and immunotherapy has recently been explored in NSCLC to improve clinical outcomes. Carboplatin-based chemotherapies in combination with pembrolizumab have been studied in the frontline setting in KEYNOTE-021 [9–11] and KEYNOTE-189 for nonsquamous NSCLC [12] and in KEYNOTE-047 for squamous NSCLC [13]. Platinum-based chemotherapy was also studied in combination with atezolizumab in IMpower133 in small cell lung cancer patients [14]. These clinical trials demonstrated superior response rates and survival benefits of combination regimes compared with monotherapies with acceptable toxicity profiles, although mechanisms responsible for their clinical successes have not been fully elucidated.

In MM patients whose diseases have progressed after pembrolizumab treatment, we observed a higher objective response to subsequent chemotherapies (approximately 26%), including carboplatin and paclitaxel, compared with that in chemotherapy-only historic controls [8]. Based on these observations, we have employed chemo-immunotherapy (CIT) combination to treat MM patients who have disease progression on anti-PD-1 based-therapy. The initial success of this treatment regimen led to our recent identification of a novel subset of CD8+ T-cells, CX3CR1+CD8+ T-cells, in the peripheral blood that can withstand chemotherapy during CIT with increased anti-tumor activities [15]. The increased frequencies of CX3CR1+CD8+ T-cells are responsive for the therapeutic benefit in patients who responded to CIT, providing a potential biomarker to monitor treatment response and to guide rational combination strategies [15]. In this retrospective study, we summarized the clinical outcomes of MM patients who received CIT and demonstrated improved response rates and overall survival (OS) of salvage CIT compared with immunotherapy or chemotherapy alone after anti-PD-1 therapy failure, with no additional toxicities. The dynamic changes of CX3CR1+ therapy-responsive CD8+ T-cells during CIT are also monitored in a subset of the patients. Thus, our results, consistent with our pre-clinical model, provide clinical evidence to support the use of CIT as an effective salvage therapy in patients after progression on anti-PD-1 therapy, and the utilization of CX3CR1+ therapy-responsive CD8+ T-cells in monitoring the T-cell immune responses and treatment outcomes during CIT.

Methods

Patient information and study design

The medical records of patients with MM who had disease progression upon anti-PD-1 therapy and received subsequent CIT, ICI or chemotherapy alone, seen at Mayo Clinic, Rochester, between 1 January 2012 and 30 June 2018 were reviewed. The patients were divided into two cohorts based on the subsequent therapy [treatment of interest (TOI)] received immediately after PD-1 blockade failure. Patients treated with subsequent chemotherapy-immunotherapy combination were included in the CIT cohort. Patients treated with subsequent ICI alone includes pembrolizumab or nivolumab (either alone or in combination with ipilimumab), or chemotherapy alone were included in the ICI/chemotherapy cohort. The study was approved by the Mayo Clinic Institutional Review Board, and conducted in accordance with the Declaration of Helsinki. Informed consent was obtained for biospecimen collection from all patients.

Only patients who had received at least one complete cycle of the TOI (i.e. CIT, ICI, or chemotherapy) following failure of anti-PD-1 therapy were considered evaluable. Response to treatment was assessed based on direct review of scan images and radiology reports according to the RECIST 1.1 and irRECIST criteria [16] as applicable according to the TOI. In the CIT cohort, first disease response assessment was performed upon the completion of 2–3 cycles of combination treatment, or earlier if clinically indicated. Objective response rate (ORR) was assessed by measuring the rate of a partial response and complete response. The irAEs were collected from review of the EMR retrospectively graded according to the National Cancer Institute Common Terminology for Adverse Events version 4.0 [17].

All time-to-event analyses were performed from the date of first-line systemic therapy initiation using the Kaplan–Meier method and the log-rank test. Event-free survival (EFS) was defined as the time from the TOI initiation to the time of first event (progression of disease, initiation of a new treatment, or death). Patients who did not reach an event were censored for EFS at the date of last follow-up. In the MM setting, EFS is a clinically relevant endpoint that correlates very closely to progression-free survival (PFS) and was selected as our time-to-event endpoint given its lower likelihood of bias in retrospective data analysis.

10X sample processing, cDNA library preparation, sequencing, and single-cell RNA-Seq analysis

Peripheral blood mononuclear cell (PBMC) samples were collected from the subjects treated. PBMCs were stained with CD8-PE-Cy7 (BD Pharmingen, San Jose, California, clone RPA-T8, catalog 304006), CD3-BV421 (BioLegend, San Jose, California, clone OKT3, catalog 317344) and CD45-FITC (BioLegend, clone San Jose, California, HI30, catalog 304006). Cells were sorted on a FACSAria IIu SORP (BD Biosciences, San Jose, California). The sorted samples for the 10X droplet generation and the subsequent cDNA libraries were prepared as outlined by the 10X Genomics Single Cell 3' v2 Reagent Kit user guide with a target capture of 2000 cells.

cDNA libraries were sequenced on an Illumina HiSeq 4000. A median sequencing depth of 80 000 reads/cell was targeted for each sample.

Fastq files generation from raw sequencing data (bcl files), alignment to the human genome reference sequences (build GRCh38), and a digital gene expression matrix generation were all performed using the 10X Genomics cellranger commands. Cells and gene filtering were done as follows: cells with low number of detected genes (<200) and a very high (>0.4) mitochondrial genome transcript ratio were excluded. Genes detected (Unique molecular identifier count > 0) in less than three cells were removed. The integrated analyses of samples from two-time points of a patient (baseline and post-therapy) were carried out using Seurat package under the default setting.

Flow analysis of human T-cells isolated from peripheral blood

The following panel of antibodies was used for analysis of PBMC populations: CD8-PE-Cy7 (BD Pharmingen, clone RPA-T8, catalog 304006), CD11a-APC (BioLegend, clone HI111, catalog 301212), PD-1 FITC (BioLegend, clone EH12.2H7, catalog 32990), CX3CR1-APC/Cy7 (BioLegend, clone 2A9-1, catalog 341616), Bim-PE (Cell Signaling Technology, Danvers, Massachusett, clone C34C5, catalog 12186S), Ki-67-BV421(BD Biosciences, clone B56, catalog 562899), Granzyme B-PerCP (Novus Biologicals, Danvers, Massachusett, clone CLB-GB11, catalog NBP1-50071PCP). CD8+ T-cells were first stained for surface markers followed by intracellular staining. Flow cytometry data were collected on a CytoFLEX LX (Beckman Coulter, Atlanta, Georgia). Flow cytometry analysis was performed with FlowJo software 10.4 (Tree Star, Palo Alto, California).

