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Permalink

https://escholarship.org/uc/item/22x0s9n4

Journal

Cancer Immunology, Immunotherapy, 68(9)

ISSN

0340-7004

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Publication Date

2019-09-01

DOI

10.1007/s00262-019-02384-y

Peer reviewed

Tumor-Induced Peripheral Immunosuppression Promotes Brain Metastasis in Patients with Non-Small Cell Lung Cancer

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Abstract

Introduction: Brain metastases are a significant source of morbidity and mortality for patients with lung cancer. Lung cancer can induce local and systemic immunosuppression, promoting tumor growth and dissemination. One mechanism of immunosuppression is tumor-induced expansion of programmed death-ligand 1 (PD-L1) expressing myeloid cells. Here, we investigate the immune phenotype in peripheral blood from NSCLC patients with or without brain metastasis.

Methods: Peripheral blood was collected from patients with lung metastatic brain tumors and pre-metastatic lung cancer. Immunosuppressive monocytes, myeloid-derived suppressor cells (MDSCs), and regulatory T cells (Tregs) were quantified through flow cytometry. T cell reactivity was analyzed via ELISpot. Brain metastasis conditioned media was collected from tumor-derived cell cultures and analyzed for cytokines by ELISA. Naïve monocytes were stimulated with brain metastasis conditioned media to evaluate PD-L1 stimulation.

Results: Patients with brain metastatic lung carcinoma demonstrated increased peripheral monocyte PD-L1, MDSC abundance, and Treg percentage compared to early stage pre-metastatic

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patients and healthy controls. Patients with elevated peripheral monocyte PD-L1 had less reactive T cells and worse survival. Brain metastasis conditioned media stimulation increased monocyte PD-L1, and conditioned media IL-6 levels correlated with PD-L1 induction. Treatment with anti-IL-6 or anti-IL-6 receptor antibodies reduced PD-L1 expression. In summary, patients with lung cancer and brain metastases exhibit multiple markers of peripheral immunosuppression.

Conclusions: Tumor-derived IL-6 was capable of inducing immunosuppressive PD-L1$^+$ myeloid cells, which correlated with worse outcomes. Therefore, monitoring of immunosuppressive factors in peripheral blood may suggest new targets for therapeutic intervention in selected patients.

Précis:

In brain metastatic non-small cell lung cancer patients, increased monocyte PD-L1 expression correlated with decreased T-cell activity and worse survival. In vitro studies suggest tumor-derived IL-6 is a primary driver of monocyte PD-L1 expression.

Keywords

non-small cell lung cancer; brain metastasis; interleukin 6; IL-6; PD-L1; immune checkpoint

Introduction

Lung cancer is the leading cause of cancer death in the United States and worldwide.[2] Of all forms of lung cancer, 80 to 85% of cases are histologically identified as non-small cell lung cancer (NSCLC), the majority of which are adenocarcinoma or squamous cell carcinoma.[3] NSCLC has a high propensity to metastasize to the brain, with 15-30% of patients developing brain metastases (BM).[4] The standard of care for patients diagnosed with early stage (I-II) NSCLC often consists of surgical resection with occasional adjuvant radiation or chemotherapy.[3] However, once patients progress to stage IV disease with distant metastases, including BM, curative therapy becomes nearly impossible and the focus shifts to life prolonging treatments, primarily systemic chemotherapy. Specific management of BM with whole brain radiation therapy, stereotactic radiosurgery, or surgical resection is required as penetration of chemotherapeutic agents through the blood-brain barrier is limited.[5] For patients with BM, neurologic morbidity from the disease and from treatment constitutes a significant source of decreased quality of life.

Recent advances in immunotherapy for NSCLC have shown favorable results.[6,7] The most successful immunotherapy to date has been the introduction of immune checkpoint inhibitors targeting the programmed death-ligand 1 (PD-L1)/programmed death 1 (PD-1) pathway.[8] [9] Multiple agents targeting PD-1 (nivolumab, pembrolizumab) and PD-L1 (durvalumab, atezolizumab) have demonstrated significant improvement in overall survival for NSCLC patients compared to conventional chemotherapy in phase III clinical trials. [6,10-15] These agents are now Food and Drug Administration (FDA) approved for the treatment of stage III/IV NCSLC. Yet, while the benefits of immune checkpoint inhibition are clear for advanced malignancy, the role of these agents for early stage disease remains less well defined. Patients with stage I-II NSCLC who undergo surgery and adjuvant

chemotherapy have a 5-year survival rate of 55%.[16] Furthermore, the serious (grade 3-4) adverse reaction rate from checkpoint inhibition is in excess of 15%.[12,13,17] For advanced disease, response to checkpoint inhibitors can be followed radiographically using the response evaluation criteria in solid tumors (RECIST) criteria, but no criteria exist for monitoring early, completely resected disease. Efforts to evaluate the efficacy of immune checkpoint inhibitors in the adjuvant setting are ongoing, however there remains no way to identify which patients are at greatest risk for development of distant metastases to the brain and who would benefit from early treatment.

