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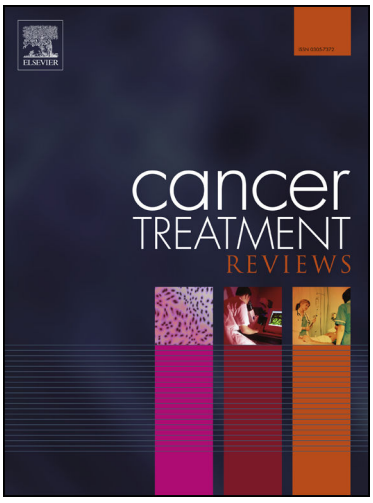

Hot Topic

Immunotherapy in NSCLC patients with brain metastases. Understanding brain tumor microenvironment and dissecting outcomes from immune checkpoint blockade in the clinic

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Review

Title: Immunotherapy in NSCLC patients with brain metastases. Understanding brain tumor microenvironment and dissecting outcomes from immune checkpoint blockade in the clinic.

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Immunotherapy in NSCLC patients with brain metastases. Understanding brain tumor microenvironment and dissecting outcomes from immune checkpoint blockade in the clinic.

Abstract

Background: Brain metastases are frequent complications in patients with non-small-cell lung cancer (NSCLC) associated with significant morbidity and poor prognosis. Our goal is to give a global overlook on clinical efficacy from immune checkpoint inhibitors in this setting and to review the role of biomarkers and molecular interactions in brain metastases from patients with NSCLC.

Methods: We reviewed clinical trials reporting clinical outcomes of patients with NSCLC with brain metastases as well as publications assessing the tumor microenvironment and the complex molecular interactions of tumor cells with immune and resident cells in brain metastases from NSCLC biopsies or preclinical models.

Results: Although limited data are available on immunotherapy in patients with brain metastases, immune checkpoint inhibitors alone or in combination with chemotherapy have shown promising intracranial efficacy and safety results. The underlying mechanism of action of immune checkpoint inhibitors in the brain niche and their influence on tumor microenvironment are still not known. Lower PD-L1 expression and less T $CD8^+$ infiltration were found in brain metastases compared with matched NSCLC primary tumors, suggesting an immunosuppressive microenvironment in the brain. Reactive astrocytes and tumor associated macrophages are paramount in NSCLC brain metastases and play a role in promoting tumor progression and immune evasion.

Conclusions: Discordances in the immune profile between primary tumours and brain metastases underscore differences in the tumour microenvironment and immune system interactions within the lung and brain niche. The characterization of immune phenotype of

brain metastases and dissecting the interplay among immune cells and resident stromal cells along with cancer cells is crucial to unravel effective immunotherapeutic approaches in patients with NSCLC and brain metastases.

Keywords: Brain metastases, non-small-cell lung cancer, tumor infiltrating lymphocytes (TILs), tumor microenvironment, PD-L1 expression, astrocytes, macrophages.

Background

Brain metastases are the most frequent cancer-related neurological complication and are associated with a negative impact in neurocognitive function, quality of life deterioration, and poor prognosis [1]. Lung cancer is the most common tumor to metastasize to the brain. About one third of patients with non-small cell lung cancer (NSCLC) will develop brain metastases and approximately fifty percent of brain metastases are diagnosed synchronously with the primary lung tumor [2].

Surgical resection and stereotactic radiotherapy are treatment options for selected patients with limited number of brain metastases [3]. For patients with multiple or symptomatic brain metastases, whole brain radiotherapy (WBRT) is considered the standard of care, despite the limited number of randomized clinical trials and the high risk to develop treatment-related neurocognitive decline [4]. However, in patients with multiple synchronous and asymptomatic brain metastases, systemic therapies can be an effective alternative approach to WBRT [5].

In patients with oncogene-addicted NSCLC brain metastases (20%) [6], next generation epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) tyrosine kinase inhibitors (TKIs) have been shown to be better than first-generation TKIs at penetrating the central nervous system (CNS) [7, 8] and have a higher intracranial response rate (iCRR) (66%–78% vs. 29%–43%) [9-11]. Third generation TKIs were effective in patients without brain metastases but also in patients with disease progression in the brain during treatment with first-generation TKIs [10-12]. First-line treatments with new generation TKIs such as osimertinib in *EGFR*-mutated patients or alectinib in *ALK*-rearranged patients showed a reduction in the cumulative incidence of brain metastases compared with first-generation TKIs [10].

Blocking the programmed death protein-1 (PD-1)/PD-L1 axis with immunotherapy has revolutionized the treatment landscape of patients with locally advanced or advanced NSCLC. Nivolumab and pembrolizumab (antibodies against PD-1) or atezolizumab (antibody against PD-L1) have been approved in the second-line setting of patients with advanced NSCLC [13-18]; while frontline pembrolizumab or atezolizumab are approved as monotherapy in patients with advanced NSCLC with PD-L1 expression equal or superior to 50% [17-19]. In addition, immunotherapy in combination with conventional chemotherapy is approved as first-line treatment of patients with NSCLC regardless of PD-L1 expression [16-18, 20-22]. In patients with stage III unresectable NSCLC treated with definitive concurrent chemoradiotherapy, consolidation with durvalumab (antibody against PD-L1) improved progression-free survival (PFS) and overall survival (OS) compared with placebo and is also an approved treatment in this setting [23-25].

