Targeting MDSCs using ATRA: a phase I/II clinical trial combining pembrolizumab and all-trans retinoic acid for metastatic melanoma

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Targeting MDSCs using ATRA: a phase I/II clinical trial combining pembrolizumab and all-trans retinoic acid for metastatic melanoma

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Abstract: Myeloid-derived suppressor cells (MDSCs) are potent suppressors of antitumor immunity and are commonly associated with poor outcomes in melanoma patients treated with immune checkpoint inhibitors. Inducing the differentiation of MDSCs using all-trans retinoic acid (ATRA) alters their activity and reduces MDSC frequency. This trial seeks to assess the safety and efficacy of combining ATRA and pembrolizumab in metastatic melanoma patients. In 24 stage IV melanoma patients, treatment with pembrolizumab Q3W plus the supplemental treatment of ATRA orally for three days surrounding each of the first four pembrolizumab infusions effectively lowered the frequency of circulating PMN-MDSCs and enhanced melanoma-specific T cell activity. The combination was well tolerated. Median progression free survival was 20.3 months, and the overall response rate was 71%, with 50% of patients experiencing a complete response. Targeting MDSCs remains a promising mechanism to enhance the efficacy of anti-PD-1 therapies and this combination merits further investigation.
**Introduction**

While immune checkpoint inhibitors (ICIs) have improved the outlook for many melanoma patients, a significant proportion of patients exhibit resistance to these therapies. A variety of mechanisms have been associated with resistance to ICIs\(^1\). One resistance mechanism of particular interest is the accumulation of a heterogeneous population of immature immunosuppressive myeloid lineage cells called myeloid-derived suppressor cells (MDSCs)\(^2, 3\). MDSCs are potent suppressors of antitumor immunity and represent a significant obstacle to the success of ICI treatment in several cancer types\(^3, 4\). Therefore, overcoming the suppressive activity of MDSCs has the potential to enhance the efficacy of ICIs.

MDSCs diminish the efficacy of ICIs by inhibiting several different mechanisms, including direct cell:cell interactions as well as through the production of immunosuppressive factors\(^3, 4, 5, 6\). By secreting high levels of the immunosuppressive cytokines interleukin (IL)-10, transforming growth factor (TGF)-β, and reactive oxygen species (ROS) MDSCs inhibit antitumor immunity\(^3, 4, 5, 6\). In addition, MDSCs secrete arginase-1 (ARG1) and indoleamine 2,3-dioxygenase 1 (IDO1), depleting important nutrients required for effective T cell functions\(^3, 4, 5, 6\). By inhibiting the functions of CD8\(^+\) T cells, MDSCs eliminate a highly effective mechanism of antitumor immunity, as CD8\(^+\) T cells express the targets of ICIs and are important effectors of ICI activity.

MDSCs are distinguished from other myeloid cells based on the expression of HLA-DR and are characterized by their highly immunosuppressive functions\(^4, 5\). MDSCs can be divided into three subpopulations: monocytic MDSCs (MO-MDSCs, CD14\(^+\)CD15\(^-\)), polymorphonuclear-like MDSCs (PMN-MDSCs, CD14\(^-\)CD15\(^+\)Lox1\(^+\)), and early MDSCs (eMDSCs, CD14\(^-\)CD15\(^-\))\(^4, 5\). Due to their immature nature, MDSCs are susceptible to modification or reduction by differentiating agents such as all-trans retinoic acid (ATRA)\(^6, 6, 7\). ATRA, a vitamin A derivative, is part of the standard of care therapy for acute promyelocytic leukemia. Although the mechanism resulting in ATRA-mediated MDSC differentiation is still under investigation, ATRA has been shown to decrease both the frequency and function of MDSCs through activation of ERK1/2, upregulation of glutathione...
In previous clinical trials combining ATRA with a dendritic cell (DC) vaccine, IL-2, or the anti-CTLA-4 ICI ipilimumab, ATRA decreased the frequency of circulating MDSCs and improved the antitumor functions of T cells. In addition, our prior clinical trial combining ipilimumab with ATRA found that the 150mg/m² dose of ATRA appeared to be safe and well tolerated. Thus, in the current clinical trial, we hypothesized that combining ATRA with the anti-PD-1 monoclonal antibody pembrolizumab, would decrease the frequency of MDSCs and thereby improve the efficacy of pembrolizumab. The primary objective of this study was to establish the maximum tolerated dose (MTD) and recommended phase II dose (RP2D) of ATRA in metastatic melanoma patients treated with pembrolizumab. The secondary objectives were to describe the safety and toxicity of combined treatment with pembrolizumab and ATRA in melanoma patients and to assess the antitumor activity in terms of a) the reduction in MDSC frequency and suppressive function in peripheral blood of metastatic melanoma patients and b) progression free survival (PFS).

