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Melanoma brain metastasis globally reconfigures chemokine and cytokine profiles in patient cerebrospinal fluid

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The aggressiveness of melanoma is believed to be correlated with tumor–stroma-associated immune cells. Cytokines and chemokines act to recruit and then modulate the activities of these cells, ultimately affecting disease progression. Because melanoma frequently metastasizes to the brain, we asked whether global differences in immunokine profiles could be detected in the cerebrospinal fluid (CSF) of melanoma patients and reveal aspects of tumor biology that correlate with patient outcomes. We therefore measured the levels of 12 cytokines and 12 chemokines in melanoma patient CSF and the resulting data were analyzed to develop unsupervised hierarchical clustergrams and heat maps. Unexpectedly, the overall profiles of immunokines found in these samples showed a generalized reconfiguration of their expression in melanoma patient CSF, resulting in the segregation of individuals with melanoma brain metastasis from nondisease controls. Chemokine CCL22 and cytokines IL1α, IL4, and IL5 were reduced in most samples, whereas a subset including CXCL10, CCL4, CCL17, and IL8 showed increased expression. Further, analysis of clusters identified within the melanoma patient set comparing patient outcome suggests that suppression of IL1α, IL4, IL5, and CCL22, with concomitant elevation of CXCL10, CCL4, and CCL17, may correlate with more aggressive development of brain metastasis. These results suggest that global immunokine suppression in the host, together with a selective increase in specific chemokines, constitute a predominant immunomodulatory feature of melanoma brain metastasis. These alterations likely drive the course of this disease in the brain and variations in the immune profiles of individual patients may predict outcomes. *Melanoma Res* 24:120–130 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The ability of melanoma to successfully metastasize to the brain, like malignant tumors elsewhere, depends on the complex tumor microenvironment they establish to facilitate their survival and progression [1,2]. Genetic abnormalities within the melanoma cells themselves, such as *AKT* overexpression and *BRAF*, *p53*, *CDKN2A*, and *PTEN* mutations, as well as epigenetic silencing [3,4], are considered to promote cellular transformation. However, melanoma metastases require the additional interplay between the cancer cells and stromal cells, together with the presence of various elements within the tissue environment that they invade, for successful tumor translocation, extravasation, survival, and proliferation [5,6]. A key requirement for the subversion of normal cells and tissue within the metastatic site involves the recruitment of myofibroblasts, innate, and adaptive immune cells [7]. Many of these cell types are of bone marrow origin and may be recruited to tumors in response to cytokine and chemokine (collectively referred to here as immunokines) production by the melanoma, stromal cells, or both. In turn, these newly recruited cells also secrete immunokines that attract additional cells and/or modulate the activity of cells within the tumor [8,9]. In particular, melanoma cells have been shown to secrete immunokines that modulate the activity of regulatory T cells (Tregs) that exert immune suppressive functions and tumor-associated macrophages that facilitate neovascularization, invasion, and metastasis [10–12]. Furthermore, the administration of immunomodulatory cytokines, such as recombinant IL2, and immune-checkpoint inhibitors, such as ipilimumab, nivolumab, and lambrolizumab, can eradicate melanomas, and immunotherapy is a major and rapidly evolving modality of treatment for metastatic melanomas [13–16]. These treatment successes therefore support the importance of immune participation in tumor maintenance, survival, and growth. Thus, the specific activities of immunokines likely have a significant influence on the pathophysiology of the tumor and determination of their relative levels of expression may yield important clues to its behavior.

Traditionally, the cerebrospinal fluid (CSF) has been considered to merely maintain the homeostatic environment required for normal functions of the brain.

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However, recent data have emerged to challenge this view. In addition to providing a homeostatic environment, the CSF also provides a conduit for transmitting signals during neurodevelopment and progression of primary and metastatic brain tumors [17–19]. Therefore, given the potential importance of immune signaling in modulating the function of metastatic melanoma of the brain, we postulated that immunokines could be detected in the CSF and may shed light on the biology of melanoma brain metastasis. By analyzing immunokines that are known to be associated with inflammation [9,10], we found that the presence of melanoma markedly altered the background levels of most assayed. Importantly, all melanoma patients in our set showed a generalized suppression of multiple immunokines while often showing an elevation of a subset including CXCL10, CCL4, CCL17, and IL8. Unsupervised clustering of patients on the basis of their immunokine profiles showed clusters that appear to correlate with patient outcome. Therefore, our data suggest that the relationship between the immune system and melanoma is an important factor in determining patient outcome and that CSF immunokine profiles may serve as potential diagnostic biomarkers for the detection of melanoma brain metastasis.

Materials and methods

Patients and cerebrospinal fluid

Individual CSF samples from 22 patients with melanoma brain metastases and five nondisease controls were collected at the time of neurological evaluation when there was an indication for lumbar puncture or sampling from a ventricular reservoir. Informed consent was obtained from patients for CSF storage and analysis under institutional review board-approved protocols at the Beth Israel Deaconess Medical Center. CSF samples were stored at -80°C until analysis.

Individual and multiplexed enzyme-linked immunosorbent assay

Twelve-Plex Cytokine and Chemokine Infrared Searchlight enzyme-linked immunosorbent assay (ELISA) kits (Aushon Biosystems, Billerica, Massachusetts, USA) were used to quantify the levels of CSF immunokines, most of which were known to interact with melanoma [9,10]. Cytokines analyzed included interleukins such as IL1α, IL1β, IL2, IL4, IL5, IL6, IL8, IL10, IL12, and IL13, as well as interferon-γ (IFN-γ), and tumor necrosis factor-α, which are common mediators of inflammation. The frequent inflammatory chemokines analyzed included CCL2 (monocyte chemotactic protein 1, MCP1), CCL3 (macrophage inflammatory protein 1α, MIP1α), CCL4 (macrophage inflammatory protein 1β, MIP1β), CCL5 (regulated upon activation, normal T cell expressed and secreted, RANTES), CCL11 (eotaxin), CCL17 (thymus and activation-regulated chemokine, TARC), CCL22 (macrophage-derived chemokine, MDC), CCL23 (myeloid progenitor inhibitory factor 1, MPIF1), CXCL1 (growth-regulated oncogene α, GROα), CXCL5 (epithelial neutrophil-activating peptide 78), CXCL9 (monokine induced by IFN-γ, MIG1), and CXCL10 (induced protein 10, IP-10).