However, among patients with non–oncogene addicted NSCLC with brain metastases, there are limited data available on intracranial efficacy of immunotherapy because those patients have generally been excluded or underrepresented in clinical trials [26]. The presence of tumor infiltrating lymphocytes (TILs) and PD-L1 expression has been observed in brain metastases from patients with NSCLC; however, their expression is generally not concordant among the matched primary tumor and brain metastases samples [27]. The unique organ-specific interplay between the tumor microenvironment and immune system may explain this difference. Moreover, other cells of the brain microenvironment like macrophages and astrocytes which surround brain metastases are involved in tumor progression and immune evasion [28].

The main goal of this review is to summarize the evidence for immunotherapy efficacy in patients with NSCLC and brain metastases and to compile the studies that have

reported the immune-phenotype characteristics and microenvironment interactions in the niche of NSCLC brain metastases in order to better understand and help optimize immunotherapy treatments in patients with brain metastases.

Immunotherapy treatment in patients with NSCLC brain metastases

Currently, there are limited results on immunotherapy efficacy and safety in patients with NSCLC and brain metastases. These patients have been underrepresented in most clinical trials evaluating immunotherapy. Only 6.2–17.5% of patients enrolled in these studies had asymptomatic or previously treated and stable brain metastases; however, patients with symptomatic brain metastases were excluded from all trials [26] (**Table 1**). Available data of immunotherapy efficacy in patients with NSCLC and brain metastases come from single-arm phase I/II trials [29-31], expanded access programs (EAP) [32, 33], pre-planned analyses of phase III clinical trials and retrospective series [31, 34-36].

The proof of concept for the intracranial activity of immunotherapy in patients with NSCLC came from a non-randomized, open-label, phase II trial evaluating the efficacy of pembrolizumab in patients with NSCLC with brain metastases. Eligibility criteria required at least one or more untreated or progressive brain metastases between 5 and 20 mm in patients without associated neurological symptoms or requiring corticosteroids. Cohort 1 (N=37) enrolled patients with PD-L1 expression ≥1% and cohort 2 (N=5) enrolled patients with previously treated NSCLC without PD-L1 expression or without tissue evaluable for PD-L1 expression. In the overall NSCLC cohort 57% of patients had received prior radiotherapy. Pembrolizumab showed an iCRR of 29.7% (95% CI 15.9–47.0) with 6 patients showing discordances between CNS and systemic responses.

Strikingly, none of the patients in the cohort 2 had a brain metastases response. Durable intracranial responses were observed (median 5.7 months, 95% CI 4.0–17.7) and 34% of the patients were alive at two years (95% CI 21–54) [29,30].

CheckMate 012, a phase I multicohort study assessing the safety and tolerability of nivolumab alone or combined with other therapies in patients with advanced NSCLC, included 12 patients with at least 1 asymptomatic and untreated brain metastasis in cohort M [31]. Two intracranial responses were observed (iCRR 16.7%; 95% CI 2.1–48.4) and the median PFS was 1.6 months (95% CI 0.92–2.50) and median OS was 8.0 months (95% CI 1.38–15.50). No treatment-related nervous system adverse events were reported.

The Italian and French nivolumab EAPs included 409 and 130 patients with NSCLC, who had brain metastases that were asymptomatic, stable and did not require corticosteroids. In the Italian EAP, 118 patients had received corticosteroids and 74 received concomitant brain radiotherapy. The overall response rate (ORR) was 17% and 12% and the disease control rate (DCR) was 40% and 37% in the Italian and French EAPs, respectively. Median OS was 8.1 months in the Italian EAP and 6.6 months in the French EAP [32, 33].

In a pooled analyses of patients with NSCLC and pretreated stable brain metastases enrolled in three clinical trials with nivolumab (CheckMate 063, 017 and 057) there were 46 patients with brain metastases who received nivolumab and 42 who received docetaxel as second-line treatment. Most patients had been previously treated with brain radiotherapy (74% of patients receiving nivolumab and 83% receiving docetaxel). A third of patients receiving nivolumab had no evidence of CNS progression (stable/decreased CNS lesions). Nivolumab was generally well tolerated and treatment-

related neurological adverse events occurred in 5 of 46 (11%) patients and were all grade 1 or 2 [31].

Two pooled analysis of large pembrolizumab monotherapy trials (KEYNOTE 001, 010, 024 and 042) and pembrolizumab plus chemotherapy trials (KEYNOTE 021, 189 and 407) have shown an improved survival with pembrolizumab (alone or in combination with chemotherapy) compared with chemotherapy alone, irrespective of the presence of brain metastases at baseline [34, 35].

A multicenter retrospective series with 1,025 patients with advanced NSCLC treated with immunotherapy included a cohort of 255 patients with brain metastases (39.2% active, 14.3% symptomatic and 29.4% being treated with corticosteroids). This study reported similar ORR between patients with brain metastases (20.6%) and without brain metastases (22.7%). The iCRR in patients with active brain metastases (n = 73) was 27.3%. Median OS was 8.6 months (95% CI 6.8–12.0) in patients with brain metastases and 11.4 months (95% CI 8.6–13.8) in patients without brain metastases [36].