Statistical analysis

Two-sided Wilcoxon rank-sum test and Fisher's exact test were used to compare the medians of continuous and categorical variables, respectively. The Kaplan–Meier method was utilized for all time-to-event analyses. The Cox regression method was used for all multivariate analysis. A P-value <0.05 was considered statistically significant and analysis was performed using JMP 10.0 software (SAS Institute, Cary, North Carolina, USA).

Results

Cytotoxic chemotherapy induces clinical response after disease progression upon anti-PD-1 treatment

Treatment options for MM patients whose disease progressed after PD-1 blockade are very limited. We have previously reported that cytotoxic chemotherapy remains clinically effective after PD-1 blockade failure [8]. Based on the clinical benefits and acceptable toxicities of CIT combination reported in NSCLC studies [12,18], we have employed the addition of cytotoxic chemotherapy to ongoing PD-1 blockade as the salvage regimen in MM patients who have failed anti-PD-1 treatment with no other viable treatment options available.

Figure 1 demonstrates the representative imaging findings from two patients treated with CIT combination in the salvage setting. Both patients have BRAF V600E mutated MM and had failed BRAF/MEK inhibitors. They had further disease progression upon subsequent anti-PD-1 therapy and presented with severe symptoms including worsening of fatigue and abdominal pain (Fig. 1a and c). Carboplatin (area under the curve of five) and paclitaxel (175 mg/m^2) were administered every 3 weeks in combination with anti-PD-1 therapy, aiming to achieve symptomatic control and disease response. Both patients tolerated combination therapy well without unexpected adverse effects, and experienced significant symptomatic improvement after two cycles of therapy, with radiographic evidence of disease response (Fig. 1b and d). Chemotherapy was discontinued after a total of four and eight cycles in combination with anti-PD-1 therapy, respectively, while anti-PD-1 therapy was continued.

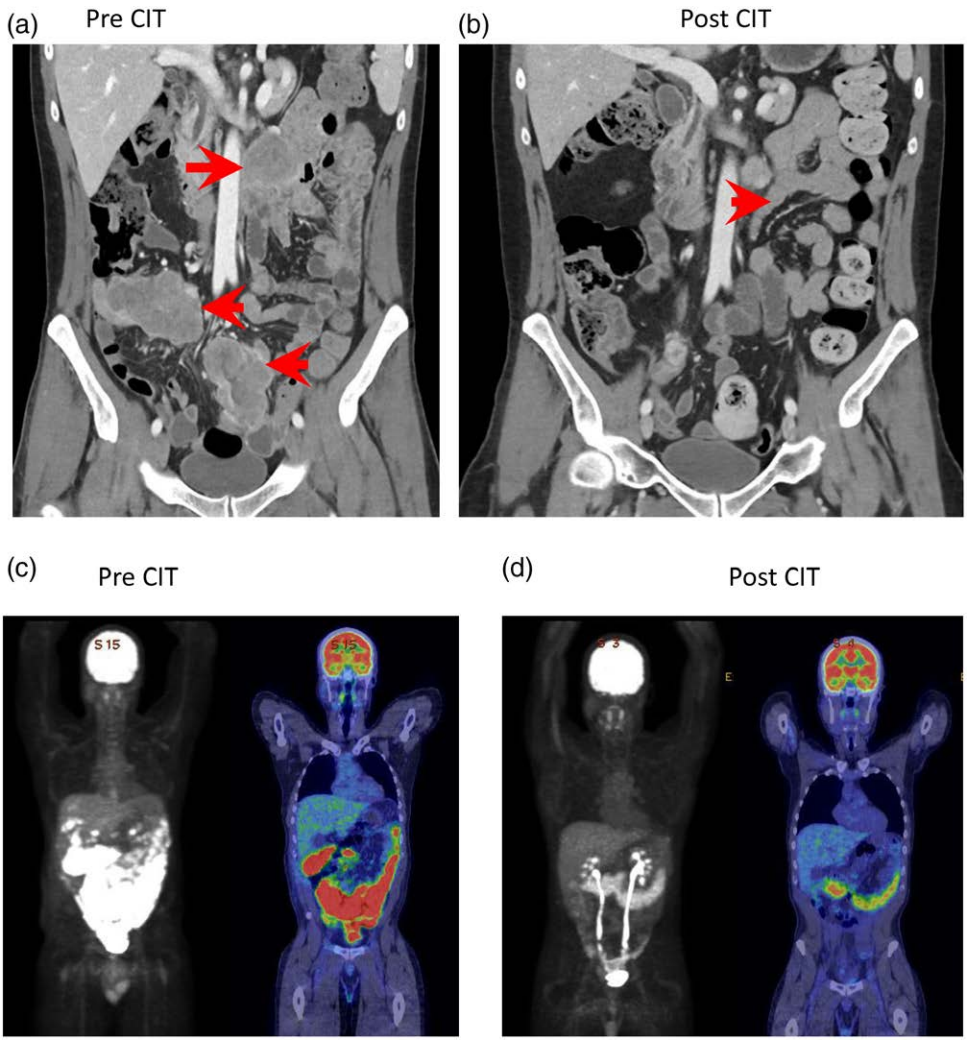
Patients and disease characteristics

To examine the clinical benefit of CIT in the setting of disease progression upon PD-1 blockade therapy, we retrospectively reviewed clinical outcomes of MM patients who received subsequent salvage CIT, chemotherapy or ICI therapy, immediately after PD-1 blockade failure. A total of 60 patients were identified, among which 33 (55%) patients were treated with CIT (CIT cohort) while the remaining 27 (45%) patients were treated with either ICI alone (n = 9) or chemotherapy alone (n = 18) (ICI/chemotherapy cohort). Baseline characteristics are described in Table 1. The disease characteristics were similar between the two cohorts with respect to age at diagnosis, sex, primary site of disease, LDH, development of brain metastasis, and prior anti-PD1 based therapy that failed to provide durable response. The median lines of systemic therapy received for the entire cohort were 5 (range 2–10), and are similar between the two cohorts [CIT cohort: median of five lines (range 2–12); ICI/chemotherapy alone cohort: median four lines (range 2–8), P = 0.08]. Ocular and mucosal melanoma patients were equally distributed between the cohorts, as was the presence of BRAF mutations. The non-V600 BRAF mutations were: pQ626T, pQ209P, and pN581S.

