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Abstract: Background: Brain metastases are frequent complications in patients with non-small-cell lung cancer (NSCLC) associated with significant morbidity and poor prognosis. Anecdotal intracranial responses with immunotherapy have been reported in this patient population. Tumor infiltrating lymphocytes may have prognostic value in patients with NSCLC with brain metastases; however, the predictive value of programmed death ligand-1 expression and tumor mutational burden are less known in the context of brain metastases.
Methods: We summarized clinical outcomes of immunotherapy in NSCLC clinical studies in patients with brain metastases. We revised the immune-phenotype characteristics and the comparison of paired human primary tumors and brain metastases in patients with NSCLC as well as the complex interactions of tumor and resident cells in the brain microenvironment.
Results: Limited data are available on intracranial efficacy of immunotherapy in NSCLC and on the modulation of tumor microenvironment exerted by immunotherapy in the brain niche. 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. Glial cells, reactive astrocytes and tumor associated macrophages were detected surrounding and infiltrating 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. Further research on brain microenvironment and cancer cells interactions and their influence on tumor immune responses is needed to design novel and effective immunotherapeutic approaches.

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Cover Letter

Editor *Cancer Treatment Reviews*
Prof. Rolf Stahel
University of Zurich, Zurich, Switzerland

April 10th, 2020

Dear Editor,

We are pleased to submit our manuscript, entitled "Immune phenotype of NSCLC brain metastases in the age of immune checkpoint inhibitors" to *Cancer Treatment Reviews* for your consideration as review article. We reviewed and summarized the evidence for immune checkpoint inhibitors efficacy in patients with advanced non-small cell lung cancer (NSCLC) with brain metastases, and we also compiled the most relevant studies examining the immune phenotype and the complex interplay among the cells that constitute the tumor microenvironment in the brain niche of patients with NSCLC. This review was motivated by our research that focuses on the characterization of the tumor microenvironment in brain metastases of patients with lung cancer. In this sense, we are currently leading a clinical trial exploring the safety and efficacy of PD-L1 blockade in combination with platinum-based chemotherapy in patients with untreated synchronous brain metastases (ATEZO BRAIN study, NCT03526900). We consider that this review will be of interest for the readers of *Cancer Treatment Reviews* as brain metastases are an important complication in patients with NSCLC that urgently requires novel and effective therapeutic approaches.

All the authors contributed to this manuscript and approved the final version submitted. None of the work included in this manuscript has been previously published in a peer-reviewed journal and is not under consideration for publication elsewhere.

Thank you for your consideration of this manuscript.

Sincerely yours,

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Review

Title: The immune phenotype of NSCLC brain metastases in the age of immune checkpoint inhibitors

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The immune phenotype of NSCLC brain metastases in the age of immune checkpoint inhibitors

Abstract

Background: Brain metastases are frequent complications in patients with non-small-cell lung cancer (NSCLC) associated with significant morbidity and poor prognosis. Anecdotal intracranial responses with immunotherapy have been reported in this patient population. Tumor infiltrating lymphocytes may have prognostic value in patients with NSCLC with brain metastases; however, the predictive value of programmed death ligand-1 expression and tumor mutational burden are less known in the context of brain metastases.

Methods: We summarized clinical outcomes of immunotherapy in NSCLC clinical studies in patients with brain metastases. We revised the immune-phenotype characteristics and the comparison of paired human primary tumors and brain metastases in patients with NSCLC as well as the complex interactions of tumor and resident cells in the brain microenvironment.

Results: Limited data are available on intracranial efficacy of immunotherapy in NSCLC and on the modulation of tumor microenvironment exerted by immunotherapy in the brain niche. 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. Glial cells, reactive astrocytes and tumor associated macrophages were detected surrounding and infiltrating 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. Further research on brain microenvironment

and cancer cells interactions and their influence on tumor immune responses is needed to design novel and effective immunotherapeutic approaches.

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 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]. In the QUARTZ study, patients with advanced NSCLC with brain metastases were randomized to best supportive care (BSC) including dexamethasone plus WBRT versus BSC alone. No differences were observed between the two arms in overall survival (OS) (HR 1.06, 95% CI 0.90–1.26), overall quality of life, or dexamethasone use. In patients with multiple synchronous and asymptomatic brain metastases, systemic therapies can be an alternative approach to WBRT. Upfront WBRT versus delayed WBRT with platinum-based chemotherapy in patients with advanced non-oncogene addicted NSCLC and synchronous brain metastases was comparable in terms of intracranial response rate (iCRR) and OS, supporting the use of chemotherapy and suggesting that delaying WBRT was not deleterious in terms of OS [5]. In a separate study, cisplatin plus pemetrexed chemotherapy in the setting of multiple asymptomatic and untreated

NSCLC brain metastases was well tolerated and achieved an encouraging iCRR of 42% and an overall response rate (ORR) of 35% [6].