These studies showed promising and similar efficacy in terms of OS with immunotherapy across the subgroup of patients with brain metastases and without brain metastases **(see Figure 1)**, but the available evidence has important limitations. Globally, patients with brain metastases have been underrepresented in clinical trials and this population was highly selected, including only those patients with stable, previously treated, or asymptomatic brain metastases. Furthermore, the available data comes mostly from retrospective studies or post hoc analysis of clinical trials that were not preplanned and did not adjust for multiple testing. In addition, in most phase III clinical trials, brain metastases were not a stratification factor and the studies were not designed specifically

to determine the intracranial efficacy of immunotherapy. Hence, brain imaging at the time of randomization and during follow-up were not prospectively defined or required.

Several ongoing single-arm phase II clinical trials are evaluating the role of immunotherapy in patients with untreated brain metastases (Clinicaltrials.gov NCT02681549, NCT02886585, NCT03526900). The intracranial efficacy will be measured by modified RECIST in the first study while Response Assessment in Neuro-Oncology Brain Metastases (RANO-BM) criteria [37] will be used in the other two studies. Furthermore, there are several ongoing clinical trials assessing the safety and efficacy of combining immunotherapy with brain radiotherapy (especially with stereotactic radiosurgery); however, prospective data are not available yet and these studies have not been included in this review.

In the following sections we describe the role of the microenvironment and how it is regulated by the interactions with TILs, astrocytes and macrophages in the NSCLC brain metastases niche.

Molecular and tumor microenvironment differences between NSCLC primary tumors and brain metastases

Genomic studies using next-generation sequencing have shown significant heterogeneity between matched primary lung tumors and brain metastases in terms of somatic mutations and copy number alterations [38, 39]. Mutations detected in samples obtained from brain metastases were not identified in more than half of their matched primary lung tumors, suggesting that metastatic tumor cells undergo a branched evolution [38].

Brain metastases samples have genomic alterations in molecular pathways that play a critical role in cancer progression, such as PI3K, EGFR, and in the cell cycle, such as

CDK [38]. Recently, *MYC*, *YAP1* and *MMP13* gene amplifications and *CDKN2A/B* gene deletions were also identified as metastatic drivers in lung adenocarcinoma brain metastases [40]. Gene polymorphisms or genomic alterations of the PI3K-AKT-mTOR pathway are associated with a higher risk of developing brain metastases in patients with NSCLC [39, 41]. In patients harboring PI3K pathway aberrations, all brain metastases samples showed a pattern of gene expression consistent with *PTEN* loss [39]. Similarly, loss of *PTEN* nuclear expression by immunohistochemistry (IHC) was more common in brain metastases samples than in matched primary lung tumors [42]. Gene expression analysis performed in breast and melanoma cell lines derived from patient samples showed that *PTEN* loss is common in tumor cells from brain metastases regardless of *PTEN* expression level in primary tumor cells. The loss of *PTEN* expression in brain metastases but not in other metastatic locations such as lung metastases suggests that *PTEN* loss in the brain is a secondary event imposed by the brain microenvironment [43]. The divergent evolution of metastatic cells that have settled in the brain can likely be explained by the significant pressure generated by the brain microenvironment [44].

The brain microenvironment is a unique niche that does not share many similarities with other organs and previous reviews have already excellently described the properties that set it apart [44]. Features that are unique to the brain include the presence of the blood brain barrier, exclusive environmental cells (including microglia, astrocytes, oligodendrocytes and neurons), a lymphatic system that drains to local cervical lymph nodes, and the composition of the extracellular matrix [45, 46]. In addition, the CNS has a specialized immunological microenvironment, and was classically considered an immune-privileged niche [47]. The healthy brain contains almost no lymphocytes; although there is evidence for immune surveillance of the normal human CNS by $CD3^+/CD8^+$ lymphocytes [48] and TILs have been found surrounding NSCLC brain

metastases [49]. Moreover, two major glial cells, astrocytes and microglia, have been detected surrounding and, in the case of the resident macrophages, also infiltrating NSCLC brain metastases [50]. These stromal-host cells interactions modulate tumor progression and tumor immune-evasion [51].

Characterization of immune phenotype in brain metastases

Cohorts with brain metastases samples from different solid tumors including NSCLC

The presence of tumor immune cells infiltration and their potential prognostic role has been assessed in brain metastases lesions from tumors of different origin, including lung cancer (**Table 2**).

A study characterizing the immune infiltrate in the brain metastases in 17 human autopsy tissue samples observed that it was mainly driven by activation of innate immunity, basically CD163+ and CD68+ macrophages in the peritumoral area. Whereas low burden of CD20+ and CD79+ B lymphocytes was observed and mainly CD3+, CD8+ and CD4+ T lymphocytes were detected infiltrating and surrounding the brain metastases. Moreover, a small fraction of CD8+ T lymphocytes had granzyme B expression, suggesting a low cytotoxic activation of adaptive immunity within brain metastases [50].