Treatment characteristics and outcome analysis

Among the 33 patients included in the CIT cohort, the TOI consisted of carboplatin/paclitaxel (n = 29), nab-paclitaxel (n = 2), paclitaxel (n = 1), or temozolomide (n = 1) in combination with PD-1 blockade. All chemotherapy was given at standard recommended dosing schedule [2]. Among the 27 patients included in the ICI or chemotherapy alone cohort, the TOI consisted of carboplatin/paclitaxel (n = 11), temozolomide (n = 4), nab-paclitaxel (n =

Fig. 1



Clinical benefits of chemoimmunotherapy (CIT) in metastatic melanoma patients after progressed on PD-1 blockade. (a and b) A patient with BRAF V600E mutation had symptomatic disease progression after receiving Dabrafenib plus Trematinib and Nivolumab plus Ipilimumab. He then received Pembrolizumab (200 mg Q 3 weeks) in combination with Carboplatin and Paclitaxel (AUC of 5 and 175 mg/m^2, respectively, Q 3 weeks). CT of the abdomen and pelvis obtained before (a) and after five cycles of CIT (b) are shown. Red arrows indicate large para-aortic and pelvic lesions. (c and d) A metastatic melanoma patient with BRAF V600E mutation was previously treated with targeted therapy and experienced significant disease progression after pembrolizumab therapy with peritoneal carcinomatosis. He subsequently received salvage CIT therapy with pembrolizumab (2 mg/kg Q 3 weeks) and carboplatin and paclitaxel (AUC of 5 and 175 mg/m^2, respectively). PET scans obtained before (c) and after two cycles of CIT (d) were shown. AUC, area under the curve.

3), ipilimumab/nivolumab (n = 4), pembrolizumab (n = 4), or nivolumab (n = 1) (Table 1). In the CIT cohort, the TOI ranged between the second and tenth line of therapy (median fourth line of therapy), suggesting that most of the patients were heavily treated before receiving CIT. Similarly, in the ICI or chemotherapy alone cohort, the TOI ranged between the second and sixth line (median fourth line of therapy), $P = 0.67$. Among patients harboring a BRAF mutation, exposure to BRAF/MEK inhibitors prior to the TOI was similar in both cohorts [CIT cohort: 11 (91%) patients; ICI or chemotherapy alone cohort: seven (78%) patients, $P = 0.36$].

Response assessments to the TOI were available in 59 (98%) of the patients and are described in Table 1. The ORR following the TOI was higher in the CIT cohort (59%) compared to the ICI or chemotherapy alone cohort (15%, $P = 0.0003$).

After a median follow-up of 3.9 years, the median OS for all 60 patients was 2 years [95% confidence interval (CI) 1.7–3.6]. Patients in the CIT cohort had a median OS of 3.5 years (95% CI 1.7–NR; 3-year OS 59%) compared to 1.8 years (95% CI 0.9–2; 3-year OS 32%) in the ICI/chemotherapy alone cohort, $P = 0.02$ (Fig. 2a). The median OS of patients with ocular melanoma was shorter

Table 1 Patient characteristics and response rates

	CIT (n = 33)	ICI or Chemotherapy Alone (n = 27)	*P* value
Age, median (range)	56 (23–77)	58 (21–77)	0.97
Male sex, n (%)	23 (70)	16 (59)	0.40
Primary site, n (%)			0.44
Cutaneous	21 (64)	19 (70)	
Ocular	6 (18)	4 (15)	
Mucosal	4 (12)	4 (15)	
Unknown	2 (6)	0	
Brain metastasis, n (%)	12 (36)	8 (30)	0.58
Median (range) LDH prior to TOI (U/L)	230 (138–127)	288 (134–1609)	0.07
BRAF mutation, n (%)	12 (36)	9 (33)	0.80
V600	10 (30)	8 (30)	
Other	2 (6)	1 (3)	
Failed ICI line, n (%)			0.41
Pembrolizumab	23 (70)	22 (81)	
Nivolumab	4 (12)	1 (4)	
Ipilimumab/Nivolumab	6 (18)	4 (15)	
Chemotherapy, n (%)[a]			
Carboplatin/paclitaxel	29 (88)	11 (61)	
Nab-paclitaxel	2 (6)	3 (16)	
Temozolomide	1 (3)	4 (23)	
Paclitaxel	1 (3)	0	
ORR, n (%)	19 (59)	4 (15)	**0.0003**
Response, n (%)			
CR	5 (15)	2 (7)	
PR	14 (43)	2 (7)	
SD	1 (3)	1 (4)	
PD	12 (36)	22 (82)	
Unknown	1 (3)	0	

CIT, chemo-immunotherapy; CR, complete response; ICI, immune checkpoint inhibitors; ORR, objective response rate; PD, progression of disease; PR, partial response; SD, stable disease; TOI, treatment of interest.
[a]Chemotherapy regimen used as part of the TOI in the CIT cohort or chemotherapy alone group (n = 18).

[median 1.5 years (95% CI 0.3–1.7)] compared to other patients [median 3.2 years (95% CI 2–5), $P = 0.002$]. On a multivariate analysis of OS including TOI and primary site location (ocular vs. other), both variables were independently associated with survival. However, the longer OS seen in the CIT cohort remains even after the exclusion of patients with ocular melanoma [median 5 years (95% CI 2.4–NR)] compared to the ICI or chemotherapy alone cohort [median 1.9 years (95% CI 1.3–2.2), $P = 0.006$].

The median EFS following CIT was 7.6 months (95% CI 6–10) compared to 3.4 months (95% CI 2.8–4.1) following either ICI or chemotherapy alone, $P = 0.0005$ (Fig. 2b). When considering only the ICI or chemotherapy alone cohort, the EFS following ICI alone [median 4.1 months (95% CI 2–4.6)] or chemotherapy alone [median 3.3 months (95% CI 2.2–3.8)] were similar ($P = 0.68$) (Fig. 2c). A trend towards longer median EFS with CIT regimen was seen in BRAF wild-type patients [median 9 months (95% CI 6–12)] compared to those harboring a BRAF mutation [median 6.5 months (95% CI 1.8–9.1), $P = 0.29$] (Fig. 3a–c).