Metastatic events likely occur long before disseminated disease is recognized. In many instances, effective immune surveillance can eliminate early malignancy and microscopic metastases before progression to clinical disease.[18] However, it has been recognized that many cancers have the potential to induce local and systemic immunosuppression[19], which then allows lymphatically or hematogenously transported tumor cells to establish metastatic tumors throughout the body. Systemic immunosuppression in patients with malignancy has largely been attributed to expansion of suppressive immune cells. Among the myeloid lineage, expansion of suppressive myeloid cells, such as myeloid derived suppressor cells (MDSCs) has been reported and associated with poor prognosis.[20,21] We previously demonstrated that in patients with glioblastoma, circulating myeloid cells have increased PD-L1 surface expression[22], which was associated with worse survival in patients receiving vaccine immunotherapy.[23] In addition, Tregs have been found to be elevated in stage III/IV chemotherapy-naïve NSCLC patients compared to healthy donors, with the percentage of naïve Tregs associated with poor clinical outcome.[24] Based on these findings, we hypothesized that lung cancer can induce systemic immunosuppression during progression, allowing for the formation of distant metastases in the brain. In this study, we sought to identify the suppressive factors correlated with brain metastases by comparing the peripheral immune phenotype in patients with early stage (I/II) pre-metastatic NSCLC versus patients with established brain metastases. We aimed to identify immunologic changes to serve as potential therapeutic targets for patients at risk of developing disseminated metastatic disease. Having demonstrated that such immunologic changes occur in patients with glioblastoma in response to tumor-derived interleukin 6 (IL-6),[25] we also evaluated the role of IL-6 production in patients with NSCLC.

Materials and Methods

Patient cohorts, clinical tissue samples, and peripheral blood samples

Brain metastatic lung adenocarcinoma patient specimens collected at Northwestern Memorial Hospital between 2014 and 2017 were analyzed based on the availability of matched tissue for immunohistochemical analysis and peripheral blood mononuclear cells (PBMCs) for immunophenotyping. Formalin-fixed, paraffin-embedded (FFPE) tissue specimens and frozen PBMCs of patients with brain-metastatic lung adenocarcinoma were obtained from the Northwestern University Nervous System Tumor Bank. Frozen PBMCs from patients with early stage lung adenocarcinoma were obtained from the Northwestern University Pathology Core. Clinical information such as sex, tumor location, type of surgery,

extent of resection, treatment history, and outcome was obtained from review of the medical records.

Flow cytometry

Frozen PBMCs were once-thawed in a water bath at 37°C prior to use. PBMCs obtained from the Northwestern University Pathology Core had significant red blood cell contamination and were treated with a lysis buffer (154.4 mM ammonium chloride, 10 mM sodium bicarbonate, 97.3 μM EDTA) prior to staining. Cells were washed, re-suspended in a 2% bovine serum albumin solution, and stained with antibodies following manufacturer recommendations (eBioscience). The following anti-human antibodies were used for cell surface and intracellular staining: CD45 FITC (clone HI30), CD163 APC (clone eBioGHI/61), CD33 FITC (clone HIM3-4), HLA-DR APC (clone LN3), CD11b APC-eFluor 780 (clone ICRF44), PD-L1 PE (clone MIH1), CD3 PerCP-eFluor 710 (clone OKT3), CD4 PE (clone OKT4), CD25 APC (clone BC96), FoxP3 FITC (clone PCH101). For assays requiring intracellular stains, cells were first fixed in a 2% paraformaldehyde solution (Thermo Scientific) and permeabilized with a saponin-based Perm/Wash buffer (BD Biosciences). Sample data were collected by flow cytometry on an Attune NxT cytometer with Attune NxT Software (Thermo Scientific). OneComp eBeads were used for compensation controls (Invitrogen). Fluorescence-minus-one (FMO) controls were used for appropriate gating of subsets. For optimal gating, fluorescence minus one (FMO) controls were included for each patient sample. Post-hoc analyses were performed with the FlowJo Software v10.3 (TreeStar).

Immunofluorescent staining

The following primary antibodies were used in this study: IL-6 (1:400, ab6672, Abcam), CD68 (1:100, ab199000, Abcam), TTF-1 (1:50, 180221, ThermoFisher). Tissue sections from FFPE blocks (5 μm thickness) were deparaffinized in xylene and rehydrated in serial baths of graded ethanol (100%, 95%, 70%). Heat induced epitope retrieval was performed with an EDTA buffer pH 8.0 in a decloaking chamber (Biocare Medical). Sections were permeabilized with 0.5% Triton X-100 for 15 min at room temperature. Blocking of non-specific binding of primary antibody was performed using a solution of 10% normal goat serum (Sigma-Aldrich). Slides were incubated at 4°C overnight for all primary antibodies. Slides were then incubated for 1 hour at room temperature with secondary goat anti-mouse (A-11032, ThermoFisher Scientific) antibodies conjugated to Alexa Fluor 594. IL-6 staining was developed with tyramide signal amplification (Alexa Fluor 488 Tyramide SuperBoost, ThermoFisher). Coverslips were then applied using a mounting media containing DAPI (ab104139, Abcam). Slides were imaged with the Nikon A1R confocal microscope. Quantification of IL-6, CD68, and TTF-1 positivity was done manually, averaging across 3 high powered fields per tissue section.

Cell culture and brain metastasis conditioned media (BMCM) collection

Explant cell cultures were generated from brain metastasis tumor tissue. RPMI 1640 supplemented with 10% FBS (GE Healthcare), 1% sodium pyruvate, 1% non-essential amino acids, and 1% penicillin-streptomycin (Corning) was used for culture. BMCM was collected from 10 cm petri dishes at 90-100% confluency following 72 hours of

conditioning. The BMCM was subjected to differential centrifugation to remove cellular debris. BMCM was then concentrated (20X, 20kDa filter, Millipore) and stored at −80°C in aliquots to reduce freeze/thaw cycles.