In one of the largest mixed cohorts (N=252), the immune infiltrates distribution was analyzed. These authors described highly variable distribution of the immune infiltrates across brain metastases with three major patterns described: perivascular stromal infiltration (typically observed in NSCLC and other carcinomas with prominent fibrovascular stroma), peritumoral infiltration (lymphocytes surrounding brain

metastases like an inflammatory wall), and homogenously diffuse infiltration (often seen in melanoma). No association between TIL and OS was identified [52].

Other studies have also suggested that a TIL infiltration can be observed in the stroma and peritumoral parenchyma of brain metastases [49, 53]. In contrast with previously mentioned studies, a high density of CD3+, CD8+ and CD45RO+ cells and a high immunescore was positively correlated with OS [49].

Cohorts with matched NSCLC primary tumor and brain metastases specimens

Remarkable spatial heterogeneity of immune infiltrates in matched primary lesions and corresponding brain metastases has been observed in patients with NSCLC. Analysis of PD-L1 and CD3+ T-cell expression in 146 NSCLC primary tumor and brain metastases paired samples reported that PD-L1 expression was discordant in 14% ($\kappa = 0.71$, 95% CI 0.55–0.87) of cases based on PD-L1 expression in tumor cells and 26% ($\kappa = 0.38$, 95% CI 0.17–0.59) of cases based on PD-L1 expression in immune cells. Significantly more brain metastases (n = 35, 24%; 95% CI 18–32) than primary tumors (n = 23, 16%; 95% CI 11–23; p = 0.009) were lacking TILs and PD-L1 expression. Primary lung tumors (n = 22, 15%; 95% CI 10–22) had more positive TILs and PD-L1 expression than brain metastases (n = 13, 9%; 95% CI 5–15; p = ns). Taken together, these results suggested that the tumor microenvironment of brain metastases is more likely to be immunosuppressive compared with primary lung tumors [27].

In a cohort of 20 paired NSCLC primary tumor and brain metastases samples, T-cell receptor sequencing showed a significant reduction of T-cell clones in brain metastases compared with paired primary tumors (median 1540 vs. 4551; p = 0.0005) [54]. A

minimal overlap in T-cell clones was observed between paired lesions, suggesting that most T-cell clones were unique to the lesion in which they were detected. Like other studies, fewer T cells (CD3+) were observed in brain metastases than in primary lung tumors ($p = 0.003$); however significantly higher tumor mutational burden (TMB) was observed in brain metastases than in the paired lung lesions (median 24.9/Mb vs. 12.5/Mb, $p < 0.0001$). Despite the higher TMB detected in brain metastases samples, predicted neoantigen load was not significantly higher in brain metastases compared with matched primary tumors (median 898 vs. 874 respectively; $p = 0.20$). Spatial intratumor heterogeneity and accumulation of subclonal mutations along with divergent tumor immunogenicity associated with the metastatic process could explain the disparities between matched samples [54].

Lower T-cell richness and T-cell densities were reported in brain metastases compared with primary lung tumors after sequencing T-cell receptor beta (TCRβ) in a cohort of 78 samples from NSCLC primary tumors and paired brain metastases [55]. However, in contrast with the previous study, the authors found a high frequency of shared T-cell clones between matched samples. The immune profiling analyses using a 770-immune gene expression panel reported significantly lower Th1, CD8+ and TILs and high fraction of monocyte-derived macrophages in brain metastases compared with primary tumors.

The previous studies evaluated the brain metastases immune phenotype from retrospective cohorts of patients who had not been treated with immunotherapy; therefore, the predictive value of these markers cannot be assessed. In addition, these studies were highly heterogenous in terms of the biomarkers evaluated, antibodies clones employed, cut-offs used for PD-1/PD-L1 positive definition, and methods used for their

quantification may limit the conclusions obtained. The discrepancies about the prognostic role of TILs in brain metastases may be explained by differences in the study population and prior systemic and local treatments received.

Interactions between tumor microenvironment and tumor cells in brain metastases

Many studies have shown that brain metastases specimens from patients with NSCLC are surrounded and infiltrated by activated astrocytes and microglia [50, 56]. In the next section and in **Figure 1** we aim to summarize the molecular mechanisms employed by glial cells to modulate tumor progression and tumor immune response [51].

Astrocytes

Astrocytes are the most abundant glial cells in the brain metastases microenvironment. After injury, astrocytes change their phenotype upregulating the levels of GFAP and inducing a transcriptional program known as reactive astrogliosis [57]. Reactive astrocytes (RA) play a dual role. At initial steps of brain metastases development, RA produce deleterious signals that compromise the viability of metastases-initiating cells [58, 59]. However, once metastatic cells are established, RA facilitate tumor progression [60]. In the brain microenvironment, RA are a major source of plasminogen activator (PA) converting plasminogen into plasmin endopeptidase. In response to brain injury, astrocytes also express high levels of proapoptotic cytokine FasL in their membrane. Plasmin suppresses brain metastases by transforming membrane-bound astrocytic FasL into a paracrine death signal targeting cancer cells and by inactivating the adhesion molecule L1CAM expressed by tumor cellsand used for spreading along brain capillaries. Brain metastases from lung and breast cancers have been shown to prevent

plasmin activation by producing high levels of anti-PA serpins, mainly neuroserpin and serpin B2. These anti-PA serpins block RA-mediated plasmin activation and reverse the metastasis-suppressive effects of plasmin [28].