In patients with oncogene-addicted NSCLC brain metastases (20%) [7], 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) [8, 9] and have a higher iCRR (66%–78% vs. 29%–43%) [10-12]. 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 [11-13]. 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 [11].

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 [14-19]; while frontline pembrolizumab is approved as monotherapy in advanced NSCLC patients with PD-L1 expression of 1% or greater [19, 20]. In addition, immunotherapy in combination with conventional chemotherapy is approved as first-line treatment of patients with NSCLC regardless of PD-L1 expression [18, 19, 21-23]. 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 OS compared with placebo and is also an approved treatment in this setting [24-26].

However, among patients with non–oncogene addicted NSCLC with brain metastases, limited data on intracranial efficacy of immunotherapy are available because those patients have generally been excluded from clinical trials [27]. The presence of 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 [28]. 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 [29].

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 [27] (**Table 1**). Available data of immunotherapy in patients with NSCLC and brain

71 metastases come from one single-arm phase I/II trials, expanded access programs
72 (EAP), pre-planned analyses of phase III clinical trials and retrospective series.

73 The proof of concept of intracranial activity of immunotherapy in patients with NSCLC
74 came from a non-randomized, open-label, phase II trial evaluating the efficacy of
75 pembrolizumab in patients with NSCLC with brain metastases. Eligibility criteria
76 required at least one or more untreated or progressive brain metastases between 5 and
77 20 mm in patients without associated neurological symptoms or requiring
78 corticosteroids. Cohort 1 enrolled patients with PD-L1 expression ≥1% and cohort 2
79 enrolled patients with previously treated NSCLC without PD-L1 expression or without
80 evaluable tissue. In the NSCLC cohort (n = 34), 87% of patients were PD-L1 positive
81 and 51% had received prior radiotherapy. Pembrolizumab showed an iCRR of 29.4%
82 (95% CI 15.1–47.5) with 7 patients showing discordances between CNS and systemic
83 responses. Durable intracranial responses were observed (median 7.5 months, 95% CI
84 1.3–23.3) and 31% of the patients were alive at two years (95% CI 19–54%) [30, 31].

85 CheckMate 012, a phase I multicohort study assessing the safety and tolerability of
86 nivolumab alone or combined with other therapies in patients with advanced NSCLC,
87 included 12 patients with at least 1 asymptomatic and untreated brain metastasis in the
88 M cohort [33]. Two intracranial responses were observed (iCRR 16.7%; 95% CI 2.1–
89 48.4) and the median PFS was 1.6 months (95% CI 0.92–2.50) and median OS was 8.0
90 months (95% CI 1.38–15.50). No treatment-related nervous system adverse events
91 were reported. The Italian and French nivolumab EAPs included 409 and 130 patients
92 with NSCLC, respectively, who had brain metastases that were asymptomatic, stable
93 and did not require corticosteroids. The overall ORR was 17% and 12% and the disease
94 control rate (DCR) was 40% and 37% in the Italian and French EAPs, respectively. In

the Italian EAP, 118 patients received corticosteroids at baseline and 74 received concomitant brain radiotherapy. Median OS was 8.1 months in the Italian EAP and 6.6 months in the French EAP [34, 35].

An analysis was conducted of patients with NSCLC and pretreated stable brain metastases enrolled in three clinical trials with nivolumab (CheckMate 063, 017 and 057). In the pooled analyses there were 46 patients with brain metastases who received nivolumab and 42 who received docetaxel as second-line treatment. Most patients were previously treated with brain radiotherapy (74% of patients receiving nivolumab and 83% receiving docetaxel). Intracranial disease control rate (iDCR) was reported in 33% of patients receiving nivolumab. The median OS for nivolumab vs. docetaxel in patients with brain metastases at baseline from CheckMate 017 was 4.99 vs. 3.86 months (HR not determined) and in CheckMate 057 it was 7.61 vs. 7.33 months (HR 1.04, 95% CI 0.62–1.76). In the pooled analysis, patients with pretreated brain metastases achieved longer OS (8.4 months; 95% CI 4.99–11.6) with nivolumab than with docetaxel (6.2 months; 95% CI 4.4–9.23). Among patients without documented brain metastases, median OS was 10.4 months (95% CI 9.1–12.9) vs 8.4 months (95% CI 7.33–9.33) with nivolumab vs docetaxel, respectively. Nivolumab was generally well tolerated and treatment-related neurological adverse events occurred only in 5 of 46 (11%) patients and were all grade 1 or 2 [33].