Longer EFS from the TOI was indeed associated with a longer OS in all patients. Using a landmark analysis for OS at 18 months, patients with a EFS greater than 6 months had a longer OS (median 60 months [95% CI 39–NR] vs. 25 months [95% CI 24–49], $P = 0.03$). Using a landmark analysis for OS at 24 months, again a longer OS was seen in patients with an EFS greater than 6 months (median 60 months [95% CI 39–NR] vs. 36 months [95% CI 25–NR], $P = 0.02$).

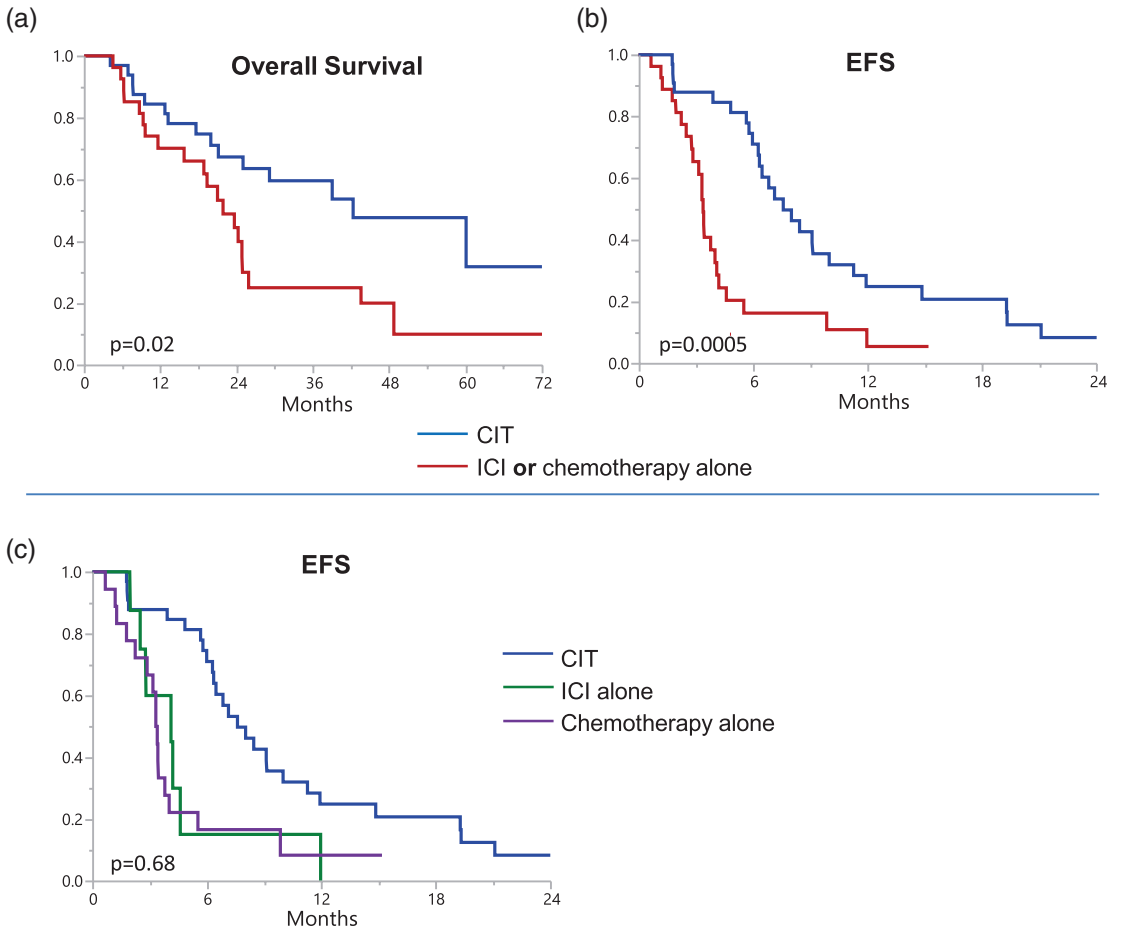
Chemo-immunotherapy combination demonstrates acceptable safety profiles

Common side effects associated with chemotherapy and immune-related adverse events (irAE) associated with immunotherapy were also reviewed. Table 2 summarizes toxicities that were attributable to the TOI. A similar rate of adverse events was seen in both cohorts. Cytopenias (anemia, leukopenia, neutropenia, or thrombocytopenia) were the most common grade 3 or higher AE, however, no cases of neutropenic fever were reported in neither cohort. In addition, the rate of irAE is not significantly increased in the CIT cohort compared with patients who received ICI alone. No additional toxicities were observed with the CIT regimen.

Dynamic changes in CX3CR1+ therapy-responsive CD8+ T-cells upon chemo-immunotherapy

We have previously reported that CX3CR1+ identifies anti-PD-1 therapy-responsive CD8+ T-cells in peripheral blood, with lower frequency in non-responders compared with responders after anti-PD-1 monotherapy. This subset of CD8+ T-cells can withstand the addition of chemotherapy with preserved anti-tumor activity that can be potentiated with concurrent anti-PD-1 therapy, and is thought to be responsible for the clinical benefit of CIT [15]. To confirm our preclinical findings, first,