Gap junctions are also involved in the communication between astrocytes and tumor cells. Protocadherin-7 expression in tumor cells promotes conexin43-dependent gap junction formation in lung and breast adenocarcinoma models. Once these junctions are formed, cancer cells from brain metastases transfer cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) to astrocytes activating the STING pathway, an innate immune response pathway able to sense cytosolic double-stranded DNA; thereby producing inflammatory cytokines such as TNF-α and IFN-α. These paracrine signals activate STAT1 and NF-κB pathways in cancer cells promoting tumor growth and increased chemoresistance [61]. Gap junctions are also involved with transferring small non-coding RNAs (ncRNAs) from astrocytes to lung cancer cells. These ncRNAs promote resistance to chemotherapy and are overexpressed in human lung tumor cells co-cultured with astrocytes compared with lung cancer cells cultured without them [62].

Activation of the endothelin-axis orchestrates the pro-survival transcriptional program in lung cancer cells through gap junctions. Specifically, heterotypic gap junctions between cancer cells and astrocytes stimulate upregulation of IL-6 and IL-8, which increase endothelin-1 (ET-1) production from astrocytes and ET receptor expression (ET_AR and ET_BR) on cancer cells. This was associated with activation of the phosphorylated kinases AKT and MAPK and induction of anti-apoptotic genes, such as *BCL2L1*, *GSTA5* and *TWIST1* in cancer cells [63]. As expected, a dual antagonist of ET_AR and ET_BR signaling in combination with paclitaxel prevented astrocyte-mediated protection of cancer cells

leading to a significant reduction in cell division with increased apoptosis, and to increased survival in mice harboring brain metastases [64].

STAT3 activation (Tyr705 phosphorylation, pSTAT3) in a subpopulation of RA associated with lung-derived brain metastases cells induces a pro-metastatic phenotype. Increased STAT3 signaling in RA promotes metastasis viability and modulates innate and acquired immune responses. In this sense, pSTAT3+RA negatively influence CD8+ T-cell activation presumably through PD-L1 expression and through secretion of immunosuppressive molecules such as vascular endothelial growth factor-A (VEGF-A), lipocalin-2, tissue inhibitor of metalloproteinases-1 (TIMP-1), and proteins of the extracellular matrix (ECM) that could act as a physical barrier limiting the access of CD8+ T-cells. Additionally, pSTAT3+RA also promote expansion of CD74+ microglia and macrophages by increasing the levels of CD74 and macrophage migration inhibitory factor (MIF). Patients with higher levels of pSTAT3+RA in brain metastases had shorter survival. In a cohort of 18 patients with NSCLC with previously treated brain metastases, treatment with an oral STAT3 inhibitor yielded intracranial responses [60].

Moreover, astrocytes are well-known secretory cells with the ability to release extracellular vesicles into the brain microenvironment [43]. Models of brain injury showed that extracellular vesicles released from RA were able to attract peripheral leukocyte cells to the brain through regulation of acute cytokine production in the liver [65]. The ability of astrocytes to attract peripheral leukocytes in the brain metastases context is unknown.

Tumor-associated macrophages and microglia

Microglial cells constitute highly specialized resident tissue macrophages of the CNS which are renewed by local proliferation and act as a major component of the brain immune system [66]. After a brain injury, microglial cells exhibit phagocytic and cytotoxic properties and can release several factors like nitric oxide (NO) and pro-inflammatory cytokines, which have anti-tumor properties [67]. However, upon certain CNS disturbances such as glioblastoma invasion, microglial cells release immunosuppressive factors such as interleukins, transforming growth factor-β (TGF-β), monocyte chemoattractant protein (MCP-1) and prostaglandin E2 (PGE-2) which promote tumor growth [68]. Most data on microglia behavior in brain lesions come from primary brain tumors, fundamentally glioblastoma; whereas the role of microglia in the brain metastases context has been less studied.

Until recently, there were no specific biomarkers to differentiate macrophages from tissue-resident microglia and bone marrow-derived macrophages (BMDMs) [69]. CD49D and TMEM 119 have been identified as differential markers between BMDMs and microglia [69, 70]. These studies also suggested that most tumor associated macrophages (TAM) in the brain metastases were derived from peripheral monocytes and not from resident microglia [69, 71].

In established NSCLC brain metastases, TAM and microglia are the most abundant non-cancerous cells types surrounding and infiltrating the tumor mass. The TAM/microglia cells adopt a tumor-supportive phenotype and promote tumor progression by decreasing not only their cytotoxic activity, but also TNF-α and iNOS expression [50, 72]. Co-culture studies of breast cancer cell lines with macrophages showed that WNT signaling was a key regulator of tumor invasion which can be reversed with a WNT antagonist

[73]. Similarly, this molecular pathway, through *LEF1* and *HOXB9* target genes, has been identified as major determinant of lung adenocarcinoma dissemination to the brain [74].

A recent study using a lung-derived brain metastases xenograft model found that immune checkpoints Lag3 and Havcr2 (Tim3) were overexpressed in macrophages present in the brain stroma. Authors hypothesized that these immune checkpoints may contribute to brain metastasis progression due to a reciprocal neuroinflammatory response of the stroma [75].