A pooled analysis of pembrolizumab monotherapy clinical trials (KEYNOTE 001, 010, 024 and 042) included 293 patients with PD-L1 positive NSCLC with pretreated stable brain metastases. Pembrolizumab improved outcomes compared with chemotherapy irrespective of the presence of brain metastases at baseline. The median OS in patients with brain metastases was 13.4 months with pembrolizumab vs. 10.3 months with

chemotherapy (HR 0.83, 95% CI 0.62–1.10) for patients with PD-L1 ≥1% and 19.7 months with pembrolizumab vs. 9.7 months with chemotherapy (HR 0.78, 95% CI 0.71–0.85) for patients with PD-L1 ≥50%. Pembrolizumab showed a manageable safety profile in both subgroups of patients [36].

In a pooled analysis of clinical trials assessing pembrolizumab plus platinum-based chemotherapy (KEYNOTE 021, 189 and 407), the combination arm was superior to chemotherapy alone in terms of ORR, PFS and OS. Patients with asymptomatic and untreated or pretreated brain metastases were enrolled if they were clinically stable and did not require steroids. Overall, 171 patients with brain metastases were included in the pooled analysis of the 3 studies. Treatment with pembrolizumab plus chemotherapy improved survival compared with chemotherapy alone, both in patients with brain metastases (median OS 18.8 vs. 7.6 months; HR 0.48, 95% CI 0.32–0.70) as in patients without brain metastases (median OS 22.5 vs. 13.5 months; HR 0.63, 95% CI 0.53–0.75) [37].

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 [21, 38].

These studies showed promising efficacy of immunotherapy in patients with 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 with stable, previously treated, or asymptomatic brain metastases. Furthermore, the available data have many limitations because it 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 [39] 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 NSCLC brain metastases.

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 [40, 41]. 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 [40].

Brain metastases samples have genomic alterations in molecular pathways that play a critical role in cancer progression, such as *PI3K*, *EGFR*, and cell cycle, *CDK* [40]. Recently, *MYC*, *YAP1* and *MMP13* gene amplifications and *CDKN2A*/*B* gene deletions were also identified as metastatic drivers in lung adenocarcinoma brain metastases [42]. 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 [41, 43]. In patients harboring *PI3K* aberrations, all brain metastases samples showed a pattern of gene expression consistent with *PTEN* loss [41]. Similarly, loss of *PTEN* nuclear expression by immunohistochemistry (IHC) was more common in brain metastases samples than in matched primary lung tumors [44]. 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

188 [45]. The divergent evolution of metastatic cells that have settled in the brain can likely
189 be explained by the significant pressure generated by the brain microenvironment [46].

190 The brain microenvironment is a unique niche that does not share many similarities
191 with other organs and previous reviews have already excellently described the
192 properties that set it apart [46]. Features that are unique to the brain include the
193 presence of the blood brain barrier, exclusive environmental cells (including microglia,
194 astrocytes, oligodendrocytes and neurons), a lymphatic system that drains to local
195 cervical lymph nodes, and the composition of the extracellular matrix [47, 48]. In
196 addition, the CNS has a specialized immunological microenvironment, and was
197 classically considered an immune-privileged niche [49]. The healthy brain contains
198 almost no lymphocytes; although there is evidence for immune surveillance of the
199 normal human CNS by $CD3^+/CD8^+$ lymphocytes [50] and TILs have been found
200 surrounding NSCLC brain metastases samples [51]. Moreover, two major glial cells,
201 astrocytes and microglia, have been detected surrounding and, in the case of the
202 resident macrophages, also infiltrating NSCLC brain metastases [52]. These stromal-
203 host cells interactions modulate tumor progression and tumor immune-evasion [53].

204

205 **Characterization of immune phenotype in brain metastases**

206 **Cohorts with brain metastases samples from different solid tumors**

207 The presence of tumor immune cells infiltration has been assessed in brain metastases
208 lesions from tumors of different origin, including cohorts of lung cancer (**Table 2**).

209 The inflammatory infiltrate inside and surrounding brain metastases was characterized
210 using IHC to assess a panel of 21 inflammatory markers in 17 human autopsy tissue

211 samples, including 5 from patients with NSCLC. The inflammatory infiltrates
212 comprised mainly activation of CD163+ and CD68+ macrophages and glial fibrillary
213 acidic protein (GFAP)-positive astrocytes in the peritumoral region. In terms of TILs, a
214 low burden of CD20+ and CD79+ B lymphocytes was observed while CD3+, CD8+
215 and CD4+ T lymphocytes were detected infiltrating and surrounding the brain
216 metastases. A small fraction of CD8+ T lymphocytes had granzyme B expression,
217 suggesting low cytotoxic activation. The authors concluded that the inflammatory
218 reaction to brain metastases was mainly driven by activation of innate immunity,
219 basically macrophages in the peritumoral area, but did not appear to be sufficient to
220 activate adaptive immunity [52].