**Results**

**Patients**

A total of 24 patients were enrolled in this prospective, single arm, single institution clinical trial between 10/31/2017 and 07/30/2020 (Table 1). At enrollment, the median age was 66 years (42 – 94), median BMI was 28.5 (19 – 36), and 21 (87%) of the patients were male. All patients had an ECOG performance status ≤1, and 17% (n = 4) had elevated lactate dehydrogenase (LDH) levels on the date of informed consent. Among enrolled patients, 75% (n = 18) had received no previous treatment for melanoma, with 13% (n = 3) receiving prior ipilimumab, 4% (n = 1) receiving prior radiation, 8% (n = 2) receiving prior plaque brachytherapy, and none of the patients receiving prior targeted therapy (Table 1). Sixty seven percent (n = 16) of patients had a cutaneous primary melanoma, 8% (n = 2) had a uveal primary melanoma, 4% (n = 1) had a mucosal primary melanoma, and 21% (n = 5) had an unknown primary melanoma. BRAF V600 mutations were observed in 50% (n = 12) of patients, and NRAS mutations were observed in 20% (n = 5) of patients. The median time from initial
melanoma diagnosis to trial screen was 11.5 months (range, 0 – 62 months). At trial screen, the patients had an average of two metastatic sites with the most common sites of metastases being lungs (50%, n = 12), lymph nodes (38%, n = 9), soft tissue (33%, n = 8), and liver (17%, n = 4). Follow-up began at the time of enrollment, lasting until death, withdrawal due to enrollment in a different clinical trial, change in treatment, or data cut off, with a median duration of follow-up at the time of data cutoff of 15 months (3 – 35 months).

### Table 1. Patient demographics and disease characteristics at baseline

| Characteristic                                      | All patients (n = 24) |
|----------------------------------------------------|-----------------------|
| Median age, years (range)                          | 66 (42 – 94)          |
| Male sex, n (%)                                    | 21 (87)               |
| Median BMI (range)                                 | 28.5 (19 – 36)        |
| ECOG performance status, n (%)                     |                       |
| 0                                                   | 14 (58)               |
| 1                                                   | 10 (42)               |
| Median time from diagnosis to trial screen, months (range) | 11.5 (0 – 62)        |
| Median follow-up, months (range)                   | 15 (3 – 34)           |
| Elevated LDH level, n (%)                           | 4 (17)                |
| Prior therapies, n (%)                              |                       |
| Ipilimumab                                          | 3 (13)                |
| Radiation                                           | 1 (4)                 |
| Plaque brachytherapy                                | 2 (8)                 |
| No prior therapies                                  | 18 (75)               |
| Primary melanoma subtype, n (%)                    |                       |
| Cutaneous                                           | 16 (67)               |
| Uveal                                               | 2 (8)                 |
| Mucosal                                             | 1 (4)                 |
| Unknown                                             | 5 (21)                |
| BRAFV600 mutational status, n (%)                   |                       |
| WT                                                  | 9 (38%)               |
| Mutant                                              | 12 (50%)              |
| Undetermined                                        | 3 (12%)               |
| Sites of metastasis at trial screen, n (%)          |                       |
| Lung                                                | 12 (50%)              |
| Lymph nodes                                         | 9 (38%)               |
| Soft tissue                                         | 8 (33%)               |
| Liver                                               | 4 (17%)               |
| Adrenal gland                                       | 2 (8%)                |
| Subcutaneous                                        | 2 (8%)                |
| Skin                                                | 2 (8%)                |
| Brain                                               | 2 (8%)                |
| Breast                                              | 1 (4%)                |
| Bone                                                | 1 (4%)                |