Fig. 2

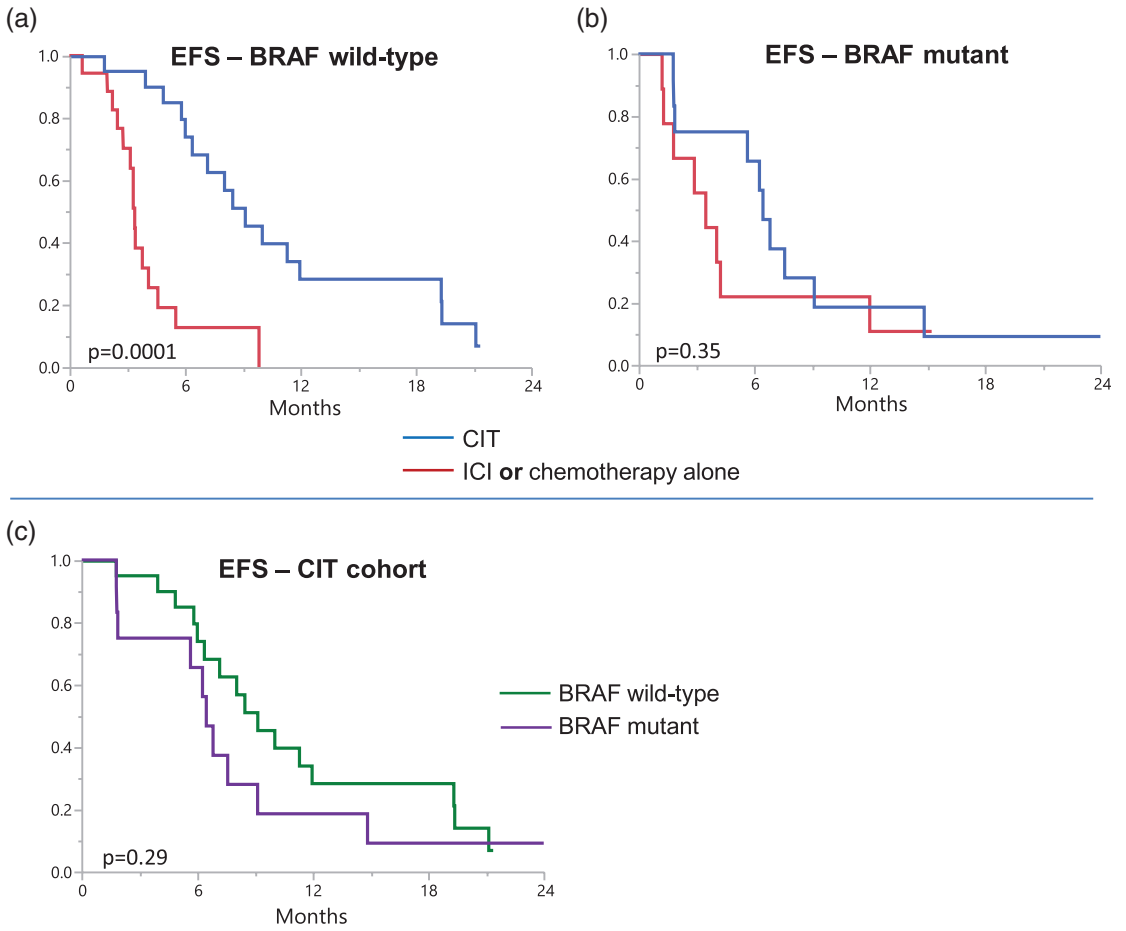


Clinical outcomes of chemo-immunotherapy (CIT), chemotherapy, or immune checkpoint inhibitors (ICI) in metastatic melanoma patients after disease progression on anti-PD1 therapy. EFS, event-free survival.

we performed single-cell RNA analysis using sorted CD3+CD45+CD8+ cells T-cells from patients' peripheral blood before (baseline) and after anti-PD-1 treatment (post-therapy). As shown in Fig. 4, after anti-PD-1 therapy, the frequency of CX3CR1-expressing cells is decreased from baseline in non-responders compared with responders, consistent with our previous finding that the levels of CX3CR1 in tumor-reactive CD8+ T-cells are lower in patients who are resistant to PD-1 blockade.

Next, we examined the dynamic change in CX3CR1+ therapy-responsive CD8+ T-cells upon CIT using archived PBMC from patients in the CIT cohort (Fig. 5a). Three patients who responded to CIT had multiple blood collections throughout the entire treatment course starting from the initiation of pembrolizumab (blue arrow). Upon disease progression, they received carboplatin and paclitaxel in combination with pembrolizumab (red arrow). The frequencies of CX3CR1+ Granzyme B+ T-cells throughout the treatment were measured. Of note, the frequency of CX3CR1+ Granzyme B+ CD8+ T cells in peripheral blood is comparable between healthy donors and untreated MM patients (Fig. S1, Supplemental digital content 1, *http://links.lww.com/MR/A229*), suggesting the change of this subset of CD8+ T cells may be induced by therapeutic regimens but not by the disease status. An increase in frequency of CX3CR1+ Granzyme B+ CD8+ T cells was associated with patients responded to CIT treatment (Fig. 5a). In contrast, similar increase of the frequency of CX3CR1+ Granzyme B+ CD8+ T cells were not observed in patients who did not respond to subsequent CIT after their diseases have progressed on PD-1 blockade (Figure S2, Supplemental digital content 2, *http://links.lww.com/MR/A230*). We previously found a decrease of the frequency of Bim+ tumor-reactive CD8+ T-cells in patients who responded to anti-PD-1 therapy [19]. In line with this observation, the frequency of Bim+ tumor-reactive T-cells did not decrease in this new cohort of patients who failed to respond to their initial

Fig. 3



Clinical outcomes of chemo-immunotherapy (CIT), chemotherapy, or immune checkpoint inhibitors (ICI) in metastatic melanoma patients after disease progression on anti-PD1 therapy according to BRAF status. (a) EFS in CIT cohort compared to ICI or chemotherapy alone cohort in BRAF wildtype MM patients. (b) EFS in CIT cohort compared to IC or chemotherapy alone cohort in BRAF mutant MM patients. (c) EFS in BRAF wild-type patients vs. BRAF mutant patients in CIT cohort. EFS, event-free survival; MM, metastatic melanoma.