Statistical analysis

The prognostic factors of our cohort, such as age <60 and ≥ 60 years, initial cutaneous melanoma stage from 0 to 4, and Breslow depth measured in centimeters, were evaluated using the Wilcoxon rank-sum test. Analysis of the ELISA data on the 24 immunokines was carried out by, first, normalizing each data point to a Gaussian distribution using *Z*-scores. These normalized values were then input into the MATLAB Bioinformatics Toolbox (MATLAB, Natick, Massachusetts, USA) to generate unsupervised heat maps and clustergrams, with the former showing the relatedness of patients on the basis of their chemokine and cytokine profile and the latter showing the relatedness of each marker relative of all markers tested. Distinct clusters were defined on the basis of a relative metric unit distance away from the origin of the corresponding patient dendrogram that allowed the segregation of noticeable subgroups. In addition, a K-means hierarchical cluster analysis was carried out using R to validate the initial cluster analysis carried out by the MATLAB Bioinformatics Toolbox. Ward's method was used to compute the linkage between clusters, which minimizes the variance within clusters, and a dendrogram of the results was created.

Each individual cluster of the heat map was then compared with patient outcomes, including (a) survival time from the diagnosis of melanoma to the date of first brain metastasis, (b) survival time from the date of first brain metastasis to the date of death, (c) the overall survival time from the diagnosis date of melanoma to the date of death, and (d) response to biologics treatment. The Wilcoxon rank-sum test was used to determine whether any significant differences in patient outcome exist between individual clusters on the basis of their overall immunokine profiles.

Results

The melanoma cohort has known clinical prognostic factors

The clinical characteristics of our cohort are summarized in Table 1. The median age of the patients was 54 (range 25–84) years. Patient age (<60 vs. ≥ 60) did not have a statistically significant effect on the overall survival ($P = 0.2740$). We next assessed our patient population to confirm that it conformed to known prognostic variables for metastatic melanoma. Although only 16 of 22 patients had data on Breslow depth of the cutaneous melanoma at initial diagnosis, there was a trend associating increased Breslow depth with decreased time from diagnosis to brain metastasis ($R^2 = 0.3051$) and with

Table 1 Characteristics of patients with melanoma brain metastasis and nondisease controls

Sample ID	Age	Sex	Stage	Breslow depth (cm)	Cutaneous melanoma to first brain metastasis (months)	Brain metastasis to the date of death or last follow-up (months)	Overall survival (months)
M1	64	M	3	9.0	17.2	5.2	22.4
M2	51	F	3	1.1	20.5	3.3	23.8
M3	72	M	2	5.0	38.9	13.3	52.3
M4	72	M	2	1.8	70.7	16.6	87.3
M5	58	M	2	1.4	59.9	4.1	64.0
M6	46	M	1	0.5	53.1	17.0	70.1
M7	51	F	1	1.5	27.6	6.3	33.9
M8	45	M	3	3.8	9.3	13.3	22.6
M9	69	F	1	1.1	81.9	39.0	120.9
M10	60	F	1	0.6	22.6	15.1	37.7
M11	28	F	3	5.0	45.7	28.0	73.8
M12	38	F	4	7.7	0.6	18.5	19.1
M13	33	F	3	5.0	4.9	13.2	18.1
M14	44	F	2	3.9	21.7	2.6	24.3
M15	25	F	3	2.7	10.3	5.4	15.7
M16	56	F	4	N/A	23.6	1.9	25.6
M17	47	M	1	N/A	34.7	34.5	69.2
M18	67	F	1	N/A	73.5	12.4	85.9
M19	84	M	4	N/A	0.0	73.3	73.3
M20	44	F	1	0.5	69.8	29.6	99.4
M21	60	M	1	N/A	306.1	18.5	324.6
M22	64	F	2	N/A	17.5	2.1	19.7
N1	57	F	NA	NA	NA	NA	NA
N2	39	F	NA	NA	NA	NA	NA
N3	75	M	NA	NA	NA	NA	NA
N4	88	F	NA	NA	NA	NA	NA
N5	22	M	NA	NA	NA	NA	NA

N/A, not available; NA, not applicable.

decreased overall survival ($R^2 = 0.2349$) (Table 1 and Fig. 1). The median overall survival of patients, from initial melanoma diagnosis to death, was 78.0 (range 33.9–324.6) months for stage 1 disease ($n = 8$), 349.5 (range 19.7–87.3) months for stage 2 disease ($n = 5$), 22.5 (range 15.7–73.8) months for stage 3 disease ($n = 6$), and 25.6 (range 19.1–73.3) months for stage 4 disease ($n = 3$). Patients with stage 1 disease had a significantly prolonged overall survival compared with those with stage 3 disease ($P = 0.0080$). Furthermore, there was a significantly prolonged interval from the time of first melanoma diagnosis to brain metastasis in stage 1 patients (median = 61.5 months, range 22.6–306.1 months) as compared with stage 3 patients (median = 13.8 months, range 4.9–45.7 months) ($P = 0.0047$) and stage 4 patients (median 0.6 months, range 0.0–23.6 months) ($P = 0.0242$). There was no statistical difference between patients with stage 1 versus 2 ($P = 0.2844$), stage 2 versus 3 ($P = 0.0519$), stage 2 versus 4 ($P = 0.1429$), or stage 3 versus 4 ($P = 0.3810$) disease. The findings on the clinical characteristics of our cohort are, therefore, consistent with respect to known prognostic factors for cutaneous melanoma.