Myeloid cell stimulation and IL-6 measurement

PBMCs were collected from healthy donors through Ficoll (GE Healthcare) density gradient separation. CD14$^+$ magnetic selection (Stemcell) was used to isolate monocytes according to manufacturer's instructions. Monocytes were stimulated with BMCM (1X concentration, diluted in RPMI 1640 supplemented with 2.5% FBS) for 24 hours prior to analysis by flow cytometry. Treatment conditions included anti-IL6 antibody (siltuximab, 10 μg/mL, Janssen Biotech), anti-IL6-receptor antibody (tocilizumab, 1 μg/mL, Genentech), and IgG1 isotype control (1-10 μg/mL, QA16A12 Biolegend). Assessment of IL-6 in both BMCM and plasma was accomplished using an ELISA kit according to manufacturer's instructions (Abcam, ab46042).

ELISpot assay

To compare the relative reactivity of patient T cells, the IFN-γ ELISpot assay (Cellular Technology Limited) was used. Precoated plates were blocked with RPMI 1640 supplemented with 10% FBS for 2 hours at 37°C. Patient T cells were isolated using a T cell magnetic enrichment kit (Stemcell) and plated at 1,000 cells per well in serum-free CTL-test media (Cellular Technology Limited). Propidium monoazide (PMA) (Sigma) and ionomycin (Sigma) were used to stimulate cells for 48 hours. All cultures were carried out in duplicate. The plate was washed and Biotin anti-human IFN-γ detection antibody (Cellular Technology Limited) was added. After 2 hours of incubation at room temperature, the plate was washed and avidin-alkaline phosphatase (Cellular Technology Limited) was added. Plates were developed using 80μL/well of substrate solution for 15 minutes at room temperature. Spots were imaged using the AID Classic ELISpot reader and counted using AID ELISpot software version 7 (AID ELISpot, Autoimmun Diagnostika GmbH).

Survey of The Cancer Genome Atlas (TCGA)

Lung adenocarcinoma tumor samples with mRNA data provided by TCGA (Provisional data set, National Cancer Institute) were queried. IL-6 and CD274 (PD-L1) mRNA expression determined by RNA sequencing (RNASeq Version 2) were downloaded from the cBioPortal for Cancer Genomics (Memorial Sloan Kettering Cancer Center).[26,27] Samples with IL-6 mRNA data were divided at the median into IL-6^{low} and IL-6^{high} groups and compared across CD274 expression and for differences in progression free survival (PFS) and overall survival (OS).

Statistical analysis

Statistical analyses were performed using Prism7 (GraphPad Software). Expression of various markers between groups were compared using the two-sided Student's *t* test or ANOVA. The Kaplan-Meier method was used to estimate survival distributions. Survival differences between groups were assessed using the log-rank test, and hazard ratios were calculated by a univariate analysis. P-values <0.05 were considered statistically significant.

Results

Patients

To study the correlation between peripheral immune phenotype and metastasis to the brain, banked blood samples from adenocarcinoma patients undergoing surgical resection of their primary lung tumors (stage I/II) or brain metastases (stage IV) were obtained. Peripheral blood samples were available from a total of 49 patients; 15 patients with stage I/II lung adenocarcinoma and 34 patients with stage IV brain-metastatic lung adenocarcinoma. Patient characteristics are presented in Table 1. The median age for patients with early stage NSCLC was 68 years versus 66 years for patients with brain-metastatic NSCLC (p=0.16). Of the 15 early stage patients, 11 (73%) were female; of the 34 patients with brain metastases, 21 (62%) were female. Smoking history and prevalence of driver mutations were not significantly different between patients with early stage or brain-metastatic NSCLC.

Peripheral immunosuppression in patients with brain-metastatic NSCLC

To evaluate the degree of peripheral immunosuppression, PD-L1 expression on circulating myeloid cells (CD45$^+$,CD11b$^+$,PD-L1$^+$), MDSC abundance (CD33$^+$,CD11b$^+$,HLA-DR^{low}), and Treg percentage (CD3$^+$,CD4$^+$,CD25$^+$,FoxP3$^+$) in patients with brain metastases were evaluated via flow cytometry. Patients with brain metastases had significantly elevated peripheral monocyte PD-L1 expression compared to healthy controls and non-metastatic NSCLC patients (p<0.0001) (Fig 1A, B). Due to limited availability of PBMC samples, MDSC abundance and Treg percentage were only analyzed in patients with brain metastases and healthy controls. Mean MDSC abundance was 5.7% in healthy controls and 18.0% in patients with brain metastases (p=0.0002) (Fig 1C, D). Mean Treg percentage among CD4$^+$ cells was 4.6% in healthy controls and 7.9% in patients with brain metastases (p=0.029) (Fig 1E, F).

Peripheral monocyte PD-L1 is independently associated with outcome

To evaluate the impact of immunosuppressive markers on clinical outcomes in patients with brain metastatic disease, Kaplan-Meier progression free and overall survival curves stratified by relative expression of each marker were generated. High and low subgroups for myeloid PD-L1 expression, MDSC abundance, and Treg percentage were determined relative to a cut-off around the median. Elevated peripheral myeloid PD-L1 expression was associated with significantly worse progression free (p=0.01) and overall survival (p=0.02) (Fig. 2A, B). Neither MDSC abundance nor Treg percentage were associated with differences in progression free or overall survival (Fig. 2C-F).

Patient characteristics at the time of surgery were also evaluated for impact on survival. Male sex and Eastern Cooperative Oncology Group (ECOG) performance score greater than 2 were highly associated with a significantly shorter overall survival (p=0.02 and 0.0001, respectively; Supplemental Fig. 1). Age and smoking history were not predictive of survival (data not shown).