Table 2 Toxicities associated with the treatment-of-interest

	CIT		ICI or chemotherapy alone		
Toxicity, %	All	Grade ≥ 3	All	Grade ≥ 3	*P* value[a]
Cytopenias	56	19	34	15	0.13
Peripheral neuropathy	25	0	15	0	0.36
Immune-related AE	25	6	12	4	0.48

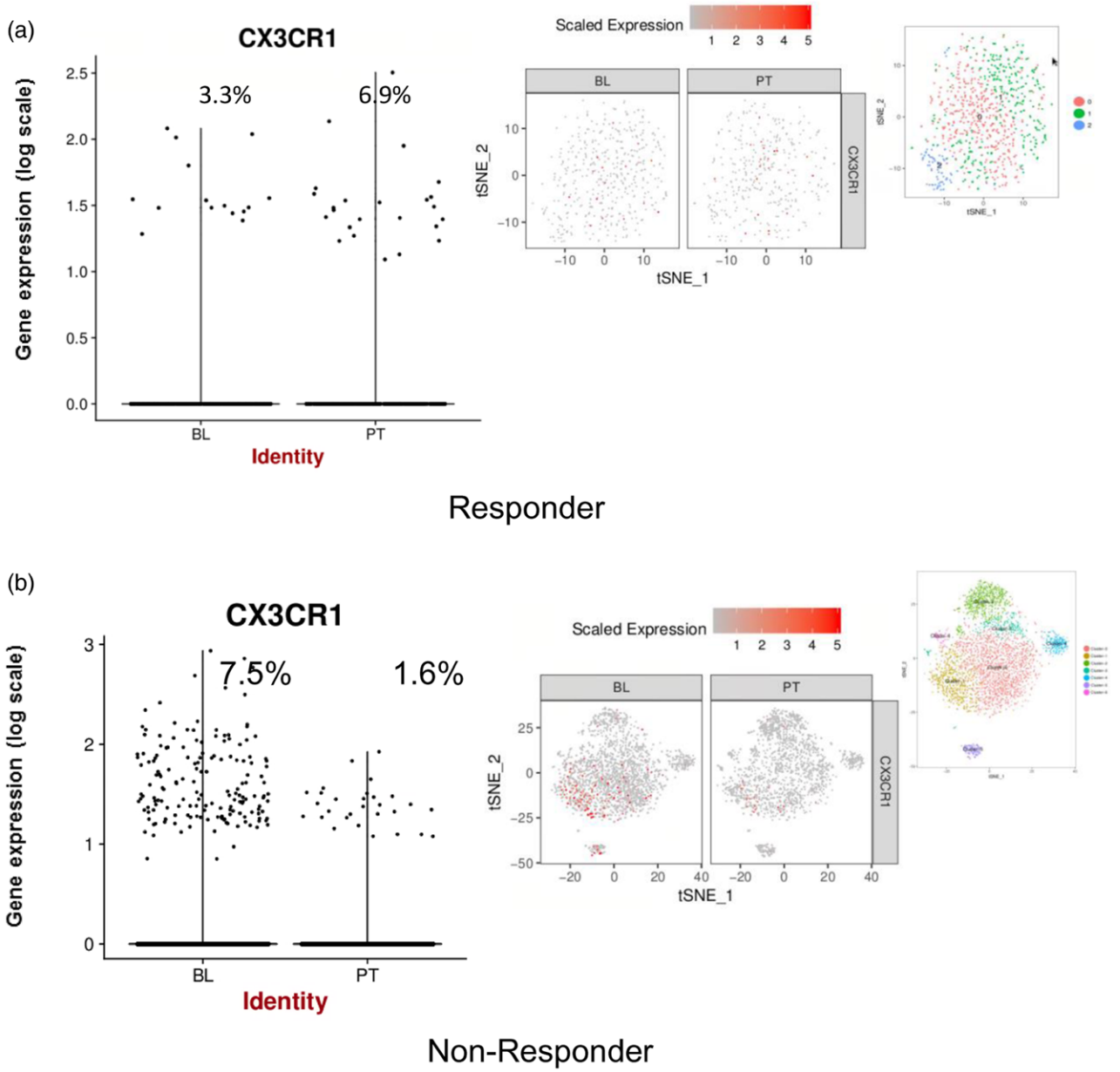
CIT, chemo-immunotherapy; ICI, immune checkpoint inhibitors.
[a]For AE of any grade.

anti-PD-1 therapy (Fig. 5b). Our results suggest that Bim expression may not be directly regulated by chemotherapy in T cells although Bim expression can be regulated by PD-1 blockade therapy as we previously reported [19]. Taken together the change of CX3CR1+ granzyme B+ CD8+ T cells in peripheral blood may reflect a dynamic T cell response to CIT in association with clinical outcomes.

Discussion

ICI have revolutionized the treatment of MM and have dramatically improved patient outcomes. However, for patients who have failed anti-PD-1 therapy, clinical management remains challenging, with poor outcomes. Here, we demonstrated that CIT can induce clinical responses in patients with disease progression after anti-PD-1 treatment, resulting in prolonged ORR and OS compared