221 In a cohort of 252 samples from brain metastases of different primary tumors including
222 62 NSCLC specimens, the distribution of immune infiltrates and PD-L1 expression was
223 evaluated by IHC on tissue microarrays. Immune infiltrates were highly variable across
224 brain metastases with three major patterns described: perivascular stromal infiltration
225 (typically observed in NSCLC and other carcinomas with prominent fibrovascular
226 stroma), peritumoral infiltration (lymphocytes surrounding brain metastases like an
227 inflammatory wall), and homogenously diffuse infiltration (often seen in melanoma).
228 Neither in the whole cohort nor in the subgroup of NSCLC brain metastases, was there
229 an association of TIL and PD-L1 expression with OS [54].

230 In a mixed cohort of 116 brain metastases samples, including 61 from lung tumors, TIL
231 distribution and their prognostic role was evaluated by IHC using CD3, CD8,
232 CD45RO, FOXP3, PD-1 and PD-L1 markers. Overall TIL infiltration was high, with
233 TILs found in nearly all samples (115/116). CD3+ cells distribution in the stroma and
234 peritumoral parenchyma was more prominent than within the metastatic lesion where

only sparse infiltration was observed. A high density of CD3+ and CD8+ and CD45RO+ cells and a high immune score (40.2% of the samples, based on automated calculation of the CD3+/CD8+ ratio) was positively correlated with OS [51].

The prognostic role of immune cells in NSCLC brain metastases was further confirmed in a homogeneous cohort of 208 samples of brain metastases from lung adenocarcinoma. Mononuclear cells were evaluated by hematoxylin-eosin staining in the peritumoral area (called mononuclear ring) and the intratumoral stroma. More than half (55.7%) showed peritumoral mononuclear ring presence. The lack of mononuclear ring infiltration showed a borderline tendency toward worse survival (HR 1.73, 95% CI 0.58–2.99; p = 0.05). No correlation was observed between PD-1 or PD-L1 expression in tumor cells and PD-1 expression in immune cells and OS [55].

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 [28].

In a cohort of 20 paired NSCLC primary tumor and brain metastases lesions, T-cell receptor sequencing showed a significant reduction of T-cell clones in brain metastases compared with paired primary tumors (median 1540 vs. 4551; $p = 0.0005$) [56]. 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 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 [56].

A recent study reported lower T-cell richness and T-cell densities 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 [57]. However, in contrast with the previously mentioned 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;

283 therefore, the predictive value of these markers cannot be assessed. In addition, these
284 studies were highly heterogenous in terms of the biomarkers evaluated, antibodies
285 clones employed, cut-offs used for PD-1/PD-L1 positive definition, and methods used
286 for their quantification may limit the conclusions obtained. The discrepancies about the
287 prognostic role of TILs in brain metastases may be explained by differences in the
288 study population and prior systemic and local treatments received.

289

290 **Interactions between tumor microenvironment and tumor cells in brain**
291 **metastases**

292 Many studies have shown that brain metastases specimens from patients with NSCLC
293 are surrounded and infiltrated by activated astrocytes and microglia [52, 58]. In the next
294 section and in **Figure 1** we aim to summarize the molecular mechanisms employed by
295 glial cells to modulate tumor progression and tumor immune response [53].

296 **Astrocytes**

297 Astrocytes are the most abundant glial cells in the brain metastases microenvironment.
298 After injury, astrocytes change their phenotype upregulating the levels of GFAP and
299 inducing a transcriptional program known as reactive astrogliosis [59]. Reactive
300 astrocytes (RA) play a dual role. At initial steps of brain metastases, RA produce
301 deleterious signals that compromise the viability of metastases-initiating cells [60, 61].
302 However, once metastatic cells are established, RA facilitate tumor progression [62]. In
303 the brain microenvironment, RA area major source of plasminogen activator (PA)
304 converting plasminogen into plasmin endopeptidase. In response to brain injury,
305 astrocytes also express high levels of proapoptotic cytokine FasL in their membrane.

306 Plasmin suppresses brain metastases by transforming membrane-bound astrocytic FasL
307 into a paracrine death signal targeting cancer cells and by inactivating the adhesion
308 molecule L1CAM expressed by tumor cells and used for spreading along brain
309 capillaries. Brain metastases from lung and breast cancers have been shown to prevent
310 plasmin activation by producing high levels of anti-PA serpins, mainly neuroserpin and
311 serpin B2. These anti-PA serpins block RA-mediated plasmin activation and reverse
312 the metastasis-suppressive effects of plasmin [29].