Future studies employing specific biomarkers able to distinguish between microglia and TAMs will help us to understand the molecular pathogenesis of brain metastasis progression and to dissect the interaction between tumor cells and brain microglia.

Conclusions

Despite the increasing incidence of brain metastases in patients with NSCLC, relatively few patients have been included in clinical trials with immunotherapy. Only a highly selected group of patients with previously treated or asymptomatic brain metastases have been treated in those studies, which limits the broader applicability of these results to the clinical practice. In patients with advanced NSCLC with brain metastases, immune checkpoint inhibitors achieved an encouraging iCRR comparable to extracranial response. In terms of OS, NSCLC patients with brain metastases benefit from immunotherapy as much as the overall population. Analysis of matched primary tumor and brain lesions in small cohorts, revealed that brain metastases are more likely to be immunologically “cold", showing lower PD-L1 expression and infiltration by

lymphocytes. Differences in the genomic landscape and organ-specific particularities of tumor microenvironment and immune system may explain these discrepancies. CNS-specific cells such as glial cells, RA, microglia, and BMDMs that surround the brain metastases lesion and play pro- and anti-tumor roles in response to tumor-cell derived soluble factors. The interactions between resident CNS cells with metastatic lung cancer cells as well as the immune system have been poorly studied. In this regard, it has been shown that pSTAT3+ RA promote immune evasion by influencing CD8+ T cells and CD74+ microglia.

Due to the limited clinical and preclinical evidence, efforts should be made to increase the recruitment and development of clinical trials focusing on patients with brain metastases. Moreover, further research is needed to understand the mechanism of action of immunotherapy in the brain and the biological interactions between cancer cells, infiltrating immune cells and resident brain cells to define effective therapeutic strategies able to improve outcomes and quality of life of patients with NSCLC with brain metastases.

List of abbreviations:

NSCLC: non-small-cell lung cancer

TILs: tumor infiltrating lymphocytes

PD-L1: programmed death ligand-1

TMB: tumor mutational burden

WBRT: whole brain radiotherapy

BSC: best supportive care

OS: overall survival

iCRR: intracranial response rate

ORR: overall response rate

EGFR: epidermal growth factor receptor

ALK: anaplastic lymphoma kinase

TKIs: tyrosine kinase inhibitors

CNS: central nervous system

PD-1: programmed death protein-1

PFS: progression free survival

EAP: expanded access programs

DCR: disease control rate

iDCR: intracranial disease control rate

IHC: immunohistochemistry

RA: reactive astrocytes

cGAMP: cyclic guanosine monophosphate-adenosine monophosphate

ncRNAs: non-coding RNAs

ET-1: endothelin-1

VEGF-A: vascular endothelial growth factor-A

TIMP-1: tissue inhibitor of metalloproteinases-1

ECM: extracellular matrix

MIF: migration inhibitory factor

NO: nitric oxide

TGF-β: transforming growth factor-β

MCP-1: monocyte chemoattractant protein

PGE-2: prostaglandin E2

TAM: tumor associated macrophages

BMDM: bone marrow-derived macrophages

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Figure Legends

Figure 1. Median overall survival among patients with NSCLC and brain metastases and without brain metastases reported in pivotal phase III clinical trials with immunotherapy.

Abbreviations: BM: brain metastases; HR: hazard ratio; ICI: immune checkpoint inhibitors; OS: overall survival.

Figure 2. Interactions between stromal cells (astrocytes and tumor associated macrophages/microglia) and NSCLC brain metastases-derived cells.

Abbreviations: BCL2L1: BCL-2 like 1; CD74: cGAMP: 2′3′-cyclic GMP-AMP; Cx43: conexin43; dsDNA: double-stranded DNA; ET-1: endothelin-1; ET_AR: endothelin receptor A; ET_BR: endothelin receptor B; FasL: FAS ligand; GSTA5: glutathione S transferase alpha 5; IFN-α: interferon α; IL-1β: interleukin-1β; IL-6: interleukin 6; IL-8: interleukin 8; L1CAM: L1 cell adhesion molecule; LAG3:

lymphocyte activation gene-3; MCP-1: monocyte chemo-attractant 1; MIF: macrophage migration inhibitory factor; miRNA: microRNA; MMP-2: metalloprotease-2; MMP-9: metalloprotease-9; ncRNAs: non-coding RNAs; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; NS: neuroserpin; PA: plasminogen activator; PAI-1: plasminogen-activator inhibitor -1; PCDH7: protocadherine-7; PGE-2: prostaglandin E2; pSTAT3: phosphorylated signal transducer and activator of transcription 3; SB2: serpin B2; sFasL: soluble FAS ligand; STAT1: signal transducer and activator of transcription 1; STING: Stimulator of interferon genes; TGF-β: transforming growth factor beta; TIM3: T cell immunoglobulin and mucin domain-containing protein 3; TIMP-1: tissue inhibitor of metalloproteinases-1; TNF-α: tumor necrosis factor-α; TWIST1: TWIST related protein 1; VEGF-A: vascular endothelial growth factor A.