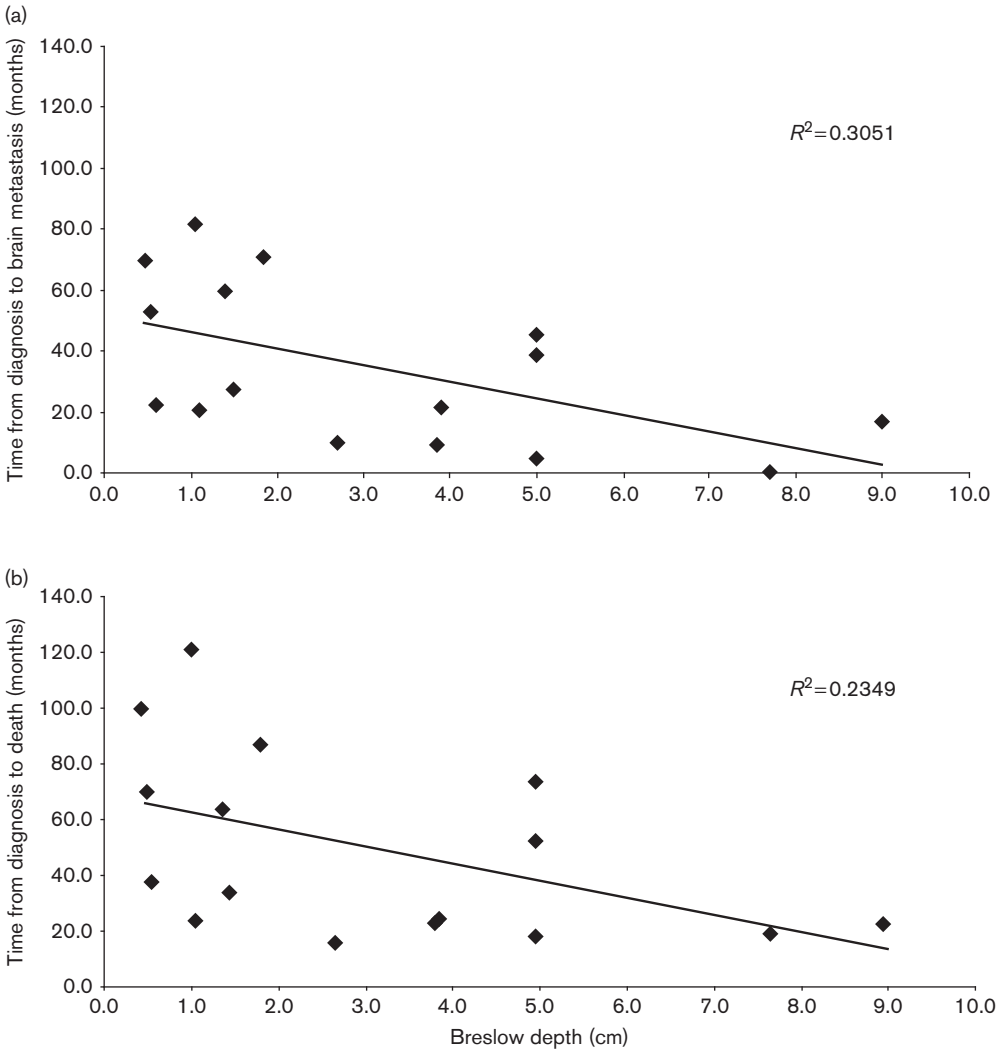
CXCL10 and IL8 chemokines are upregulated in melanoma cerebrospinal fluid

Cancerous tumors are known to secrete immunokines to effect immune subversion [20]. To test whether the presence of melanoma metastases within the brain leads to detectable alterations in the expression of immunokines within the CSF, we determined the expression profile of 24 cytokines and chemokines in the CSF (Table 2). Using a less stringent criterion of 10% chance or less, or P value less than 0.1, of the observed difference between the melanoma and control cohorts, we identified eight cytokines and nine chemokines whose levels varied significantly in our melanoma patient cohort versus our set of nondisease controls. Among them, CXCL10 and IL8 levels were significantly higher in the melanoma cohort than the controls, 92.4 (range 17.5–587.9) pg/ml versus 3.5 (range 0.0–3.9) pg/ml ($P = 0.0007$) and 53.7 (range 13.0–775.0) pg/ml versus 5.0 (range 0.0–29.5) pg/ml ($P = 0.0007$, Fig. 2a), respectively. Elevated levels of IL6 ($n = 4$), CCL17 ($n = 11$), CCL3 ($n = 5$), and CCL4 ($n = 11$) were also detected in the melanoma CSF samples.

Cerebrospinal fluid from patients with melanoma brain metastasis shows global reconfiguration of cerebrospinal fluid immunokine profiles

On examining the overall levels of immunokines in our melanoma CSF samples, we observed a generalized suppression of multiple immunokines, including IL1α, IL1β, IL4, IL5, IL10, IL12, IL13, CCL5, CXCL9, CCL11, and CCL22, relative to the levels observed in controls, but not IL2, tumor necrosis factor-α, IFN-γ, CCL2, CCL23, CXCL1, and CXCL5 (Fig. 2b). In an effort to obtain a more global view of possible differences in the relative immunokine levels that may exist between melanoma patients and controls, we generated an

Fig. 1



Breslow depth correlates with melanoma aggressiveness in the patient set. (a) Breslow depth versus time from diagnosis to brain metastasis. (b) Breslow depth versus overall survival (time from diagnosis to death).

unsupervised clustergram and heat map on the basis of the concentrations of the subset of immunokines found to vary between these two populations (Fig. 3a). The resulting analysis showed that melanoma patients were segregated away from the controls on the basis of their immunokine profiles. Generation of a hierarchical K-mean dendrogram from these data also showed a segregation of these two groups (Fig. 3b). These data suggest that the presence of tumor metastasis in the brain significantly alters host immunity within the central nervous system (CNS). Specifically, generalized suppression of IL1α, IL4, IL5, and CCL22 was detected in almost all melanoma CSF samples, suggesting the presence of global immunosuppression as part of a strategy aimed at evading host immunity against the melanoma metastasis. Furthermore, in a subset of patients, we observed the selective elevation of the three chemokines: CXCL10, CCL4, and CCL17, raising the possibility of selective chemokine activation for the purpose of oncogenesis.