Fig. 4

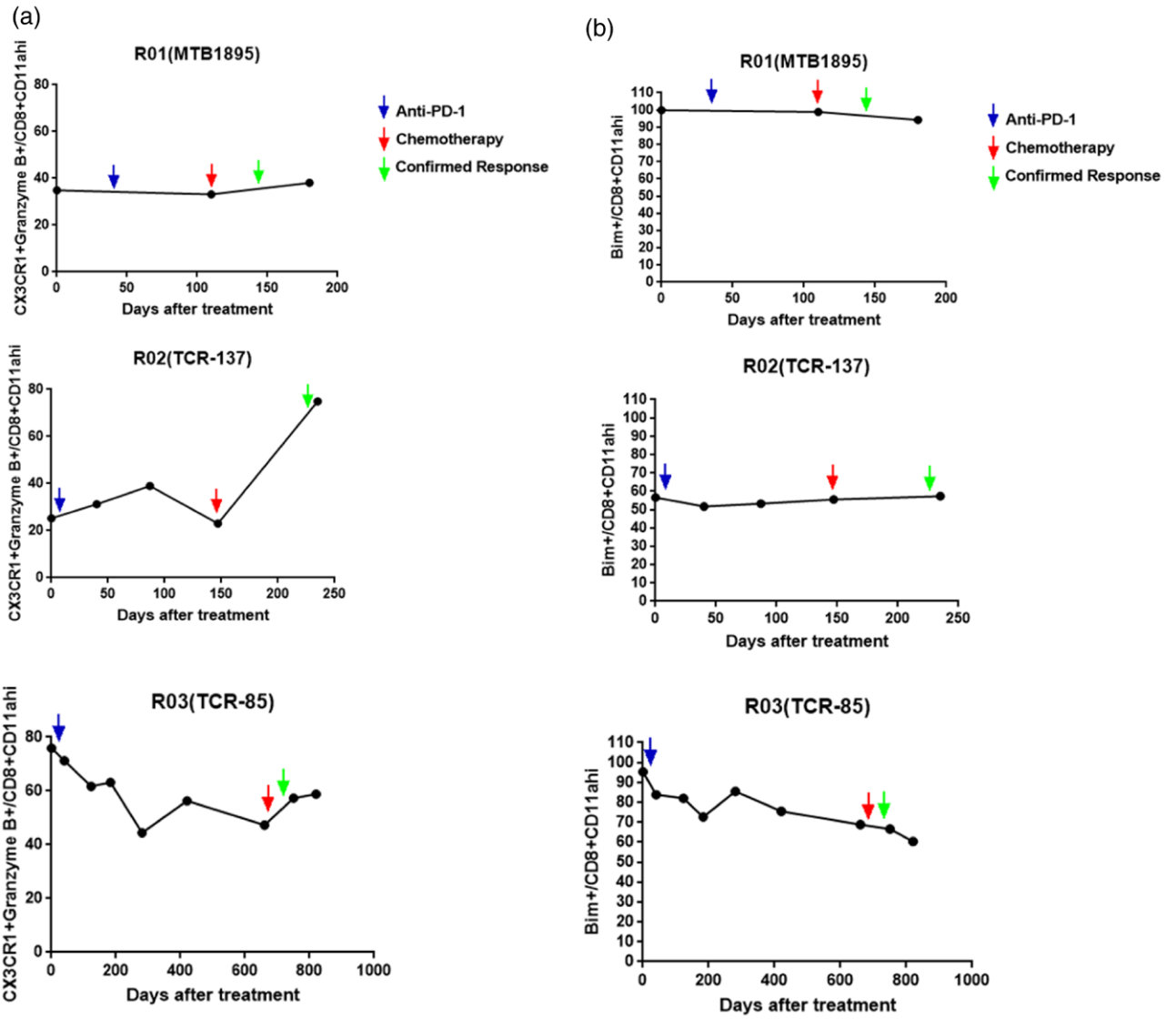


Frequency of CX3CR1+ therapy-responsive CD8+ T cells decreased after anti-PD1 therapy in non-responders. CD3+CD45+CD8+ cells were sorted and collected from PBMCs obtained from metastatic melanoma patients who have underwent subsequent anti-PD-1 therapy (BL: before anti-PD-1 therapy, PT: after anti-PD-1 therapy). Single cell RNAseq was performed and the frequencies of CX3CR1-expressing cells were compared before and after therapy in responders (a) and non-responders (b). BL, baseline; PBMCs, peripheral blood mononuclear cells; PT, post-therapy.

with chemotherapy or immunotherapy alone in the same setting, consistent with our preclinical findings in animal models.

In this study, CIT after anti-PD-1 therapy resulted in EFS of 7.6 months with an OSS of 3.5 years, suggesting a durable response provided by CIT. Anti-tumor activity of chemotherapy alone is known to be short-lasting, as demonstrated in our chemo alone cohort. We hypothesize that the durable response observed in CIT is related to chemotherapy-induced alteration of the T-cell anti-tumor immune response, by increasing the CX3CR1+ therapy-responsive CD8+ T-cells that are present in low frequencies in peripheral blood after unsuccessful anti-PD-1 therapy [15]. Concurrent PD-1 blockade in the CIT regimen can further augment the activities of this

Fig. 5



Dynamic fluctuation of CX3CR1+ therapy-responsive CD8+ T-cells in peripheral blood upon anti-PD1 and subsequent CIT therapy in patients who responded to rescue CIT. (a) Percentage of CX3CR1^{+}Granzyme B^{+} cells in CD11a^{high}CD8^{+} T-cell population isolated from the peripheral blood at multiple time points: prior to and after PD-1 therapy, the addition of chemotherapy and confirmed response to CIT (arrows indicate time points at treatment initiation and response assessment). (b) Percentage of Bim^{+} cells in CD11a^{high}CD8+ T cell population isolated from the peripheral blood obtained from the same patients at same time points as in (a). CIT, chemo-immunotherapy.

subset of effector T-cells, potentially resulting in more durable T-cell response than with chemotherapy alone, thus producing better clinical outcomes.

Combination immunotherapy has been studied in MM patients who have failed PD-1 therapy as a means to overcome treatment resistance. A retrospective study of ipilimumab to pembrolizumab after disease progression on pembrolizumab, resulted in an ORR of 21% [20]. In a recently reported phase 2 trial, pembrolizumab in combination with ipilimumab (1 mg/kg) had an ORR of 47% (CR 11.7%) after failure on pembrolizumab alone [21]. In our cohort, CIT treatment resulted in an ORR of 59% after PD-1 blockade failure, compared to 15% in those who received chemo or ICI alone. The overall disease control rate (CR, PR, and SD) was 62.5% in the CIT cohort vs. 18.5% in the chemo/ICI alone group. The lower response rates of salvage ICI reported in our study may reflect the difference in patient populations – heavily pretreated with exposure to multiple lines of systemic therapy prior to PD-1 blockade. In addition, compared to the reported phase 2 study [21], in our study, 18% of patients in the CIT cohort, and 15% in chemo/ICI cohort, had failed nivolumab/ipilimumab therapy immediately before salvage therapy. Nevertheless, our

results demonstrated CIT as an effective salvage therapy for patients who have failed multiple lines of systemic therapy with very limited therapeutic options otherwise.

Consistent with the reported CIT studies in NSCLC, our study showed that CIT demonstrated acceptable and manageable safety profiles. Most of the patients in the CIT group were able to tolerate 4–8 cycles of combination therapy. Disease assessments were performed after 2–3 cycles of CIT therapy, and patients were switched to the next line of therapy if disease progression was confirmed. Therefore, the high ORR in CIT group implies short time-to-response, suggesting that CIT can be utilized to achieve rapid disease debulking.