* Follow-up was defined as the time from trial enrollment until one of the following: death, withdrawal due to enrollment in a different clinical trial or change in treatment, two years after completing treatment (two-year regimen), or data cut off.
The mean duration of exposure to treatment was 8 months (2 – 24 months) from the date of the first dose of ATRA to the off-treatment date. Grade 3 or 4 adverse events that were attributed to pembrolizumab by the investigators occurred in 4 (17%) patients, and grade 3 or 4 events that were attributed to ATRA occurred in 1 (4%) patient (Table 2). The rate of permanent discontinuation of a study drug because of treatment-related adverse events was 25% (n = 6). Four patients (17%) discontinued the study due to pembrolizumab-related side effects; two patients (8%) experienced immune-related (IR) colitis, one patient (4%) experienced IR hepatitis, and one patient (4%) experienced polymyalgia rheumatica-like myalgias, and two patients discontinued the study due to ATRA-related side effects including intolerable headaches (n = 1, 4%) and stroke (n = 1, 4%).

### Table 2. Most commonly occurring treatment related adverse events.

| Organ category                                      | Pembrolizumab plus ATRA (n = 24) | Any grade | Grade 3 and 4 events |
|-----------------------------------------------------|----------------------------------|-----------|----------------------|
|                                                     | No. of patients | Percent of patients | No. of patients | Percent of patients |
| All Aes                                             | 24                | 100%                 | 10              | 42%                 |
| **Most common adverse events**                      |                    |                      |                  |
| Cardiovascular disorders                            | 5                  | 21%                  | 3                | 13%                 |
| Endocrine disorders                                 | 7                  | 29%                  | 1                | 4%                  |
| Eye and nervous system disorders                    | 23                 | 96%                  | 2                | 8%                  |
| Flu-like symptoms                                   | 4                  | 17%                  | 0                | 0%                  |
| Gastrointestinal disorders                          | 20                 | 83%                  | 2                | 8%                  |
| General disorders                                   | 18                 | 75%                  | 1                | 4%                  |
| Hematologic and lymphatic system disorders          | 13                 | 54%                  | 2                | 8%                  |
| Hepatic disorders                                   | 9                  | 38%                  | 2                | 8%                  |
| Infections and infestations                         | 6                  | 25%                  | 1                | 4%                  |
| Injury and surgical/medical procedures              | 4                  | 17%                  | 0                | 0%                  |
| Metabolism and nutrition disorders                  | 8                  | 33%                  | 2                | 8%                  |
| Musculoskeletal and connective tissue disorders     | 10                 | 42%                  | 0                | 0%                  |
| Neoplasms-benign, malignant, and unspecified        | 3                  | 13%                  | 0                | 0%                  |
| Psychiatric disorders                               | 4                  | 17%                  | 0                | 0%                  |
| Renal and genitourinary disorders                   | 4                  | 17%                  | 1                | 4%                  |
| Respiratory, thoracic, and mediastinal disorders    | 9                  | 38%                  | 0                | 0%                  |
| Skin and subcutaneous tissue disorders              | 16                 | 67%                  | 0                | 0%                  |

All adverse events assessed using CTCAE v.4.
The most common adverse events of any grade likely related to ATRA were headache (n = 22, 92%), skin disorders (rash/dry skin/pruritus) (n = 12, 50%), fatigue (n = 11, 46%), nausea (n = 7, 29%), vomiting (n = 5, 21%), and dry mouth (n = 4, 17%) (Table 2). The most common adverse events of any grade likely related to pembrolizumab were fatigue (n = 16, 67%), skin disorders (rash/dry skin/pruritus) (n = 10, 42%), diarrhea (n = 5, 21%), and dry mouth (n = 4, 17%). Of the events related to ATRA, one patient experienced a grade 4 event, and of the events related to pembrolizumab, all were grade 3 or lower. A full description of all adverse events observed during the study can be found in Supplementary Table 2.

**Efficacy**

With a median follow-up time of 15 months (3 – 34 months), the objective response rate (ORR) was 71% (n = 17) and the disease control rate (DCR) was 88% (n = 21) (Fig. 1B-2D, Table 3). When excluding uveal melanoma patients (n = 2), who are known to have lower response rates to anti-PD-1 \(^{10,11,12}\), the ORR was 77%. The 6-month progression-free survival (PFS) rate for all patients was 63% and the median PFS (calculated from treatment start date to data cut off) was 20.3 months (95% CI, 5.2 – 12.9) (Fig. 2E). Using RECIST v1.1 criteria, complete responses (CR) were observed in 50% (n = 12) of patients, partial responses (PR) in 21% (n = 5), stable disease (SD) in 17% (n = 4), and progressive disease (PD) or clinical progressive disease (CPD) observed in 12% (n = 3) (Fig. 1B and 1D).