Because tumors in the brain can have a disrupted blood–brain barrier, circulating chemokines in the blood could have leaked across the barrier, causing elevation of these chemokines in the CSF. Because we do not have concurrent serum samples from our cohort, we sought to answer this question by measuring the C-reactive protein (CRP), which is synthesized primarily in the liver and is present at a high concentration in the serum with the 95th percentile concentration at 9.50 μg/ml [21], but is generally excluded from the CSF. Compared with our reference nondisease serum sample with CRP measured to be 3.70 μg/ml, all of our melanoma CSF samples had

Table 2 Wilcoxon rank-sum analysis of individual immunokines

	Melanoma		Control		
	Median (pg/ml)	Range (pg/ml)	Median (pg/ml)	Range (pg/ml)	*P* value
Cytokines					
IL1α	9	0–47	509	447–626	0.0003
IL1β	0	0–7	6	4–12	0.0013
IL2	2	0–122	2	0–4	0.4129
IL4	0	0–1	26	13–47	0.0002
IL5	0	0–8	25	9–46	0.0002
IL6	8	0–532	24	13–84	0.0268
IL10	0	0–27	20	0–56	0.0061
IL12	0	0–20	0	0–4	0.0625
IL13	0	0–4	119	67–521	0.0005
IFN-γ	0	0–1	0	0–0	0.4013
TNF-α	0	0–150	0	0–1	0.4522
Chemokines					
IL8	54	13–775	5	0–30	0.0007
CCL2	399	78–1590	506	170–842	0.4880
CCL3	13	0–125	4	4–9	0.0856
CCL4	5	1–18	1	0–11	0.0655
CCL5	0	0–6	8	0–74	0.0559
CCL11	0	0–25	27	12–52	0.0010
CCL17	1	0–5	0	0–0	0.0495
CCL22	4	0–17	31	13–81	0.0005
CCL23	17	0–86	0	0–1	0.1458
CXCL1	11	2–156	8	0–25	0.1814
CXCL5	1	0–312	0	0–19	0.4761
CXCL9	10	0–439	77	62–411	0.0095
CXCL10	92	18–588	3	0–4	0.0007

Using a probability for the observed difference that was because of chance in 10% or less of the time (or $P<0.1$), there were notable differences between melanoma cerebrospinal fluid and controls in eight cytokines and nine chemokines. The eight cytokines include IL1α, IL1β, IL4, IL5, IL6, IL10, IL12, and IL13. The nine chemokines include IL8, CCL3, CCL4, CCL5, CCL11, CCL17, CCL22, CXCL9, and CXCL10.
IFN-γ, interferon-γ; IL, interleukin; TNF-α, tumor necrosis factor-α.

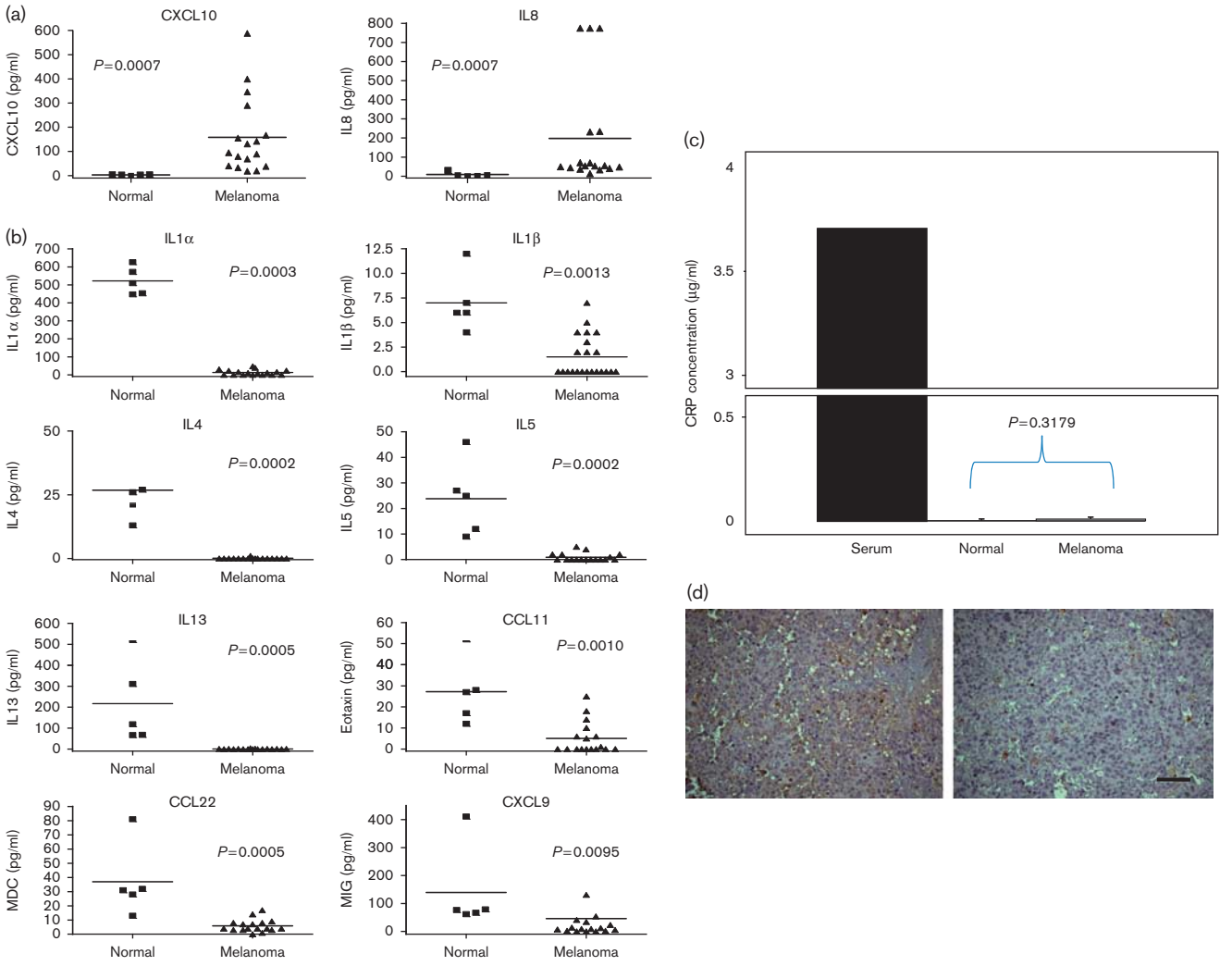
CRP of less than 0.02 μg/ml (Fig. 2c), showing that intratumoral compromise of the blood–brain barrier was insufficient to explain the observed immunokine elevation in the CSF. In addition, immunostaining of the primary melanoma tumor from patient M3, who had a high level of CXCL10, showed staining in the tumor parenchyma (Fig. 2d), further suggesting the tumor origin of this chemokine.