313 Gap junctions are also involved in the communication between astrocytes and tumor
314 cells. Protocadherin-7 expression in tumor cells promotes conexin43-dependent gap
315 junction formation in lung and breast adenocarcinoma models. Once these junctions are
316 formed, cancer cells from brain metastases transfer cyclic guanosine monophosphate-
317 adenosine monophosphate (cGAMP) to astrocytes activating the STING pathway, an
318 innate immune response pathway able to sense cytosolic double-stranded DNA;
319 thereby producing inflammatory cytokines such as TNF-α and IFN-α. These paracrine
320 signals activate STAT1 and NF-κB pathways in cancer cells promoting tumor growth
321 and increased chemoresistance [63]. Gap junctions are also involved with transferring
322 small non-coding RNAs (ncRNAs) from astrocytes to lung cancer cells. These ncRNAs
323 promote resistance to chemotherapy and are overexpressed in human lung tumor cells
324 co-cultured with astrocytes compared with lung cancer cells cultured without them
325 [64].

326 Activation of the endothelin-axis orchestrates the pro-survival transcriptional program
327 in lung cancer cells through gap junctions. Specifically, heterotypic gap junctions
328 between cancer cells and astrocytes stimulate upregulation of IL-6 and IL-8, which
329 increase endothelin-1 (ET-1) production from astrocytes and ET receptor expression

330 (ET_AR and ET_BR) on cancer cells. This results in marked activation of the
331 phosphorylated kinases AKT and MAPK and induction of antiapoptotic genes, such as
332 *BCL2L1*, *GSTA5* and *TWIST1* in cancer cells [65]. As expected, a dual antagonist of
333 ET_AR and ET_BR signaling in combination with paclitaxel prevented astrocyte-mediated
334 protection of cancer cells leading to a significant reduction in cell division with
335 increased apoptosis, and to increased survival in mice harboring brain metastases [66].

336 STAT3 activation (Tyr705 phosphorylation, pSTAT3) in a subpopulation of RA
337 associated with lung-derived brain metastases cells induces a pro-metastatic phenotype.
338 Increased STAT3 signaling in RA promotes metastasis viability and modulates innate
339 and acquired immune responses. In this sense, pSTAT3+ RA negatively influence
340 CD8+ T-cell activation presumably through PD-L1 expression and through secretion of
341 immunosuppressive molecules such as vascular endothelial growth factor-A (VEGF-
342 A), lipocalin-2, tissue inhibitor of metalloproteinases-1 (TIMP-1), and proteins of the
343 extracellular matrix (ECM) that could act as a physical barrier limiting the access of
344 CD8+ T-cells. Additionally, pSTAT3+ RA also promote expansion of CD74+
345 microglia and macrophages by increasing the levels of CD74 and macrophage
346 migration inhibitory factor (MIF). Patients with higher levels of pSTAT3+ RA in brain
347 metastases had shorter survival. In a cohort of 18 patients with NSCLC with previously
348 treated brain metastases, treatment with an oral STAT3 inhibitor yielded intracranial
349 responses [62].

350 Moreover, astrocytes are well-known secretory cells with the ability to release
351 extracellular vesicles into the brain microenvironment [45]. Models of brain injury
352 showed that extracellular vesicles released from RA were able to attract peripheral
353 leukocyte cells to the brain through regulation of acute cytokine production in the liver

[67]. 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 [68]. 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 [69]. 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 [70]. 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) [71]. CD49D and TMEM 119 have been identified as differential markers between BMDMs and microglia [71, 72]. These studies also suggested that most tumor associated macrophages (TAM) in the brain metastases were derived from peripheral monocytes and not from resident microglia [71, 73].

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 [52, 74]. 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 [75]. Similarly, this molecular pathway, through *LEF1* and *HOXB9* target genes, has been identified as major determinant of lung adenocarcinoma dissemination to the brain [76].

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 [77].

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, 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. Despite these limited data 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 revealed that brain metastases are more likely to be immunologically “cold", showing lower PD-L1 expression and infiltration by lymphocytes. Organ-specific peculiarities 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 data available, 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 NSCLC patients 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

TMA: 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.

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

Abbreviations: BCL2L1: BCL-2 like 1; cGAMP: 2′3′-cyclic GMP-AMP; Cx43: conexin43; dsRNA: 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; MCP-1: monocyte chemoattractant 1; MIF: macrophage migration inhibitory factor; miRNA: microRNA; MMP-2: metalloprotease-2; MMP-9: metalloprotease-9; ncRNAs: non-coding RNAs; NS: neuroserpin; PA: plasminogen activator; PAI-1: plasminogen-activator inhibitor -1; PCDH7: protocadherine-7; PGE-2: prostaglandin E2; SB2: serpin B2; sFasL: soluble FAS ligand; TGF-β: transforming growth factor beta; TIMP-1: tissue inhibitor of metalloproteinases-1; TNF-α: tumor necrosis factor-α; TWIST1: TWIST related protein 1.