Highlights

- The brain microenvironment, including brain metastases (BM), is “immunologically cold”
- The mechanism of action of immunotherapy in the brain or BMs is not well understood
- Intracranial responses can occur in patients with NSCLC receiving immunotherapy
- Understanding the interplay of microenvironment and BMs may improve immunotherapy outcomes

Table Legends

Table 1. Key inclusion criteria and efficacy results from pivotal phase III clinical trials with immune checkpoint inhibitors in the subgroup of patients with NSCLC and brain metastases.

Abbreviations: BM: brain metastases; HR: hazard ratio; ICI: immune checkpoint inhibitors; MRI: magnetic resonance imaging; m: months; NA: not available; NSCLC: non-small cell lung cancer; NR: not reached; OS: overall survival.

Table 2. Summary of immune-phenotype studies containing cohorts of brain metastases from NSCLC.

Abbreviations: H&E, hematoxylin and eosin; IC: immune cells; NSCLC: non-small cell lung cancer; PD-L1: programmed death ligand-1; PD-1: programmed cell death protein 1; TC: tumor cells; TIL, tumor-infiltrating lymphocytes.

Table 1. Key inclusion criteria and efficacy results from pivotal phase III clinical trials with immune checkpoint inhibitors in the subgroup of patients with NSCLC and brain metastases.

Study	ICI arm vs. control arm	Histology	PD-L1 expression	Mandatory brain MRI at screening	Number of patients with BM included	Brain metastasis inclusion criteria	ICI vs. control arm median OS (months) HR (95% CI)
Immunotherapy monotherapy							
First-line phase III trials							
Checkmate 026 [76]	Nivolumab vs. platinum doublet	NSCLC	≥1%	Yes	69 (13%)	Pretreated, off corticosteroids or on a stable or decreasing dose of ≤10 mg daily prednisone and stable	NA
KEYNOTE 024 [19, 77]	Pembrolizumab vs. platinum doublet	NSCLC	≥50%	Yes	28 (9.1%)	Pretreated, off corticosteroids and stable	HR 0.73 (0.20- 2.62)
KEYNOTE 042 [78]	Pembrolizumab vs. platinum doublet	NSCLC	≥1%	No	70 (5.5%)	Pretreated, off corticosteroids and stable	NA
Second-line phase III trials							
CheckMate 017 [14, 31]	Nivolumab vs. docetaxel	Squamous carcinoma	All comers	No	17 (6%)	Pretreated, off corticosteroids or on a stable or decreasing dose of ≤10 mg daily prednisone and stable	5.0 m vs. 3.86 m HR NA
CheckMate 057 [13, 31]	Nivolumab vs.docetaxel	Non-squamous carcinoma	All comers	No	68 (12%)	Pretreated, off corticosteroids or on a stable or decreasing dose of ≤10 mg daily prednisone and stable	7.6 m vs.7.3 m HR 1.04 (0.62–1.76)
KEYNOTE 010 [79]	Pembrolizumab vs. docetaxel	NSCLC	≥1%	No	152 (14.7%)	Pretreated, off corticosteroids and stable	NA
OAK [15, 80]	Atezolizumab vs. docetaxel	NSCLC	All comers	Yes	123 (10%)	Pretreated, off corticosteroids, stable and supratentorial	16 m vs. 11.9m HR 0.74 (0.49–1.13)
Combination of immunotherapy with chemotherapy							
First line phase III trials							
KEYNOTE 189 [20]	Carboplatin-pemetrexed + pembrolizumab vs. Carboplatin-pemetrexed + placebo	Non-squamous carcinoma	All comers	No	108 (17.5%)	Previously treated, stable and off corticosteroids or untreated, asymptomatic and off corticosteroids	HR 0.36 (0.20–0.62)
KEYNOTE 407 [21]	Carboplatin-(nab)paclitaxel +	Squamous carcinoma	All comers	No	44 (7.8%)	Previously treated, stable and off corticosteroids or untreated,	NA

Study	ICI arm vs. control arm	Histology	PD-L1 expression	Mandatory brain MRI at screening	Number of patients with BM included	Brain metastasis inclusion criteria	ICI vs. control arm median OS (months) HR (95% CI)
	pembrolizumab vs. Carboplatin-(nab)paclitaxel + placebo					asymptomatic and off corticosteroids	
CheckMate 9LA [81]	Platinum doublet (2 cycles) +. nivolumab plus ipilimumab vs platinum doublet (4 cycles)	NSCLC	All comers	Yes	122 (17.0%)	Pretreated	NR vs. 7.9 m HR 0.38
IMpower 150 [22]	Carboplatin-paclitaxel + bevacizumab + atezolizumab vs. Carboplatin-paclitaxel + bevacizumab	Non-squamous carcinoma	All comers	Yes	NA	Pretreated, off corticosteroids, stable, supratentorial or cerebellar	NA
IMpower 130 [82]	Carboplatin + nab-paclitaxel + atezolizumab vs. carboplatin + nab-paclitaxel	Non-squamous carcinoma	All comers	Yes	NA	Pretreated, off corticosteroids, stable, supratentorial or cerebellar	NA
IMpower 131 [83]	Atezolizumab + carboplatin-(nab)paclitaxel vs. carboplatin-(nab)paclitaxel	Squamous carcinoma	All comers	Yes	NA	Pretreated, off corticosteroids, stable, supratentorial or cerebellar	NA
IMpower 132 [84]	Platinum-pemetrexed + atezolizumab vs. platinum-pemetrexed	Non-squamous carcinoma	All comers	Yes	NA	Pretreated, off corticosteroids, stable, supratentorial or cerebellar	NA
Immunotherapy combinations							
CheckMate 227 [85]	Platinum doublet vs. nivolumab plus ipilimumab	NSCLC	All comers	Yes	115 (9.8%)	Pretreated, off corticosteroids or on a stable or decreasing dose of ≤10 mg daily prednisone and stable	16.8 m vs. 13.4 m HR 0.64 (0.42-0.97)