One of the interesting findings is that CIT tended to benefit more in BRAF wildtype MM patients than in BRAF mutants. We have recently shown that the PD-1/PDL1 pathway can impact the chemoresistance of melanoma tumor cells through p38MAPK pathway [22]. The majority of the BRAF mutant patients in this study had already received targeted therapy before the initiation of CIT, suggesting that resistance to targeted therapy due to persistent or over-activation of intrinsic signaling pathways, including MAP kinase pathway [23,24], has already developed. These preexisting dysregulations can potentially result in resistance to subsequent CIT, suggesting that the sequence of treatment, especially CIT in relation to targeted therapy, in BRAF mutant patients is of particular importance. Given the limited number of patients who had not received targeted therapy prior to CIT in our study, we are unable to assess the difference in response rates to CIT caused by the previous BRAF/MEK inhibitors exposure status. Further study in understanding the molecular mechanisms interplay between targeted therapy and chemotherapy in the setting of immunotherapy will provide critical insight in how to appropriately sequence targeted therapy and CIT in the setting of ICI.

Our pre-clinical studies identified CX3CR1+ CD8+ T-cells as the therapy-responsive T-cells that are responsible for the clinical benefits of CIT [15]. We have shown that after anti-PD1 therapy the proportion of the CX3CR1+ CD8+ effector T-cell subset is higher in responders compared to non-responders, suggesting that the expansion of these tumor-reactive T cells would be responsive to anti–PD-1 therapy. This subset of T-cells can withstand further chemotherapy by actively effluxing the chemotherapy drugs and preserve their CTL phenotypes [15]. CX3CR1+ CD8+ T cells express high level of PD-1, therefore they are targets of anti-PD 1 therapy and can expand with combination of PD-1 blockade and chemotherapy and are capable of effective tumor killing after CIT. Taking advantage of the archived serial peripheral blood samples obtained from patients who underwent anti-PD-1 therapy followed by rescue CIT treatment, we confirmed that the disease progression after PD-1 blockade and disease response upon CIT were associated with a decrease and followed by an increase in the frequencies of CX3CR1+CD8+ T-cells, respectively. Recently, two reports [25,26] have demonstrated that CX3CR1 identifies active effector CD8+ T cells that are capable of controlling cancer and viral infected cells. Both studies found that CX3CR1+ CD8 T cells are differentiated from stem-like cells that express PD-1 and blockade of PD-1 promotes the generation of CX3CR1+ effector CD8 T cells that are different from CX3CR1 negative exhausted CD8 T cells. These preclinical studies are in consistent with our clinical observation that increase of CX3CR1+ effector cells after PD-1 blockade therapy may contribute to the better clinical outcomes in cancer patients.

Interestingly, Bim level did not significantly change during the entire course of the treatment (from the initiation of anti-PD-1 to after CIT) in patients who responded to rescue CIT. We have previously demonstrated that Bim levels on tumor-reactive CD8+ T-cells decrease after successful anti-PD-1 therapy [19], yet remains largely unchanged in patients who did not respond to PD-1 blockade, which is consistent with our findings here since all the patients have failed anti-PD-1 therapy. The differing patterns in the changes of Bim+ and CX3CR1+ upon PD1 blockade and CIT suggest that they potentially have unique roles in serving as biomarkers in monitoring each individual therapy, however future prospective study with larger sample sizes is needed to further understand their roles as biomarkers.

CIT combination has been employed in the treatment of multiple types of solid tumors [12,27]. However, the optimal sequence of chemotherapy, immunotherapy and CIT in clinical practice is still unanswered. Our previous study in a mouse model showed that chemotherapy given during the T-cell expansion phase after anti-PD-1 therapy provided the optimal anti-tumor activity; and ongoing PD-1 blockade is required for the benefit of CIT [15]. Our clinical results in this study demonstrated an improved ORR of CIT (after previous PD-1 blockade exposure), compared with that of similar chemotherapy when given alone in historical clinical trials conducted in the pre-immunotherapy era [28]. Additionally, we showed that chemotherapy alone after anti-PD-1 therapy failure is inferior to CIT combination in the same setting. Taken together, these results strongly suggest that PD-1 blockade prior to chemotherapy is an active treatment sequence and that maintenance PD-1 blockade is necessary when chemotherapy is introduced. In line with our findings, among Merkel cell carcinoma patients who are treated with anti-PD-1/PDL1 agents, overall response rates doubles in chemo-naïve patients compared to in patients who had been exposed to previous chemotherapy [29,30].

In this retrospective study, all patients who have received salvage therapy after disease progression on PD-1

blockade (including those who are not eligible for clinical trials) were included with no pre-selection. Given the limitations of a retrospective study, our study needs to be further validated in a prospective study with a larger patient size, which can minimize the heterogeneity in the patient population.

Limitations to our study are not limited to the retrospective design. Brain metastasis is common in patients with MM with extremely poor outcomes. Clinical management is especially challenging when patients are resistant to anti-PD1 therapy. The clinical benefit of CIT in CNS disease will need to be further evaluated in trials that are specifically designed for CNS disease response assessment. In our study, ocular melanoma patients carry worse outcome even with CIT therapy, in line with their low response rates with the current standard of care. Their unique disease biology could result in resistance to CIT, which needs to be further elucidated. Additionally, most of the patients received carboplatin and paclitaxel as the backbone of CIT; however, the dosing schedule of the chemotherapy (once every 3 week vs. weekly), the length of combination, as well as the types of chemotherapy can result in various immunoregulatory effects and should be investigated in future studies. The sequence of therapy is also one of the topics of interest in melanoma treatment. Our results demonstrated a favorable clinical benefit when chemotherapy is given after anti-PD1 therapy with ongoing PD-1 blockade, consistent with our preclinical model. Further study is warranted for validation, taking special consideration of disease molecular and genetic features. Furthermore, the prognostic and predictive values of CX3CR1+CD8+ T-cells as a monitoring biomarker during CIT also needs to be validated prospectively in conjunction of clinical trials.

Conclusion

Our study provides clinical evidence supporting chemoimmunotherapy combination as an effective and safe therapeutic regimen for patients who have disease progression after anti-PD-1 therapy, especially in those who have failed multiple lines of previous systemic treatment. CX3CR1+ therapy-responsive CD8+ T-cells can be potentially used for clinical response monitoring.

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All patients provided signed informed written consent; the study was approved by the Mayo Clinic Rochester IRB and was conducted according to Declaration of Helsinki principles.

The datasets generated and analyzed during the current study are not publicly available due ongoing concomitant research projects, but are available from the corresponding author on reasonable request.

Conflicts of interest

There are no conflicts of interest.

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