It is possible that the differences observed between melanoma CSF samples and controls were because of previous therapies in the patient population. Dexamethasone use is an important temporalizing treatment for cerebral edema that is often associated with brain metastasis and the most likely therapeutic intervention that might account for our results. However, we found only nine of 22 patients were prescribed dexamethasone at the time of CSF collection, having a median daily dose of 4 (range 2–16) mg, and therefore dexamethasone is unlikely to be a contributing factor for the observed immunokine suppression. Furthermore, alkylating chemotherapies, such as dacarbazine, which is being used in conjunction with biologic agents (biochemotherapy), can potentially induce an immunosuppressive state in patients [13]. Notably, serum levels of IL6, IL10, and IFN-γ in patients treated with dacarbazine-based chemotherapy combinations are significantly lower than those treated with dacarbazine-based biochemotherapy [22]. However, in our cohort, only two patients received dacarbazine and one received thiotepa, whereas 12 patients did not receive any systemic treatment before CSF sampling. Therefore, alkylating chemotherapy is unlikely to cause the suppressed immunokine levels in our patient set.

Correlation between patient clusters and clinical outcome

We next asked whether different melanomas impose distinct immunokine signatures in the CSF. Indeed, unsupervised clustergram and heat map analyses suggested the presence of five separate clusters of patients with distinct immunokine profiles in our samples (Fig. 3a). As immunokines have been suggested to be important in driving tumor progression and metastasis [20], we therefore attempted to determine whether an association could be detected between any of these apparent clusters and patient outcome. We chose three epochs for our analysis: (a) time from diagnosis of melanoma to brain metastasis, (b) time from brain metastasis to death, and (c) overall survival. First, cluster 3 showed the shortest interval from melanoma diagnosis to brain metastasis, with a median time of 11.2 (range 0.0–306.1) months versus 31.2 (range 0.6–81.9) months for the rest of the melanoma cohort ($P = 0.2873$, Table 3A). However, on closer inspection, this cluster contains the patient M21, who had a time interval of 306.1 months or 4.1 SD from the mean, who is clearly an