**Table 3. Summary of investigator-assessed clinical efficacy parameters**

| Parameter | All Patients (n = 24) |
|-----------|-----------------------|
| Best overall response, n (%) | |
| Complete response (CR) | 12 (50%) |
| Partial response (PR) | 5 (21%) |
| Stable disease (SD) | 4 (17%) |
| Progressive disease (PD) | 2 (8%) |
| Clinical progressive disease (CPD) | 1 (4%) |
| Median treatment duration, months (range) \(^b\) | 8 (2 – 24) |
| Median duration of response, months (range) \(^c\) | 11 (0.4 – 34) |
| Clinical benefit rate (CBR), n (%) \(^d\) | 14 (58%) |
| Objective response rate (ORR), n (%) \(^e\) | 17 (71%) |
| Disease control rate (DCR), n (%) \(^f\) | 21 (88%) |
| Median follow-up time, months (range) | 15 (3 – 34) |
| Median PFS, months | 20.3 |
| 6 month PFS rate, n (%) \(^g\) | 15 (63%) |
| Progressive disease (PD) | |
| On treatment \(^i\) | 12 (50%) |
| Off treatment \(^j\) | 10 (42%) |
| Median time to progression, months (range) | 4 (1 – 10) |
| Off treatment \(^j\) | 2 (8%) |
Median time to progression, months (range)  
15 (9 – 20)

Survival rate  
75%

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; CPD, clinical progressive disease; CBR, clinical benefit rate; ORR, objective response rate; DCR, disease control rate; PFS, progression free survival.

a Progression without a scan (physician diagnosed progression based on symptoms prior to scan).
b Defined as time between first ATRA dose and last treatment dose (pembrolizumab or ATRA).
c Defined as measured from the time at which CR or PR criteria are first met until the first date of PD.
d Defined as the proportion of patients demonstrating CR, PR, or SD for a minimum of 4 months from date of first CR, PR, or SD.
e Patients who achieved a best overall response of CR or PR.
f Patients who achieved a best overall response of CR, PR, or SD.
g Patients without progression at 6 months from the date of the first treatment.
h Patients diagnosed with PD or CPD.
i Patients who progressed while on treatment.
j Patients who progressed after coming off treatment.

Response to therapy was assessed based on RECIST v1.1 criteria.

Figure 1.
Figure 1. Clinical trial design and efficacy measures. (A) Schematic depiction of the treatment and correlative sample collection schedule implemented in this clinical trial. (B) Waterfall plot depicting best overall responses (BOR) according to RECIST v1.1 criteria. (C) Spider plot depicting changes in target lesion size over time. (D) Comparisons of best of overall response, overall response rate, and disease control rate. (E) Survival curve showing the progression free survival time analyzed using the Kaplan-Meyer method, with data censored at first sign of progression, death, or last known follow-up. (F) Representative images from computerized tomography (CT) scans showing responses in the labeled organs.

In patients who responded to therapy (CR or PR) (n = 17), the median duration of response was 11 months (0.4 – 34 months) (Fig. 1C, and Table 3). At the time of data cut off, 12 patients (50%) were still responding to therapy. Overall, 58% (n = 14) had clinical benefit (CR, PR, or SD for a minimum of four months from their initial radiographically demonstrated response) from the combination. Responses to therapy were observed across metastatic sites, including lung, brain, skin, adrenal gland, and lymph nodes (Fig. 1F). Of the 58% (n = 14) of patients that showed clinical benefit, 11 patients had a known driver mutation (BRAF, n = 6; NRAS, n = 4; BRAF+NRAS, n = 1; mutational analysis was unavailable from two patients) (Supplementary Table 3). In addition, of patients who showed clinical benefit, ten were diagnosed with cutaneous primary melanomas, and four were melanomas of unknown primary. Finally, of the patients who showed clinical benefit, 14% (n = 2) received prior ipilimumab monotherapy and 86% (n = 12) received no prior therapies. Median OS was not reached; however, the OS rate (calculated from treatment start date to data cut off) was 71%.