Increased peripheral monocyte PD-L1 is associated with poor T cell function

To assess the effects of peripheral myeloid PD-L1 expression on T cell function, T cells were isolated from patient PBMC samples and stimulated in culture with PMA/ionomycin. IFN-γ production after stimulation was measured by ELISpot assay (Fig. 3). Myeloid PD-L1 expression was found to inversely correlate with T cell response and IFN-γ production (R=0.69, p = 0.038) (Fig. 3B, C). Treg percentage did not correlate with T cell reactivity (Supplemental Fig. 2).

IL-6 levels correlate with peripheral monocyte PD-L1 and progression-free survival

Having previously demonstrated that tumor derived IL-6 regulates peripheral myeloid PD-L1 expression in patients with glioblastoma,[25] we investigated the relationship between IL-6 and myeloid PD-L1 in brain metastatic NSCLC patients. Plasma IL-6 concentrations were measured by serum ELISA. Patients with brain metastases demonstrated increased IL-6 compared to non-metastatic patients (p<0.05), with an average 1.5-fold increase (Fig. 4A). In patients with early stage NSCLC, there was a positive trend toward correlation between IL-6 and peripheral myeloid PD-L1 (p=0.20; Fig 4B). In patients with brain metastases, plasma IL-6 was significantly correlated with peripheral myeloid PD-L1 expression (R=0.66, p=0.005; Fig. 4C). Similar results were observed based on analysis of the entire patient cohort (R=0.72, p<0.001; Fig. 4D).

Primary cell culture was generated from a subset of resected NSCLC brain metastases (N=6) and used to harvest BMCM. BMCM was assayed for tumor cell derived IL-6 by ELISA. IL-6 levels in each BMCM was found to correlate with peripheral myeloid PD-L1 from matched patient blood samples (R=0.83, p=0.04; Supplemental Fig. 3).

To evaluate the impact of IL-6 expression on survival, TCGA data from patients with available IL-6 mRNA expression and clinical outcomes were analyzed. In the TCGA provisional dataset, 503 lung adenocarcinoma samples had available clinical outcomes and mRNA expression levels for IL-6 and CD274 (PD-L1). Patients were divided into IL-6^{low} and IL-6^{high} groups based on mRNA expression relative to the median. Median progression free survival for patients with IL-6^{high} tumors was 28.4 months vs. 44.0 months for IL-6^{low} tumors (HR = 1.39 [95% CI, 1.036-1.852], p=0.009; Supplemental Fig. 4A). Median overall survival for patients with IL-6^{high} tumors was 49.8 months vs. 44.6 months for IL-6^{low} tumors (HR = 1.16 [95% CI, 0.869-1.55], p=0.09, Supplemental Fig. 4B). In addition, CD274 mRNA expression was greater in the IL-6^{high} group than the IL-6^{low} group (p<0.0001) (Supplemental Fig. 4C, D).

Tumor-derived IL-6 is the primary inducer of monocyte PD-L1 expression

To examine the effect of tumor derived IL-6 and other soluble factors on myeloid PD-L1 expression, naïve monocytes from healthy control subjects were cultured with BMCM for 24 hours. Analysis of induced monocyte PD-L1 expression and BCMC IL-6 levels demonstrated a strong positive correlation (R=0.91, p=0.002; Fig. 4E, F). To determine whether IL-6 was the primary driver of monocyte PD-L1 expression, monocytes were stimulated with BMCM in the presence of two clinically available IL-6 signaling inhibitors: siltuximab (SIL, an anti-IL-6 antibody) and tocilizumab (TCZ, an anti-IL-6 receptor

antibody). At clinically relevant doses, both SIL and TCZ resulted in a significant reduction in monocyte PD-L1 expression compared to isotype control (7.1% and 7.5% vs. 20.4%, respectively, p<0.001; Fig. 4G, H). No significant difference was noted between SIL and TCZ treatment effects, suggesting that the induction of PD-L1 was specific to the IL-6/IL-6R signaling pathway. Immunofluorescent staining was performed on FFPE sections of brain metastasis tissue using antibodies to IL-6, CD68, and thyroid transcription factor 1 (TTF-1) to localize the source of IL-6 in patient tumors (Fig. 5A, B). IL-6 staining predominantly colocalized with TTF-1 positive cells (p<0.0001; Fig. 5C), indicating that in the tumor microenvironment, tumor cells were the primary source of IL-6.

Discussion

Despite promising results supporting the use of immune checkpoint inhibition in patients with advanced lung cancer[12,13], the role of these agents for early stage disease remains unclear. Given the 5-year survival rate of 55%, novel treatment strategies are necessary for patients with stage I-II NSCLC beyond surgery and adjuvant radiation or chemotherapy.[16] For patients with NSCLC, the development of brain metastases is a devastating event as neurologic morbidity from the disease and treatment constitutes a significant source of decreased quality of life. However, pro-metastatic events, such as local and systemic immunosuppression[28], occur long before clinical disease is recognized. Effective immune surveillance against tumor cells requires the activation and function of cytotoxic T cells that target the neoplasm. In patients with NSCLC, various mechanisms of immune suppression have been recognized including low antigen presentation, secretion of immunosuppressive ligands, and expansion of immunosuppressive cells.[29,30] However, the majority of studies to date have focused on immunosuppression within the tumor microenvironment. In this study, we demonstrate multiple suppressive factors in the peripheral immune compartment of patients with brain-metastatic NSCLC.