Fig. 2

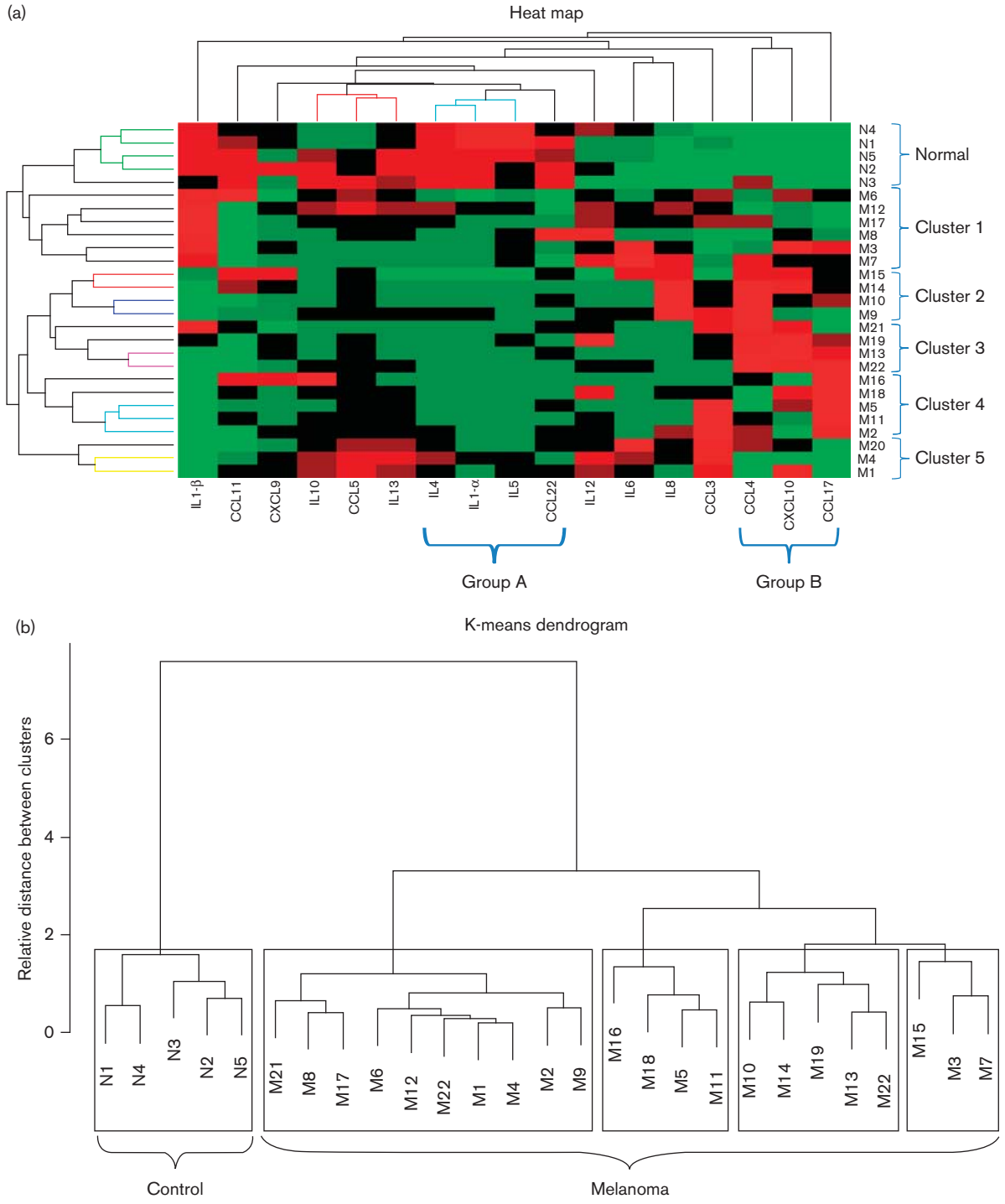


CXCL10 in the CSF is tumor derived. (a) Both CXCL10 and IL8 were significantly upregulated in melanoma CSF, whereas (b) IL1α, IL1β, IL4, IL5, IL13, CCL11, CCL22, and CXCL9 were suppressed. *P* values were derived from the Wilcoxon rank-sum test. (c) Measurement of CRP, a highly abundant serum protein not normally found in the CSF, showed 100-fold lower levels in both control and melanoma CSF samples compared with a reference nondisease serum sample, suggesting the absence of significant leak between these two compartments. The reference nondisease serum was 3.70 μg/ml, whereas the control CSF had a median CRP of 0.00 (95% CI 0.00–0.01) μg/ml and melanoma CSF had a median CRP of 0.01 (95% CI 0.00–0.01) μg/ml ($P=0.3179$). (d) Immunohistochemistry for CXCL10 was performed on primary tumor sections of patient M3 showing staining in the parenchyma of the tumor but not in tumor-infiltrating lymphocytes (left panel). The right panel showed no primary control (scale bar = 100 μm). CI, confidence interval; CRP, C-reactive protein; CSF, cerebrospinal fluid; IL, interleukin.

outlier when compared with the other patients in our sample set (range 0.0–81.9 months). Upon exclusion of M21, cluster 3 indeed possesses a statistically significant shortened time from melanoma diagnosis to brain metastasis or 4.9 (range 0.0–17.5) months ($P = 0.0307$). Notably, the remaining members in this recalculated cluster, M13, M19, and M22, all had elevated levels of CXCL10, CCL4, and CCL17, whereas IL1α, IL4, IL5, and CCL22 were markedly suppressed. Both CXCL10 and CCL4 are potent chemoattractants for CD8+ effector T cells [23–25], suggesting that these inflammatory proteins may play a role in promoting the formation of brain metastasis. Taken together, the CSF immunokine profile in these members of cluster 3 may support a propensity for the development of melanoma brain metastasis.

Second, we carried out an analysis to determine whether any of the clusters showed correlations with clinical outcome subsequent to the detection of brain metastasis. Cluster 4 showed a trend toward decreased time interval from brain metastasis to death with a median time of 4.1 (range 1.9–28.0) months versus 15.1 (range 2.6–73.3) months for the rest of the melanoma cohort

Fig. 3



Melanoma brain metastasis results in immunological reconfiguration in the CNS. (a) Unsupervised hierarchical clustering and heat map analysis of 17 relevant immunokines. The melanoma and control CSF samples were separated distinctly from each other. Significant suppression of IL1α, IL4, IL5, and CCL22 was noted in almost all melanoma CSF samples but not in controls (group A). Immunokines CCL4, CXCL10, and CCL17 seemed to aggregate together in the clustergram (group B) and both CCL3 and IL8 chemokines also appeared to cluster near them. Patients clustered into five distinct groups (clusters 1–5) on the basis of their immunokine patterns. Before CSF was sampled, patients M3, M9, M11, M12, M14, M15, M16, M17, and M21 received dexamethasone, whereas all had immunotherapy, except for M5, M6, M7, M14, M19, and M22. (b) K-mean dendrogram analysis showed distinct separation of melanoma and control CSF samples. K-means hierarchical cluster analysis was carried out using R to validate the initial cluster analysis carried out using the MATLAB Bioinformatics Toolbox. Ward's method was used to compute the linkage between clusters and a dendrogram of the results was created. CNS, central nervous system; CSF, cerebrospinal fluid; IL, interleukin.