MDSC analysis

Paired analysis comparing pretreatment (Pre-TX) to post-ATRA timepoints showed sustained decreases in absolute numbers of circulating total MDSCs (CD45⁺CD3⁻CD19⁻CD56⁻CD11b⁺CD33⁺HLA-DR⁻/low, gating strategy shown in Fig. 2A) four to six weeks after stopping ATRA (Fig. 2B). This was more striking in responding patients (CR or PR) (n = 17), where the average percent change was -49%, compared to non-responders (SD or PD) (n = 7), where the average percent change was +110% (p = 0.0168) (Fig. 2B and 2D). While the absolute numbers of PMN-MDSCs was not significantly different when comparing all patients, circulating PMN-MDSCs (CD45⁺Lox1⁺CD15⁺) (Fig. 2A) decreased in responding patients (percent change -46%), but not in non-responders [percent change +427% (p = 0.003)] (Fig. 2B and 2D). No statistically
significant changes were observed in the frequency of MO-MDSCs in either responders or non-responders (Fig. 2B and 2C).

To assess whether the decrease in MDSC populations was due to ATRA-mediated differentiation into mature HLA-DR+ myeloid cells\(^6\), we measured the frequency of HLA-DR+ myeloid cells (CD45\(^+\)CD3\(^-\)CD19\(^-\)CD56\(^-\)CD11b\(^+\)CD33\(^+\)HLA-DR\(^+\)) (Fig. 2A). We observed a statistically significant increase in HLA-DR+ myeloid cells at the cycle 2 day 0 (C2D0) timepoint \((p = 0.008)\) and a trend toward increased levels at the C4D0 timepoint \((p = 0.087)\), while no differences were observed at the post-ATRA timepoint, when the patients were no longer on ATRA (Fig. 2D). This was driven by increased numbers of HLA-DR+ myeloid cells in the responding patients but not in the non-responders (Fig. 2D).
Figure 2. The combination of ATRA and pembrolizumab decreases the number of circulating PMN-MDSCs. (A) Representative flow cytometric gating strategy to identify and quantify circulating MDSC subsets. (B) Comparisons of the number of circulating MDSC subsets in pre-treatment (pre-TX) and post-ATRA blood samples, from all patients (pts), responding (Resp) patients, and non-responding (NR) patients. (C) Summary of the percent change from pre- to post-ATRA timepoints in myeloid cell populations. (D) Comparisons of number of circulating HLA-DR+ mature myeloid cells in all patients (pts), responding (Resp) patients, and non-responding (NR) patients. Colored box denotes the time when the patients were being treated with ATRA. * Denotes $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Multiplex cytokine analysis

To determine if the decreases in MDSCs were associated with alterations in the concentrations of circulating cytokines, we performed multiplex cytokine analysis. We found that concentrations of IL-6 and IL-10 were
elevated while patients were on ATRA but returned to normal or near normal levels after the completion of ATRA treatment (Fig. 4A). IL-12p70, and TNFα were also significantly increased but rose over time and were not associated with ATRA treatment (Fig. 3A). There were no statistically significant differences or trends in the circulating concentrations of the other cytokines measured (IL-1B, IL-2, IL-4, IL-8, IL-13, and IFN-γ) (Supplementary Fig S1).

*Tumor-specific T cell analysis*

Because we have previously observed that targeting MDSCs with ATRA improves the activation of circulating tumor-specific T cells\textsuperscript{6}, we compared the frequency of tumor antigen (NY-ESO-1, tyrosinase, gp100)-specific T cells between pre-TX and post-ATRA timepoints in responding vs. non-responding patients. We found a trend towards modest increases in antigen specific CD8+ T cells in responding patients but not in non-responding patients (Fig. 3B).

*Other hematologic alterations*

As in our previous study\textsuperscript{6}, ATRA significantly reduced the number of circulating eosinophils (Fig. 3C). This observation was expected as ATRA prevents the differentiation of eosinophil hemopoietic precursors into mature eosinophils by downregulating the expression of the IL-5 receptor\textsuperscript{13}. In addition, we noted a significant increase in the number of circulating neutrophils associated with the timing of ATRA treatment (Fig. 4C). No changes in the number of circulating lymphocytes were noted (Fig. 3C). No other clinically significant changes were noted from the clinical laboratory testing (Supplementary Fig. S2).
Figure 3. The combination of ATRA and pembrolizumab promotes cytokine production and tumor-specific T cell activation. (A) Analysis of circulating cytokine concentrations. (B) Comparisons of the frequency of tumor antigen-specific (NyESO, tyrosinase, and gp100) T cells, identified as CD8+CD107+IFNγ+ T cells. (C) Analysis of the number of circulating white blood cell populations from clinical complete blood count (CBC) testing. Colored box denotes the time when the patients were being treated with ATRA. * Denotes $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Discussion