By profiling peripheral immune cells using flow cytometry, we found that patients with brain-metastatic NSCLC had significantly increased MDSC abundance and Treg percentage compared to healthy controls. These findings are consistent with the literature as systemic immunosuppression has been reported in multiple cancers including glioblastoma[22,23,31,32], prostate[33], breast[34], pancreas[34], and lung[24,21]. One prominent mediator of immunosuppression in lung cancer is expression of the immune checkpoint PD-L1.[35] Binding of PD-L1 with its receptor, PD-1, induces T cell anergy or apoptosis.[36] In addition to PD-L1 expression on tumor cells, prior investigations have shown that PD-L1 on circulating myeloid cells is a key mediator of immune suppression.[22,23] We found that NSCLC patients with brain metastases had elevated peripheral myeloid PD-L1 compared to healthy controls as well as patients with early stage disease. Using matched samples, we demonstrated that patients with increased peripheral myeloid PD-L1 had T cells with reduced reactivity upon stimulation. Furthermore, we found that peripheral myeloid PD-L1 expression was significantly associated with worse progression-free and overall survival. These data suggest a possible role for immune checkpoint inhibitors in the treatment of NSCLC patients to prevent brain metastasis, as we believe that changes in peripheral immune function facilitates tumor metastasis. Additionally, given the significant increase in myeloid PD-L1 from early stage NSCLC patients to patients with

brain metastases, we believe there may be a role for peripheral immune profiling in predicting patients at risk to develop brain metastases. As immunosuppressive changes in circulation are identified through longitudinal blood testing, immune modulating therapies, such as checkpoint inhibitors, could be initiated prior to the development of metastatic disease. We do note that the association of myeloid cell PD-L1 expression and brain metastases in this study is only correlative among unmatched patients, without proven causation. Prospective studies to track individual patients from early stage disease to the development of brain metastases are underway. We hope to identify a threshold for peripheral PD-L1 expression that is predictive of patients who will develop brain metastases, such that monitoring of peripheral immune phenotypes can help distinguish patients requiring early imaging and intervention.

Prior investigation in glioblastoma has demonstrated that tumor-derived IL-6 is similarly critical for induction of myeloid cell PD-L1 expression in gliomas.[25] Although traditionally believed to be pro-inflammatory, recent studies have shown that IL-6 also mediates various anti-inflammatory effects.[37] In this study, we demonstrated IL-6 production by brain metastatic NSCLC tumors that could induce PD-L1 in naïve monocytes. Through correlative data, we demonstrated that levels of IL-6 in plasma and BMCM were significantly associated with peripheral monocyte PD-L1 expression in patients. Blockade of the IL-6/IL-6R signaling axis with siltuximab or tocilizumab significantly reduced PD-L1 expression in monocytes stimulated with BMCM, suggesting that IL-6 is the predominant driver of myeloid PD-L1 upregulation. These data indicate a possible role for targeting of the IL-6/IL-6R pathway in the treatment of NSCLC, particularly in patients with early stage disease. Inhibition of IL-6 signaling in these patients has the potential to reduce peripheral immune suppression, delay metastatic progression, and ultimately improve survival.

Apart from its role in mediating immunosuppression, IL-6 signaling has also been implicated in other aspects of cancer progression. IL-6 has been reported to promote tumor proliferation through an autocrine signaling mechanism in NSCLC.[38] In multiple mouse models, IL-6 blockade has been shown to exhibit anti-tumor effects.[39,40] IL-6 also plays a multifaceted role in tumor metastasis: promoting cell migration and vascular leakage[41,42], mediating immune escape[43], as well as serving as a chemoattractant for circulating tumor cells.[44] Multiple studies have reported peripheral IL-6 level as a prognostic marker for patients with NSCLC.[45-47] Combined with our findings, these data implicate a multifactorial role for IL-6 blockade to impede the metastatic cascade of events.

Given the retrospective nature of this study, we are unable to demonstrate causality between IL-6-induced immune suppression and the development of brain metastases. Future studies will require prospective investigations in patients and animal models to fully demonstrate the causal effect of immunosuppression on disease progression. Such studies are now underway.

Conclusion

One in every three advanced stage NSCLC patients will develop brain metastases. In this study, we demonstrated that patients with brain metastatic NSCLC exhibit profound systemic immunosuppression with expansion of myeloid-derived suppressor cell and

regulatory T cell populations. In addition, patients with brain metastatic NSCLC had increased peripheral myeloid cell PD-L1 expression, which correlated with decreased T cell activity and worse progression-free and overall survival. We identified tumor-derived IL-6 as a primary mediator of myeloid cell PD-L1 induction. *In vitro* therapeutic blockade of IL-6 signaling resulted in decreased myeloid PD-L1 expression. As antibodies blocking the IL-6 receptor (tocilizumab) and neutralizing soluble IL-6 (siltuximab) are presently clinically available, we believe that IL-6 targeted therapy should be investigated in clinical trials to reduce tumor-associated immunosuppression and inhibit the development of brain metastases in patients with NSCLC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Abbreviations:

BM	brain metastasis
BMCM	brain metastasis conditioned media
CTLA-4	cytotoxic T lymphocyte antigen 4
ECOG	Eastern Cooperative Oncology Group
FDA	Food and Drug Administration
FFPE	formalin-fixed, paraffin-embedded
FMO	fluorescence minus one
HPFs	high powered fields
MDSC	myeloid-derived suppressor cells
NCI	National Cancer Institute
NINDS	National Institute of Neurological Disorders and Stroke
NSCLC	non-small cell lung cancer
PBL	peripheral blood leukocytes