Table 3 Analysis of clusters derived from cluster analysis and patient outcome

Cluster	*n*	Mean (months)	Median (months)	Range (months)	*P* value
(A) Time from first melanoma diagnosis to development of brain metastasis					
1	6	27.4	31.2	0.6–53.1	0.5803
2	4	34.1	22.2	10.3–81.9	0.9661
3	4	82.1	11.2	0.0–306.1	0.2873
4	5	44.7	45.7	20.5–73.5	0.3084
5	3	52.6	69.8	17.2–70.7	0.444
(B) Time from brain metastasis to death					
1	6	17.2	15.2	6.3–34.5	0.3195
2	4	15.5	10.2	2.6–39.0	0.8984
3	4	26.8	15.9	2.1–73.3	0.7657
4	5	9.9	4.1	1.9–28.0	0.1170
5	3	17.1	16.6	5.2–29.6	0.7019
(C) Overall survival					
1	6	44.5	43.1	19.1–70.1	0.5309
2	4	49.7	31.0	15.7–120.9	0.7017
3	4	108.9	46.5	18.1–324.6	0.8984
4	5	54.6	64.0	23.8–85.9	0.6383
5	3	69.7	87.3	22.4–99.4	0.3892

($P = 0.117$, Table 3B). Interestingly, only CCL17 was elevated in all members of this cluster, whereas IL1β and IL6 were suppressed in addition to the commonly observed IL1α, IL4, IL5, and CCL22 immunokine suppression. Therefore, these patients' apparent poor clinical outcome may arise from a more effectively tumor-subverted immune function relative to that in the rest of our melanoma cohort. Third, there was no detectable difference in the overall survival among the five patient clusters (Table 3C). This is likely because overall survival is influenced by the extent of the systemic malignancy rather than the number and size of the brain metastases or their treatment.

Correlation between patient clusters and previous biologics treatment

Because treatment with biologics and immune-checkpoint inhibitors can suppress or eradicate systemic melanoma [13–15], we next analyzed whether these interventions would alter patient outcomes or associate with any of the identified patients in our previous analysis. No difference was detected with respect to time from melanoma diagnosis to brain metastasis ($P = 0.6318$), time from brain metastasis to death ($P = 0.3195$), and overall survival ($P = 0.8538$) on the basis of treatment with biologics such as high-dose IL2 and/or IFN-α. However, among patients who received biologics, those in cluster 1 appeared to show a marked shortening of the interval from diagnosis to brain metastasis, with a median time of 22.0 (range 0.6–38.9) months versus 34.7 (range 4.9–306.1) months for the rest of the melanoma cohort ($P = 0.1773$). This trend may represent the selection pressure imposed onto the systemic melanomas that leaves some of the surviving clones with a higher propensity of metastasizing to the brain. Similarly, patients in cluster 4 who were treated with biologics show a trend toward shortened time from brain metastasis to death, with a median time of 7.8 (range 1.9–28.0) months versus 15.9 (range 5.2–39.0) months for the rest of the melanoma cohort ($P = 0.1275$), suggesting that the brain metastases in this cluster are particularly aggressive after selection by biologics' treatment.

Discussion

This is the first analysis of broad immunokine profiling in the CSF of patients with melanoma metastasis to the brain, a concept similar to immunoprofiling and establishing an immunoscore for systemic malignancies [26,27]. This immunoscore may predict the efficacy of cancer treatments and pave the way for personalized immunotherapy [27]. Indeed, our melanoma samples differed significantly from nondisease controls in cytokine and chemokine levels, including a marked suppression of IL1α, IL4, IL5, and CCL22 in almost all of our samples. It is important to note that although this constitutes a generalized suppression of immunokine levels as compared with control CSF, we also detected elevation of CXCL10, CCL4, and CCL17 in a large subset of our melanoma CSF. Immunostaining of the tumor origin of CXCL10, as well as our analysis of CSF versus serum CRP in control and melanoma CSF samples, also supports that these immunokine changes are specifically altered in the brain and do not emerge from the serum. Together, these data show a global response within the CNS to the presence of melanoma metastasis. There are potentially two explanations for this observation. First, this difference may reflect the altered activities of tumor-associated immune cells that impose immune suppression on the rest of the CNS through the secretion of soluble factors. This may result in suppression of resident immune cells, resulting in lowered levels of inflammatory cytokines observed in the current study [7]. Such a general suppression has been shown previously for IL1β, IL4, and IL5 in melanoma-positive sentinel lymph nodes relative to melanoma-negative controls [28]. Further analysis of this effect of melanoma metastasis to lymph node and brain could uncover more differences in immune modulation that may be specifically required for survival in these different sites. Second, it is possible that the downregulation of inflammatory cytokines that we observed could be a consequence of dexamethasone use or treatment by alkylating chemotherapies. However, our analysis showed that neither is likely to cause the observed immunosuppressive profile in the CSF. It is also possible that previous treatment with biologics may result in unpredicted responses in the immune system similar to those observed in our patient set. However, most patients, 16 out of 22, were treated with IL2 and/or IFN-α, whereas six were not, and there was no difference in the immunokine profiles between these two groups. Taken together, the immune suppression observed in our patients is likely to have been imposed by the metastases rather than arising as a result of previous therapies.

Another important observation derived from our data is that CXCL10 and IL8 are upregulated in the CNS of a majority of our melanoma patients. There is a striking, statistically significant 30-fold and 10-fold increase in CXCL10 and IL8, respectively, in melanoma CSF as compared with controls. We were able to detect CXCL10 immunohistochemical staining in the parenchyma of a primary melanoma, suggesting that the melanoma metastasis in the brain may also secrete this chemokine in an effort to recruit inflammatory effector CD8 + T cells into the tumor microenvironment for its own survival and proliferation. In addition, CXCL10 may be secreted by microglia and astrocytes. This is because CXCL10 upregulation has also been detected in Alzheimer's dementia, which has an inflammatory component likely driven by microglia resulting in a protracted course of clinical deterioration [29,30]. In experimental autoimmune encephalitis, the source of CXCL10 has been shown to originate from astrocytes within the brain, cerebellum, and spinal cord [31]. Therefore, both tumor-derived and brain-derived CXCL10 may facilitate the survival and proliferation of melanoma brain metastasis. Furthermore, the IL8 chemokine is a potent mediator for angiogenesis [32–34]. Melanoma tumor cells can also secrete IL8, but the level of expression may be regulated by the local tissue microenvironment [35]. It is also secreted by activated microglia in the brain [36] and its level is elevated in the CSF of patients with acute and chronic inflammatory neurological disorders, including HIV-associated dementia [37] and opticospinal multiple sclerosis [38]. Taken together, both tumor-derived and brain-derived IL8 may also facilitate the development of angiogenesis, which is critical to ensure the survival and proliferation of melanoma brain metastasis.