Abbreviations: BM: brain metastases; HR: hazard ratio; ICI: immune checkpoint inhibitors; MRI: magnetic resonance imaging; m: months; NA: not available; NSCLC: non-small cell lung cancer; NR: not reached; OS: overall survival.

Table 2. Summary of immune-phenotype studies containing cohorts of brain metastases from NSCLC.

Ref.	Whole cohort size (NSCLC cohort)	Inflammatory biomarkers analyzed (positivity cut-off &clones)	Locations assessed in the brain sample	Inflammatory biomarkers with a prognostic value
[50]	Mixed cohort, N = 17 (N = 5)	Astrocytes: GFAP Microglia/Macrophages: HLA ABC/MHC-I, HLA DR/MHC-II, CD68, CD163, IBA-1, AIF-1, SIGLEC-11, HMGB1, GLUT-5, iNOS, p22phox, NCF-1, NOX-1, NOXO TILs: CD3, CD4, CD8, Granzyme B, CD20, CD79A	Intratumoral Peritumoral Control tissue	No
[52]	Mixed Cohort, N = 252 (N = 62)	TILs: CD3 (A0452), CD8 (C8/144B), FOXP3 (236A/E7) TC PD-1 (≥1%, NAT105) TC PD-L1 (≥1%, E1L3N)	Intratumoral Tumor stroma Peritumoral	No
[49]	Mixed cohort, N = 116 (N = 61)	TILs: CD3, CD8, CD45RO, FOXP3 (Ventana) Immune-score* (Ventana) TC PD-1 (≥5%, Ventana) TC PD-L1 (≥5%, 5H1)	Intratumoral Tumor stroma Peritumoral	A high density of CD3+ (p=0.015), CD8+ (p=0.030) and CD45RO+ (p=0.006) cells and a high immune-score (p<0.001) was positively correlated with OS.
[53]	Lung adenocarcinoma, N = 208	Mononuclear ring (peritumoral mononuclear cells evaluated by H&E) Intratumoural stromal immune cells (<20% vs. ≥20% evaluated by H&E) IC PD-1(≥1%, ab52587) TC and IC PD-L1 (≥ 1%, SP142)	Intratumoral Peritumoral	The lack of mononuclear ring infiltration showed a borderline tendency toward worse OS (HR 1.73, 95% CI 0.58–2.99; p = 0.05)

Abbreviations: H&E, hematoxylin and eosin; IC: immune cells; NSCLC: non-small cell lung cancer; PD-L1: programmed death ligand-1; PD-1: programmed cell death protein 1; TC: tumor cells; TIL, tumor-infiltrating lymphocytes.

*Immune-score was calculated based on CD3+ and CD8+ TILs density in each region (tumor center and border region were assessed and recorded as dichotomous (high vs. low) variable. Immune-score was considered high when both CD3 and CD8 were high in the center of the metastasis and low otherwise.

Review

Title: Immunotherapy in NSCLC patients with brain metastases. Understanding brain tumor microenvironment and dissecting outcomes from immune checkpoint blockade in the clinic.

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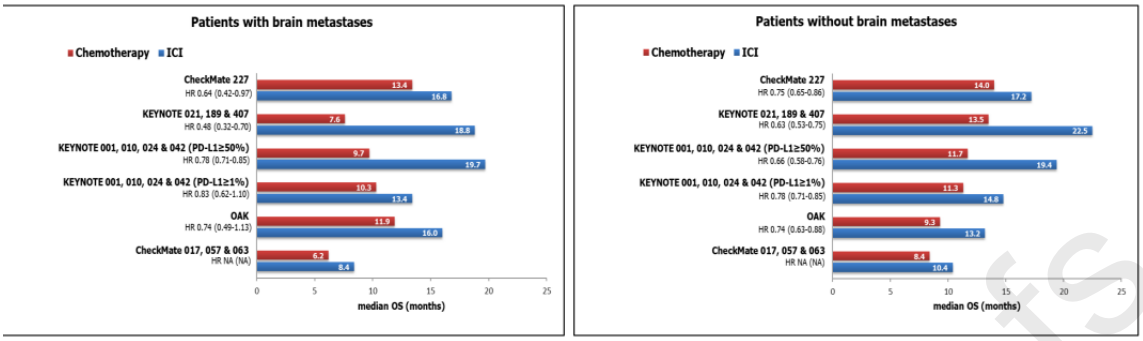

Figure 2