PBMC	peripheral blood mononuclear cells
PD-1	programmed death 1
PD-L1	programmed death-ligand 1
PFS	progression-free survival
PMA	Propidium monoazide
RECIST	response evaluation criteria in solid tumors
SIL	siltuximab, anti-IL6 antibody
TCGA	The Cancer Genome Atlas
TCZ	tocilizumab, anti-IL6-receptor antibody
Treg	regulatory T cell
TTF-1	thyroid transcription factor 1

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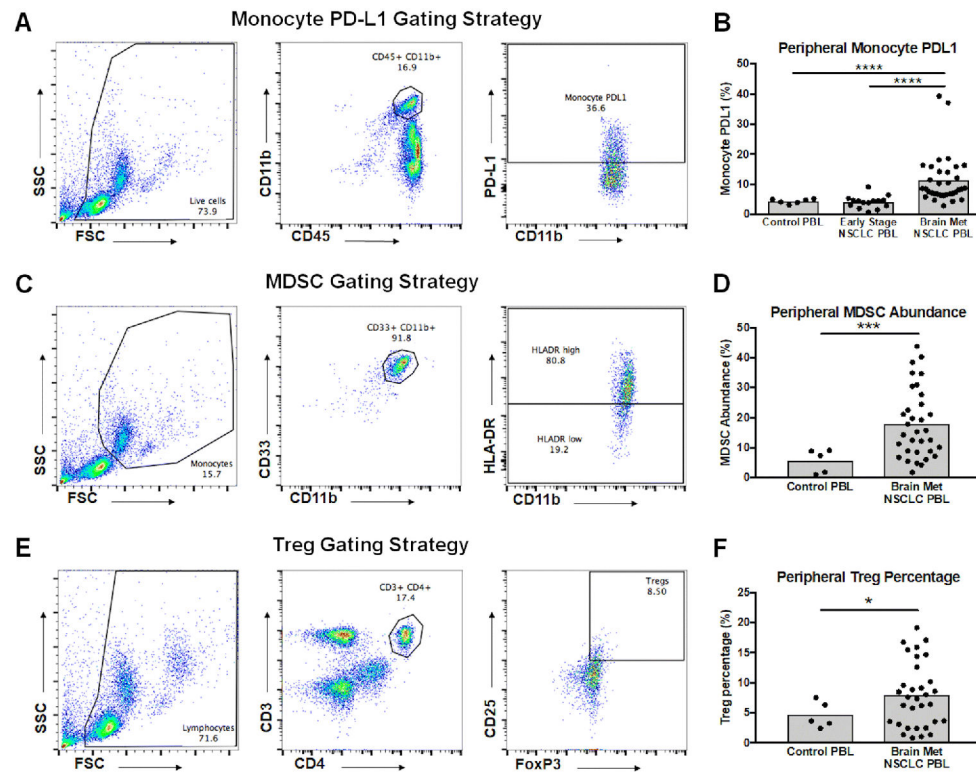
Figure 1: Patients with brain metastases exhibit increased circulating immunosuppressive cells.
(A) Representative gating scheme for identification of myeloid cells from peripheral blood leukocytes (PBL) by flow cytometry. Live cells were gated from the total population (left), followed by identification of single cells (not shown), gating for total myeloid population of CD45+/CD11b+ cells (center), and gating for PD-L1+ myeloid cells (right). (B) Summary of PD-L1 expression in monocytes from each of the 6 healthy donors, 15 early stage NSCLC patients, and 34 brain-metastatic NSCLC patients. Patients with brain metastases had increased peripheral monocyte PD-L1 (**** $p < 0.0001$). (C) Representative gating scheme for identification of MDSCs from PBLs by flow cytometry. Monocytes were gated from the total population (left), followed by identification of single cells (not shown), gating for total myeloid population of CD33+/CD11b+ cells (center), and gating for MDSCs (HLA-DR^{lo}; right). (D) Summary of MDSC abundance; patients with brain metastases had increased MDSCs (*** $p < 0.0005$). (E) Representative gating scheme for identification of Tregs from PBLs by flow cytometry. Live cells were gated from the total population (left), followed by identification of single cells (not shown), gating for CD4+ T cell population (center), and gating for Tregs (CD25+, FoxP3+; right). (F) Summary of percentage of Tregs/CD4+ T cells; patients with BM had increased Tregs (* $p < 0.05$).
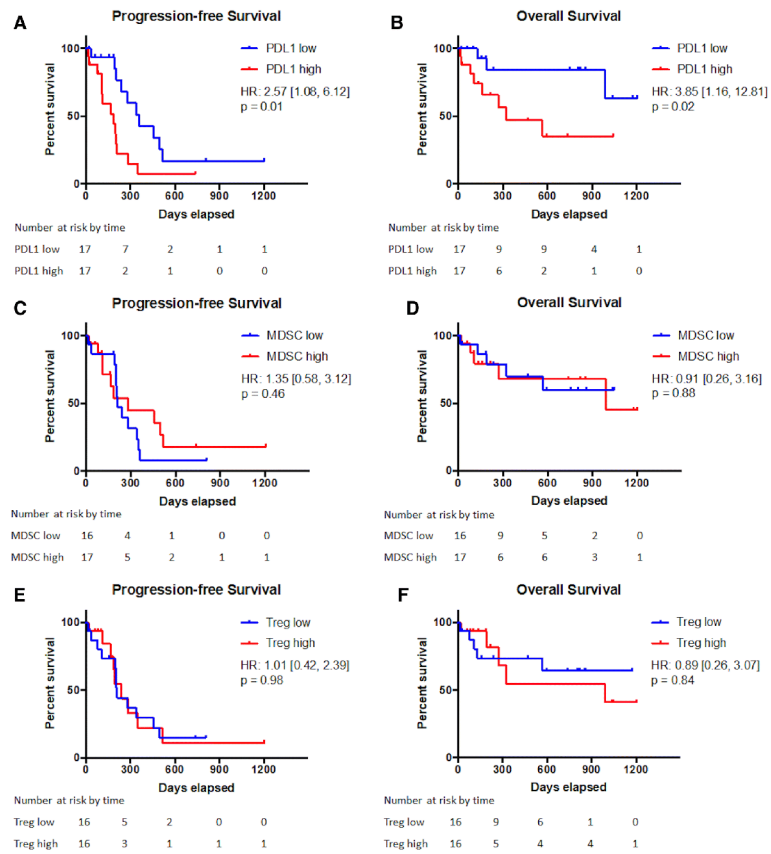
Figure 2: Elevated peripheral monocyte PD-L1 expression, but not MDSC frequency or Treg percentage, correlates with worse progression-free and overall survival.