Improving ICI-response rates remains an important goal in the immunoncology field. Because MDSCs are an important mediator of resistance to ICIs$^{3,4,5,6}$, targeting these highly immunosuppressive cells in combination with ICIs is a rational therapeutic combination. In this single arm, single institution clinical trial we found that
the combination of ATRA and pembrolizumab was safe and well tolerated. Furthermore, the combination effectively lowers the frequency of circulating MDSCs. This is the first time this combination has been tested in metastatic melanoma patients, and the encouraging clinical outcomes found, point to the importance of MDSCs in ICI responses and the potential of this combination as an effective first line therapy for metastatic melanoma patients.

Regarding the safety and tolerability of the combination, we observed that 21% (n = 5) of patients experienced grade 3 or 4 treatment-related adverse events. The most common treatment-related adverse event of any grade was headache (92%, n = 22), which was limited to and corresponded with the three-day course of ATRA treatment. Overall, this combination was well tolerated, with 25% (n = 6) of patients discontinuing treatment due to treatment-related adverse events, a rate similar to that reported for pembrolizumab monotherapy.

The ORR was 71%, with 50% of patients achieving a CR, and the median PFS was 20.3 months. The responses were durable, with the median duration of response lasting 11 months. These results are notably higher than previously published clinical trials using single agent pembrolizumab. When observed in the context of other currently available immunotherapies, such as combination ipilimumab and nivolumab, the favorable safety profile, high response rate, and extended median PFS make this a promising combination therapy for stage IV melanoma patients.

While we noted high response rates in both cutaneous and melanomas of unknown primary, the combination was less effective in patients diagnosed with uveal melanomas. Uveal melanomas are noted for their low ICI response rates, with low mutational burden and predisposition for liver metastasis making them particularly difficult to treat. Though the numbers were low in the current study, our results align with historical data in uveal melanoma patients. Despite the lack of response in uveal melanoma patients, we noted high response rates in patients diagnosed with melanomas of unknown primary, with four of the five patients (80%) with unknown primaries showing clinical benefit. This is a promising result for this patient cohort as several studies
have found conflicting data regarding their responses to ICIs\textsuperscript{16,17}. In addition, we noted several responses in difficult to treat metastatic sites, including the adrenal gland\textsuperscript{18}. Two patients in the study had adrenal metastases, with one patient achieving a PR in the adrenal gland and the other maintaining SD. While this is a very small number of patients, compared to previously observed low ICI response rates in melanoma adrenal metastases\textsuperscript{18,19,20}, this is an interesting observation.

There are a number of clinical trials testing new ICI combinations and targeting MDSCs is a novel and attractive way to enhance the efficacy of ICIs with few significant side effects\textsuperscript{21,22,23,24}. In the present study, we found that the combination of ATRA and pembrolizumab effectively reduced the frequency of total MDSCs, mainly driven by a significant reduction in circulating PMN-MDSCs. This was more apparent in patients who responded to the therapy than those who did not. The PMN-MDSC population is highly immunosuppressive and play an important role in ICI resistance\textsuperscript{4,25}. We did not observe statistically significant changes in the frequency of circulating MO-MDSCs. This may be due to the changes in MO-MDSCs being more observable in the tumor microenvironment\textsuperscript{26}. However, as the present study did not include tumor biopsies, this hypothesis will be tested in future studies.