Table Legends

Table 1. Efficacy results from pivotal phase III clinical trials with immune checkpoint inhibitors in patients with NSCLC and brain metastases.

Abbreviations: HR: hazard ratio; iCRR: intracranial response rate; iCDR: intracranial disease control rate; 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 in brain metastases derived from lung tumors.

Abbreviations: H&E, hematoxylin and eosin; IC: immune cells; NR: Not reported; 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. Efficacy results from pivotal phase III clinical trials with immune checkpoint inhibitors in NSCLC patients with brain metastases.

Study	Experimental arm vs. control arm	Histology	PD-L1 expression	Mandatory brain MRI at screening	Number of patients with brain metastases included	Brain metastasis inclusion criteria	Median OS HR (95% CI)
Immunotherapy monotherapy							
First-line phase III trials							
CheckMate 026[78]	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[20, 79]	Pembrolizumab vs. platinum doublet	NSCLC	≥50%	Yes	18 (9.1%)	Pretreated, off corticosteroids and stable	HR 0.55 (0.20–1.56)
KEYNOTE 042[80]	Pembrolizumab vs. platinum doublet	NSCLC	≥1%		70 (5.5%)	Pretreated, off corticosteroids and stable	NA
Second-line phase III trials							
Checkmate017[15, 33]	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. 3.9 m HR NR*
Checkmate 057 [14, 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 mvs.7.3 m* HR 1.04 (0.62–1.76)
KEYNOTE 010[81]	Pembrolizumab vs. docetaxel	NSCLC	≥1%	No	152 (14.7%)	Pretreated, off corticosteroids and stable	NA
OAK[16, 82]	Atezolizumab vs. docetaxel	NSCLC	All comers	Yes	123 (10%)	Pretreated, off corticosteroids, stable and supratentorial	16 m vs. 11.9 m HR 0.74 (0.49–1.13)
Combination of immunotherapy with chemotherapy							
First line phase III trials							
KEYNOTE 189[21]	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	HR 0.36 (0.20–0.62)

Study	Experimental arm vs. control arm	Histology	PD-L1 expression	Mandatory brain MRI at screening	Number of patients with brain metastases included	Brain metastasis inclusion criteria	Median OS HR (95% CI)
						off corticosteroids	
KEYNOTE 407[22]	Carboplatin-(nab)paclitaxel + pembrolizumab vs. Carboplatin-(nab)paclitaxel + placebo	Squamous carcinoma	All comers	No	44 (7.8%)	Previously treated, stable and off corticosteroids or untreated, asymptomatic and off corticosteroids	NA
IMpower 150[23]	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[83]	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[84]	Atezolizumab + carboplatin-(nab)paclitaxel vs. carboplatin-(nab)paclitaxel	Squamous carcinoma	All comers	Yes	NA	Pretreated, off corticosteroids, stable, supratentorial or cerebellar	NA
IMpower 132[85]	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[86]	Platinum doublet vs. nivolumab plus ipilimumab	NSCLC	All comers	Yes	81	Pretreated	16.8 m vs. 13.4 m HR 0.68 (0.41-1.11)

Abbreviations: HR: hazard ratio; iCRR: intracranial response rate; iCDR: intracranial disease control rate; MRI: magnetic resonance imaging; m: months; NA: not available; NSCLC: non-small cell lung cancer; NR: not reached; OS: overall survival.