Despite the presence of generalized immunokine suppression in the melanoma CSF, there is enhanced expression of certain chemokines in specific patient clusters relative to nondisease controls. High levels of chemokines CCL4, CXCL10, and CCL17 (Group B in Fig. 3a) seem to aggregate together in the clustergram, and both CCL3 and IL8 chemokines also appear to cluster near these three chemokines. Notably, cluster 3 has the highest levels of CCL4, CXCL10, and CCL17, and it has the shortest time interval from melanoma diagnosis to the development of brain metastasis. CCL17, has been shown to be expressed by brain tissue and it is a potent chemokine for T_H2-type CD4 + CD25 + Treg cells because they have the corresponding CCR4 receptor [39,40]. In patients with Vogt–Koyanagi–Harada disease, a rare autoimmune disease directed against tyrosinase and other melanocyte antigens that results in uveitis and neurological deficits, the CSF level of CCL17 was also significantly elevated when compared with control patients without the disease [41,42]. Therefore, in this setting, overexpression of CCL17 may aid the recruitment of Treg cells that provide a counter-regulatory mechanism against the inflammatory reaction within the brain and eyes. It is also noteworthy that the serum level of CCL17 was lower in Vogt–Koyanagi–Harada patients than controls [41], suggesting that CCL17 is a chemokine specifically overexpressed in the brain. In melanoma patients, however, CCL17-mediated recruitment of Treg cells to the brain may attenuate antimelanoma protective immunity and enables tolerance to melanoma metastasis. Interestingly, certain melanoma cells also have the CCR4 receptor for the CCL17 ligand [40] and they may therefore co-opt the CCL17 chemokine axis for their own migration into the brain, suggesting a more complex role for this chemokine in promoting brain metastasis.

CCL3 and CCL4 are members of the IL8 chemokine superfamily [43] and both may therefore aid the survival and proliferation of melanoma brain metastasis. They are expressed in the brain during the acute phase of experimental autoimmune encephalitis, and neutralization of CCL3 by anti-CCL3 antibody limits the extent of brain damage in this model [44]. In patients with ovarian carcinoma, elevated levels of CCL3 and CCL4 are associated with the presence of CD4 + T cells in the ascitic fluid, whereas melanoma patients had a predominance of CD8 + T cells in biopsy samples taken from the brain, lung, skin, and small bowel [45,46]. These T cells most likely have a bias toward the T_H1 response because CCL3 and CCL4 are known to activate antigen-presenting cells through the CCR5 receptor and during this process, IL12 is upregulated [47]. However, within cluster 3, where CCL4 is elevated in all patients while CCL3 is high in some patients, only one member, M19, had elevated IL12 in the CSF, whereas the rest was average or low. The high CCL4 with or without elevated CCL3, together with low IL12, suggests that there may be yet unknown mechanisms of attenuating the T_H1 response in patients with melanoma brain metastasis. Nevertheless, for patients in cluster 3, treatments that can drive down CCL3 and CCL4 may be useful therapeutic strategies. Furthermore, M21 is an outlier with the longest time interval within the entire patient set. In contrast to other members of the cluster, this patient's CSF has a low level of CCL17 and a high level of IL1β. It is possible that the relatively lower level of CCL17 in M21 impairs the migration of melanoma cells into the brain, whereas elevated IL1β may be cytotoxic to the ones that arrived there by means other than the CCL17 chemokine axis and others that survived there because of impaired T_H1 adaptive immunity [40,48]. Therefore, treatment that can lower CCL17 levels may prevent the development of melanoma brain metastasis.

Members within cluster 4 have suppressed IL1β and IL6 cytokines in addition to the generalized IL1α, IL4, IL5, and CCL22 immunokine suppression. Furthermore, only CCL17 chemokine was increased in this cluster.

Patients within this cluster have poor survival once brain metastasis is established, irrespective of previous biologics treatment. We speculate that the severe immunokine suppression in the CSF represents a similar state of immunosuppression within the brain that provides a favorable environment for melanoma brain metastases to grow and proliferate. The extent of suppression is somewhat surprising and may arise from the immunologically sequestered nature of the CNS where smaller amounts of tumor-derived immunosuppressive factors can achieve a global effect. Finally, cluster 1 has a trend for shortened time from melanoma diagnosis to brain metastasis and this only occurred in those who received biologics treatment. It is possible that biologics treatment places selection pressure on the systemic melanoma and that the surviving clones have a high propensity of metastasizing to the brain. However, both observations need to be validated in a larger population of melanoma cohort.

A major limitation of our observations is the small sample size, which is based on 22 individual CSF samples. This likely contributes toward the lack of statistical significance in the prognostic significance of our patient clusters on the basis of a set of immunokines. However, the robust segregation of melanoma CSF from controls, as seen in both the clustergram/heat map and the K-mean dendrogram analyses, strongly suggests that melanoma metastasis to the brain causes global changes in the immunokine milieu within the CNS that can be detected in the CSF. Notably, there is generalized immunokine suppression, whereas specific CXCL10 and IL8 chemokine levels are increased. Therefore, these findings provide the necessary foundation for the identification of immunokines and their relative levels of expression in the CSF, as well as their potential utility as diagnostic biomarkers for melanoma brain metastasis. Furthermore, as more is known about the immunological phenomena associated with tumors in general and within the CNS, new treatments can be developed to interrupt these tumor-associated manipulations of the immune system.

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Conflicts of interest

There are no conflicts of interest.

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