The levels of several circulating cytokine have been associated with differential clinical outcomes in patients with metastatic melanoma\textsuperscript{27,28,29}. While the changes we observed were modest, the levels of circulating IL-6 and IL-10 increased during the time when patients were on ATRA and returned to near baseline levels during the pembrolizumab monotherapy portion of the study. We also observed consistent, but small, increases in the concentrations of IL-12p70 and TNFα that remained elevated after ATRA completion. These cytokines have mixed associations with responses to ICIs reported in the literature. IL-6 and IL-10 in particular can be associated with both positive and negative outcomes\textsuperscript{27,28,29,30}. Our previous work suggests that high levels of IL-6 are associated with MDSC accumulation\textsuperscript{27}. However, other results show that changes in IL-6 levels can be associated with positive clinical outcomes\textsuperscript{31}. Likewise, while IL-10 is an immunosuppressive cytokine produced by MDSCs\textsuperscript{4}, high levels of IL-10 have been correlated with positive ICI responses and clinical outcomes\textsuperscript{30}. 
In addition to its effects on MDSCs, ATRA has been shown to activate the innate immune sensing pathway in several types of tumor cells including melanoma\textsuperscript{32}. While the current study was not designed to analyze this pathway, it is possible that the dual action of targeting MDSCs and inducing a more immunogenic tumor microenvironment may account for the high response rates in this patient cohort. Future studies that include on-treatment tumor biopsies will be required to test this hypothesis.

Overall, this pilot study demonstrates that the combination of ATRA and pembrolizumab appears to be a promising approach to enhance the efficacy of anti-PD-1 and further supports the role of MDSCs in preventing effective antitumor immunity. Several new therapies designed to target tumor-induced MDSCs are currently entering clinical trials\textsuperscript{33, 34, 35}. These include therapies that induce MDSC differentiation\textsuperscript{34}, target MDSC chemotaxis\textsuperscript{33}, and those that target the suppressive functions of MDSCs (IL-10, TGF\textbeta, Arg1, IDO1)\textsuperscript{35, 36, 37}. In addition, a recent study found that adding radiation therapy to ATRA and anti-PD-1 therapy may be an effective mechanism to further induce the differentiation of MDSCs towards a pro-inflammatory, antitumor myeloid cell phenotype\textsuperscript{38}.

Several study limitations were noted. First, this is a single arm study lacking pembrolizumab monotherapy as a control, limiting the investigators’ ability to analyze the contributions of each compound to clinical response. Future studies of this combination should include a randomized design comparing single agent pembrolizumab to the combination. Second, the low numbers of patients from a single institution and relatively short follow-up time limit the generalizability to the larger melanoma population. Third, due to the non-randomized study design, the trends observed in changes in MDSCs in responders versus non-responders may not be entirely due to targeting MDSCs with ATRA. Finally, the patients in the current study received very few prior treatments and none of these patients had previously received anti-PD-1 therapies. Future studies should also include more heavily pretreated patients and patients from multiple institutions to test the generalizability of the results from this initial study.
Methods

Study design and patients

This open label, phase I/II clinical trial in 24 stage IV melanoma patients was conducted at the University of Colorado Cancer Center (ClinicalTrials.gov # NCT03200847). Eligible patients were over the age of 18 and had not been previously treated with anti-PD-1 therapy. Other inclusion/exclusion criteria are listed in Supplementary Table 1. The primary endpoints were to establish the MTD and RP2D. Secondary endpoints were safety, reduction in circulating MDSCs, overall response rate (ORR), disease control rate (DCR), and PFS according to RECIST v1.1. The study protocol and all amendments were reviewed and approved by the Colorado Multiple Institutional Review Board (COMIRB #16-1080). The trial was conducted in compliance with the International Conference on Harmonization Good Clinical Practice guidelines and the Declaration of Helsinki. All patients provided written informed consent.

Procedures

A total of twelve subjects with a cohort size of six were tested during the safety lead in, the RP2D was established at 200mg Q3W pembrolizumab plus the supplemental treatment of 150 mg/m² ATRA orally for three days surrounding each of the first four infusions of pembrolizumab (day -1, 0, and +1), with patients continuing pembrolizumab for up to two years or until confirmed disease progression or unacceptable toxicity (Fig. 1A). All patients received the RP2D. If more than two subjects experienced DLT within the first six subjects, the next lower dose level was given to the subsequent cohort. If four or fewer subjects experienced DLT among the twelve subjects during the safety lead in, the remaining subjects would continue the same dose level. A Pocock-type boundary was used to continuously monitor for toxicity39, where the toxicity probability was set at 25%, and the desired probability of early stopping was set at 0.05.