Kaplan–Meier estimates of progression-free survival and overall survival in patients with peripheral blood analysis divided by expression of PD-L1 on peripheral monocytes (A, B), peripheral MDSC abundance (C, D), and peripheral Treg percentage (E, F). Vertical ticks indicate time points at which patients were censored.
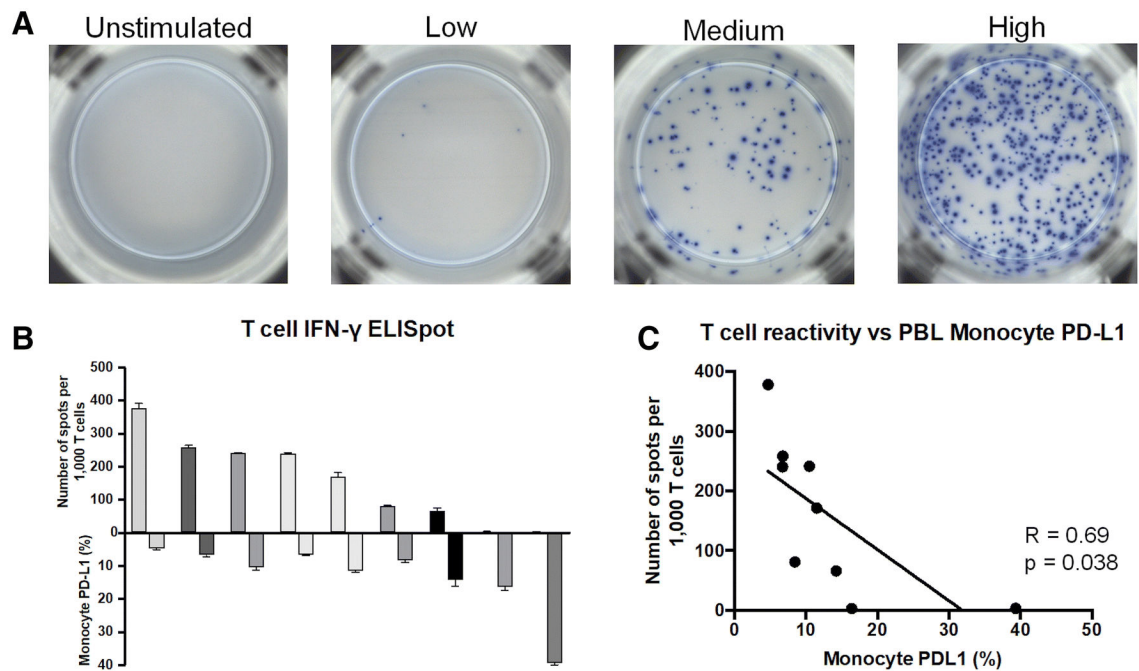
Figure 3: Peripheral monocyte PD-L1 expression correlated with T cell reactivity.
(A) Representative patient T cell responses to stimulation with PMA and ionomycin; unstimulated control (far left), low response (middle left), medium response (middle right), and high response (far right). (B) Matched patient samples (n = 9) from the IFN-γ ELISpot assay and peripheral monocyte PD-L1 profiling. Patients with increased monocyte PD-L1 expression had reduced T cell activity. (C) Linear regression comparing T cell reactivity with peripheral monocyte PD-L1 demonstrated a significant correlation (R = 0.69, p = 0.038).
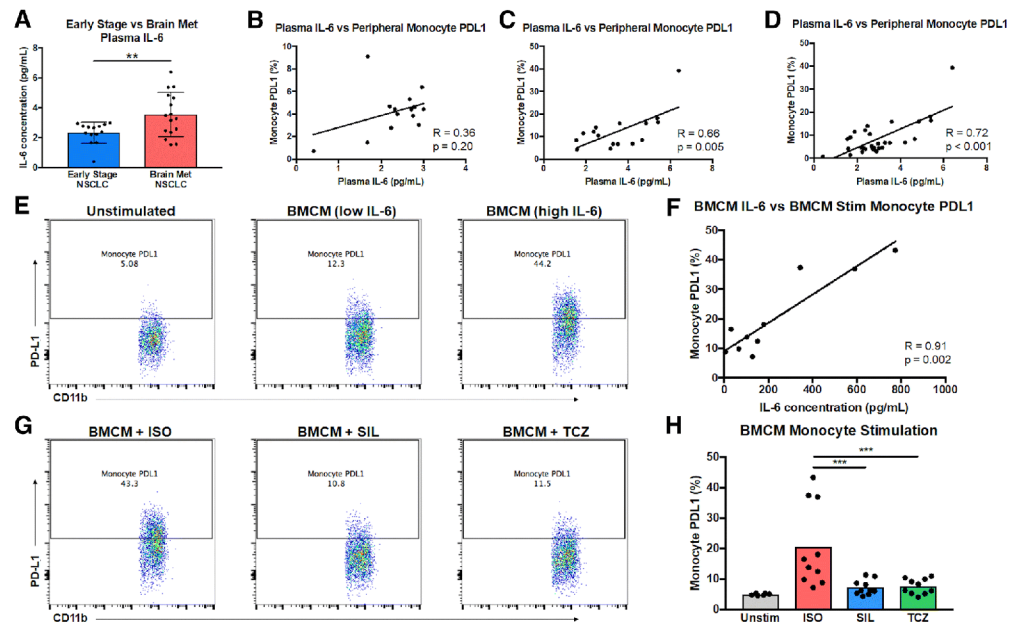
Figure 4: Tumor-derived IL-6 induces PD-L1 expression on monocytes.
(A) There was a 1.5-fold increase in plasma IL-6 in patients with brain metastases compared to non-metastatic patients (** $p < 0.01$). Correlation of plasma IL-6 with peripheral monocyte PD-L1 in early stage NSCLC patients (B) and brain-metastatic NSCLC patients (C). All patients were combined (D) and linear regression demonstrated a significant association between plasma IL-6 and monocyte PD-L1 ($R = 0.72$, $p < 0.001$). (E, F) Culture of naïve monocytes in BMCM induced upregulation of PD-L1 expression. The degree of PD-L1 expression correlated with the amount of IL-6 present in the BMCM ($R = 0.91$, $p = 0.002$). (G, H) Treatment of monocytes with siltuximab (SIL) or tocilizumab (TCZ) could prevent the increase in PD-L1 (*** $p < 0.001$) caused by exposure to BMCM (n = 10).
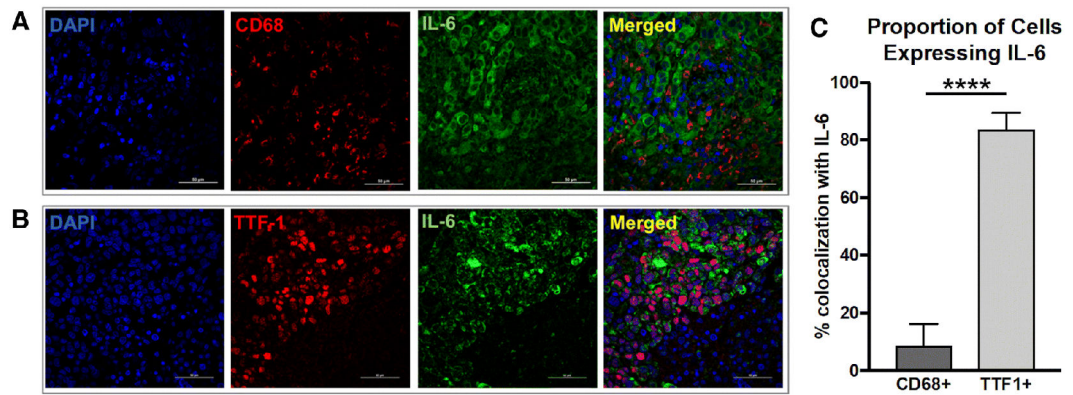
Figure 5: Tumor cells are the primary source of IL-6 secretion in the tumor microenvironment.
(A) Representative immunofluorescent staining of IL-6 and CD68 in FFPE tumor sections.
(B) Representative immunofluorescent staining of IL-6 and TTF-1 in FFPE tumor sections.
(C) Quantification of IL-6 colocalization with CD68 and TTF-1 over 3 high powered fields per tissue section. TTF-1 positive tumor cells were the predominant source of IL-6 staining (**** p < 0.0001).