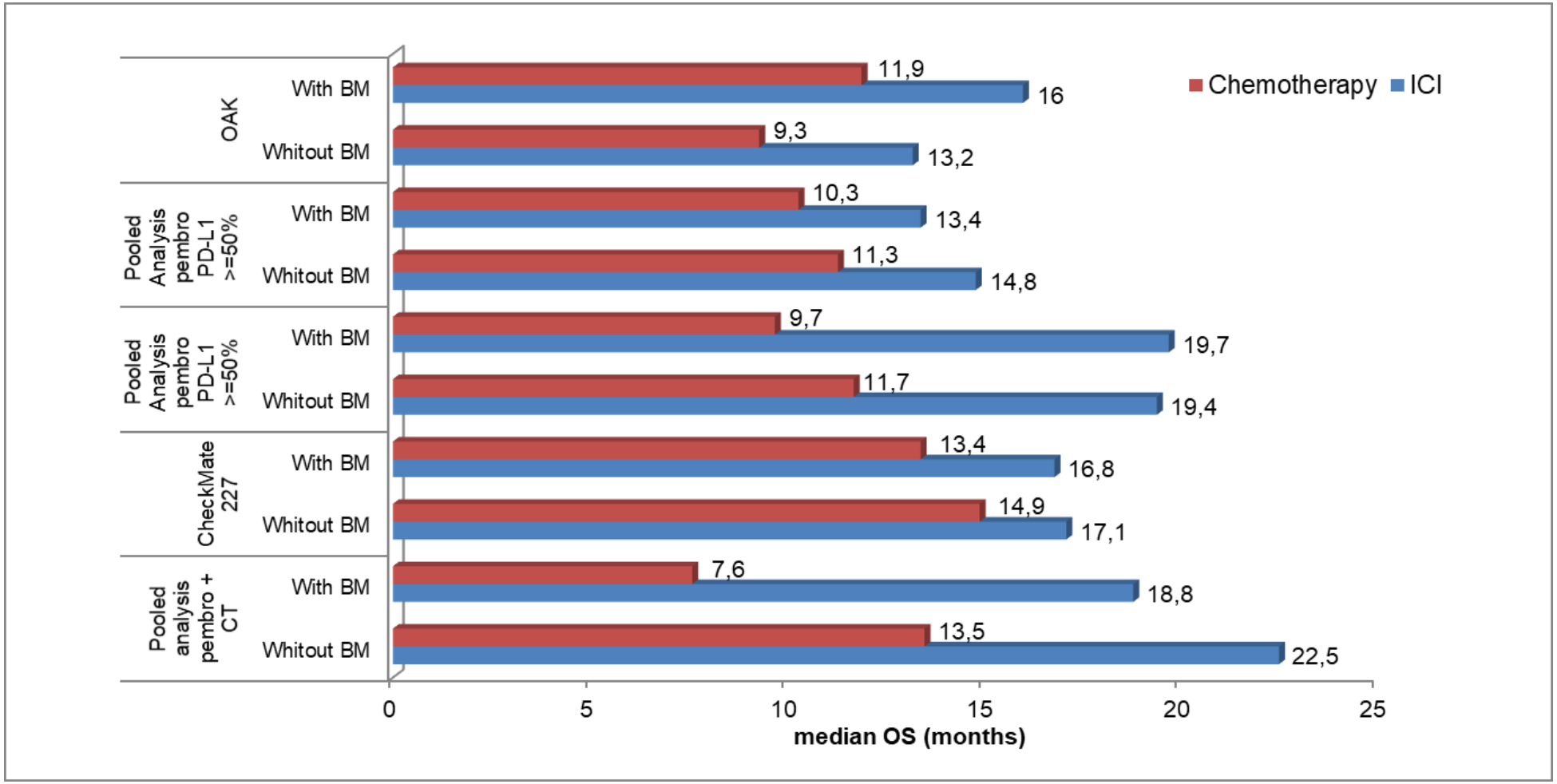
Table 2. Summary of immune-phenotype studies in brain metastases derived from lung tumors.

Ref.	Whole cohort size (NSCLC cohort)	Inflammatory markers analyzed (positivity cut-off &clones)	Locations assessed in the brain lesion	Prognostic value
[52]	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
[54]	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
[51]	Mixed cohort, N = 116 (n = 61)	TILs: CD3, CD8, CD45RO, FOXP3 (Ventana) Immunescore* (Ventana) TC PD-1 (≥5%, Ventana) TC PD-L1 (≥5%, 5H1)	Intratumoral Tumor stroma Peritumoral	High density of CD3, CD8 and CD45RO TILs High immunescore
[55]	Lung adenocarcinoma, N = 208	Mononuclear ring (peritumoral mononuclear cells by H&E) Intratumoural stromal immune cells (<20% vs. ≥20% by H&E) IC PD-1(≥1%, ab52587) TC and IC PD-L1 (≥ 1%, SP142)	Intratumoral Peritumoral	Mononuclear ring

Abbreviations: H&E, hematoxylin and eosin; IC: immune cells; NR: Not reported; 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.