Assessments
Primary outcome measures included safety assessments and flow cytometric analysis of circulating MDSCs. Safety assessments included numbers of patients experiencing DLTs, (serious) adverse events, and changes from baseline clinical laboratory parameters. Adverse events were categorized and monitored according to CTCAE v4 guidelines. Secondary outcome measures included efficacy assessments: ORR, DCR, clinical benefit rate, PFS, PFS at 6 months (6 month PFS rate), and overall survival (OS). Efficacy assessments were measured by standard of care clinical imaging, interpreted by a certified radiologist, and verified by the treating physician. Radiologic responses were categorized based on RECISTv1.1 criteria.

Sample collection

Peripheral blood from each patient was collected into tubes containing acid citrate dextrose anticoagulant (BD Biosciences). Research blood draws were performed at four time points: pre-treatment (0 to 30 days prior to the first ATRA administration), draw 2 (day 21), draw 3 (day 63), and post-ATRA treatment (84–130 days) as illustrated in Fig. 1A. Each research blood draw corresponded with standard of care clinical blood testing including complete blood counts (CBC) with automated differential count.

Flow cytometric analysis of circulating MDSCs

Peripheral blood was collected, and plasma was removed after centrifugation at 340 × g for 10 min. Peripheral blood mononuclear cells (PBMCs) were isolated by ficoll (Cytiva) density centrifugation. Freshly isolated PBMCs were stained using the following fluorescently labeled monoclonal antibodies: APC-Lineage (CD3, CD19, CD20, CD56), BV570-CD45 (clone HI30), BV421-CD11b (clone ICRF44), APC-Cy7-CD14 (clone HCD14), PE-Cy7-CD15 (clone W6D3), FITC-CD33 (clone HIM3-4), PerCP-Cy5.5-HLA-DR (clone L243), PE-Lox1 (clone 15C4) (BioLegend). Live/dead discrimination was performed using Zombie Red dye (Biolegend). Stained cells were analyzed using the Beckman Coulter Gallios flow cytometer at the University of Colorado Cancer Center Flow Cytometry Shared Resource. Data were analyzed using FlowJo software Version 10.7 (Treestar).
**T cell response assay**

PBMCs were isolated as described above and incubated for five hours in the presence of 1 µg/mL of NyESO1, tyrosinase, and gp100 peptide pools (PROIMMUNE) with FITC-anti-CD107a (Biolegend) and 1X Protein Transport Inhibitor (eBioscience) in RPMI1640 containing 10% FBS (Gemini Bio-Products), 2 mM l-glutamine (Mediatech), 100 µg/mL streptomycin (Mediatech), 100 IU/mL penicillin (Mediatech), 25 mM HEPES (Mediatech). After incubation, the cells were washed and stained with APC-Cy7-CD8 (clone HIT8a), PerCP-Cy5.5-CD19 (clone HIB19), PerCP-Cy5.5-HLA-DR (clone L243), and Zombie Red (Biolegend). The cells were then washed, fixed, and permeabilized using the BD Bioscience Cytoperm/Cytofix kit per the manufacturer’s protocol, and stained with BV421-IFNγ (clone 4S.B3, Biolegend). Flow cytometric analysis was analyzed as above.

**Circulating cytokine analysis**

The concentrations of IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, tumor necrosis factor-α (TNFα), and IFNγ in the plasma were measured using the V-PLEX Proinflammatory Panel 1 Human Kit (Meso Scale Discovery) according to the manufacturer’s protocol. The concentrations of each cytokine were quantified on a QuickPlex SQ 120 instrument (Meso Scale Discovery) located in the Human Immune Monitoring Shared Resource at the University of Colorado School of Medicine.

**Statistical analysis**

The study is powered on the endpoint of MDSCs, as this is the primary rationale for combining ATRA with pembrolizumab. Our previous study showed that the mean reduction of MDSCs was 16.5 with a variance of 121 among four subjects. Due to the small sample size, we assume conservatively that the mean reduction is 70% and the variance is 30% more than the observed values, resulting in a mean reduction of 11.58 and a variance of 157.3. We assume the response rate of this study is no lower than 50%, thus we will have twelve or more responding subjects among the 24 enrolled. These twelve subjects provide 83% power to detect a mean reduction of 11.58 with a variance of the reduction of 157.3 using a paired t-test with a two-sided alpha of 0.05.
Comparison of pre- and post-ATRA treatment data was performed using the Wilcoxon signed-rank test. Comparisons across more than two timepoints were performed using a mixed effects model with Greenhouse–Geisser correction and Dunnet’s multiple comparisons test. All statistical tests were performed using GraphPad Prism version 9.2.0.

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