Table 1

Clinical characteristics of patients included in this study

Patient characteristics	Early stage $n = 15$ (%)	Brain metastatic $n = 34$ (%)	p
Age at surgery, median (range)	68 (47–79)	66 (38–84)	0.16
Sex			
Female	11 (73.3%)	21 (61.8%)	0.53
Male	4 (26.7%)	13 (38.2%)	
Smoking history			
Never	4 (26.7%)	8 (23.5%)	0.99
<30 PY	5 (33.3%)	8 (23.5%)	0.5
30 PY	6 (40.0%)	18 (53.0%)	0.54
ECOG performance status			
0	N/A	6 (17.6%)	
1		16 (47.1%)	
2		5 (14.7%)	
3+		3 (8.8%)	
Unknown		4(11.8%)	
Mutation status			
EGFR mutation	4 (26.7%)	7 (20.6%)	0.72
KRAS mutation	5 (33.3%)	10 (29.4%)	0.99
ALK rearrangement	0 (0%)	1 (2.9%)	0.99
Negative	6 (40.0%)	16 (47.1%)	0.76
Treatment naïve at time of brain metastasis	N/A	28 (82.3%)	
Stereotactic radiosurgery after surgery	N/A	27 (79.4%)	

ALK Anaplastic lymphoma kinase, *ECOG* Eastern Cooperative Oncology Group, *EGFR* epidermal growth factor receptor, *KRAS* Kirsten rat sarcoma viral oncogene, *PY* pack-year