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

Figure 1


Chemotherapy
ICI
OAK
With BM
Whitout BM
11,9
16
9,3
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Pooled Analysis pembro PD-L1 >=50%
With BM
Whitout BM
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11,3
14,8
Pooled Analysis pembro PD-L1 >=50%
With BM
Whitout BM
9,7
19,7
11,7
19,4
CheckMate 227
With BM
Whitout BM
13,4
16,8
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17,1
Pooled analysis pembro + CT
With BM
Whitout BM
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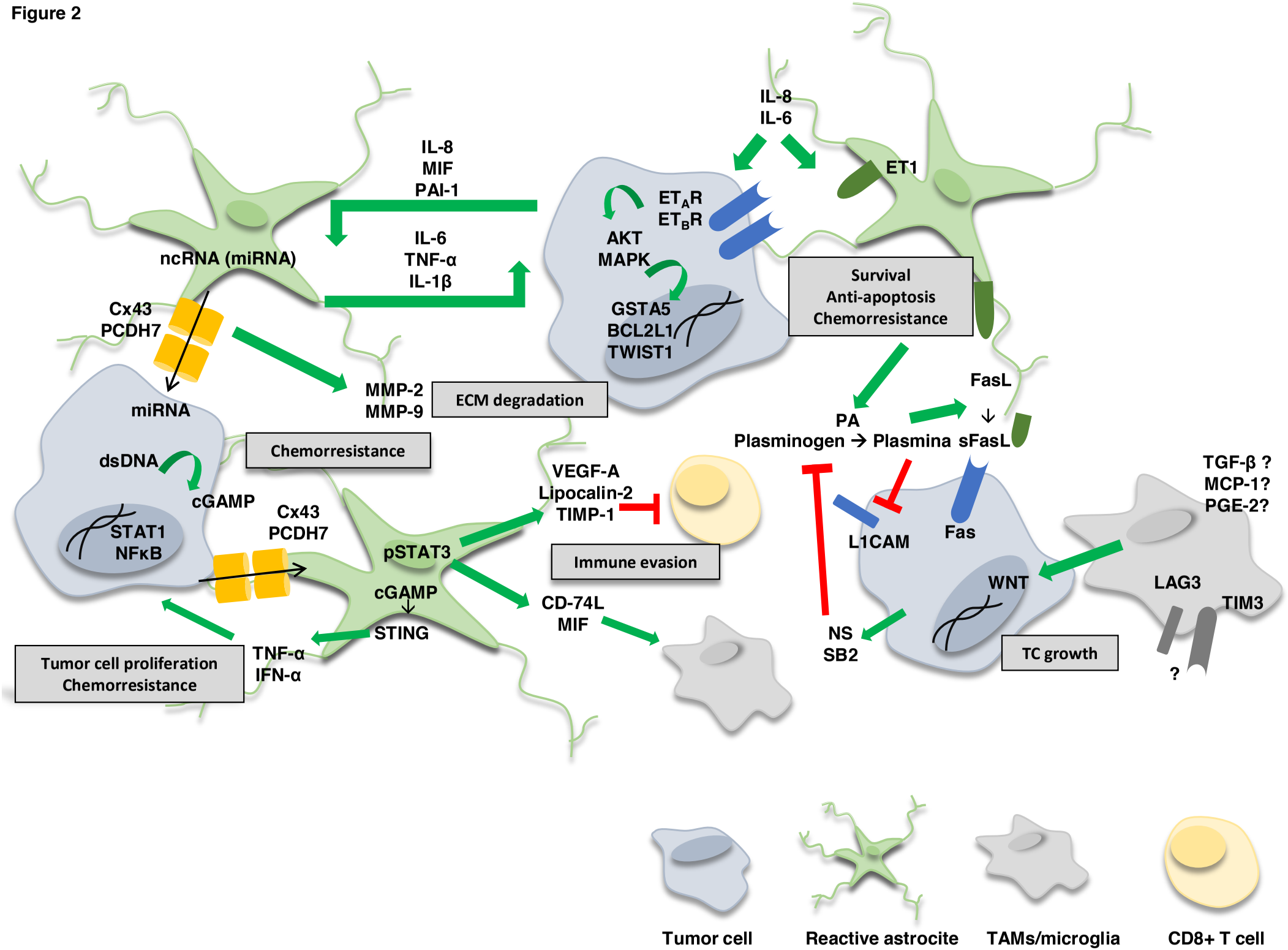
18,8
13,5
22,5
0
5
10
15
20
25
median OS (months)

Figure 2


IL-8
IL-6
ET1
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IL-6
TNF-α
IL-1β
ncRNA (miRNA)
ET$_A$R
ET$_B$R
AKT
MAPK
GSTA5
BCL2L1
TWIST1
Survival
Anti-apoptosis
Chemorresistance
Cx43
PCDH7
MMP-2
MMP-9
ECM degradation
miRNA
FasL
PA
Plasminogen → Plasmina
sFasL
Chemorresistance
dsDNA
cGAMP
STAT1
NFκB
Cx43
PCDH7
VEGF-A
Lipocalin-2
TIMP-1
TGF-β ?
MCP-1?
PGE-2?
L1CAM
Fas
pSTAT3
Immune evasion
cGAMP
STING
CD-74L
MIF
WNT
LAG3
TIM3
NS
SB2
TC growth
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Tumor cell proliferation
Chemorresistance
TNF-α
IFN-α
Tumor cell
Reactive astrocite
TAMs/microglia
CD8+ T cell

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