**ORIGINAL RESEARCH**

Transcriptional characterization of immunological infiltrates and their relation with glioblastoma patients overall survival

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**ABSTRACT**

Introduction: Several cell populations from the peripheral immune system interact to create a complex immunologic status during glioblastoma growth and response to therapy. The aim of this study was to integrate the impact of different immune cell populations present in glioblastoma tumor microenvironment on overall survival.

Methodology: Gene expression and clinical data were generated by The Cancer Genome Atlas and previously reported meta-signatures representing cells of the immune system were used. The relationship between meta-signatures was evaluated through Pearson’s correlation analyses. Survival analyses were performed through Kaplan-Meier plots and Cox regression model.

Results and discussion: Meta-signatures corresponding to infiltrating immune cells with immunosuppressive roles, such as macrophages, NK and NK T cells, MDSCs and Tregs, correlated with poorer patient prognosis. Meta-signatures related to CD8\(^+\) T cells predicted improved survival only in patients with low immunosuppressive meta-signatures. By clustering the meta-signatures we found that the cluster containing high meta-signatures of macrophages, MDSCs and Tregs demonstrated the worst prognosis.

Conclusion: Integrating the information provided by transcriptional signatures of immunological aspects is fundamental in understanding the impact of the immune system on patient survival. We found a predictive impact on survival with positive role for CD8 and negative roles for macrophages, MDSC, Tregs, NK and NK-T in glioblastoma patients. Understanding these regulatory and stimulatory factors of patients’ immune system is essential to delineate an effective strategy to increase the anti-tumor immune response and to generate potential clinical benefits.

**Introduction**

Glioblastoma (GBM) is an aggressive and incurable primary brain tumor characterized by an extensive heterogeneity at cellular and molecular levels.\(^1\) Despite the aggressive treatments, including surgical resection, radiation therapy and cytotoxic chemotherapy, the disease remains incurable with a median survival under fifteen months.\(^2\) The failure of conventional oncologic treatment to selectively eradicate glioblastoma cells has prompted investigators to look for new and more targeted therapeutic options, such as strategies that elicit an immune response against the tumor.

An increasing body of evidence suggests a significant correlation of the immune system and the pathogenesis of gliomas, with clinical implications.\(^3\) Long-term remission of secondary malignant brain tumors with postoperative infection has led to the hypothesis that an increased immune response may confer some protection against cerebral tumors.\(^4\) Moreover, several reports have also suggested that significant histories of allergy decrease the risk of patients developing glioma during their lifetime.\(^5,6\) In contrast, a compromised immune system can generate a state in which cerebral tumors can develop more easily. This is suggested by the remarkable correlation between human immunodeficiency virus (HIV)-mediated immunosuppression and intracranial glial tumors.\(^7,9\)

Several antigens have been associated with tumors capable of generating a specific response in a variety of human cancers, including malignant gliomas.\(^10,11\) These findings suggest that a T cell-dependent immune response may improve the prognosis of patients with glioma through an antigen-mediated immune response. Cytotoxic T-cell infiltration (CD8\(^+\) T lymphocytes)
found in the glioma tissue may represent antigen-dependent immune activation against glioma. Thus, the level of CD8+ T-cell infiltrates in the initial presentation and at diagnosis of GBM seems to correlate positively with the survival of patients with GBM.\textsuperscript{12,13}

However, glioblastoma tumors have been shown to induce T cell anergy and lymphopenia, impair antibody synthesis, increase circulating levels of immunosuppressive cytokines, upregulate T cell inhibiting molecules and recruit suppressive cells such as regulatory T cells (Tregs), macrophages and myeloid derived suppressor cells (MDSCs).\textsuperscript{14-20} In this manner, GBMs are able to evade the host antitumor response at the levels of antigen recognition and immune activation, producing an immunosuppressive state in the host.\textsuperscript{21-23} Thus, there is a growing need to identify predictive and prognostic biomarkers that enhance our understanding of the mechanisms underlying the complex interactions between the immune system and cancer.

High-throughput technologies are providing feasible tools for analyzing the mutation antigen and tumor molecular profiles, cell meta-signatures and epigenetic modifications of tumor and immune cells. These novel technologies will help the advancing of precision medicine\textsuperscript{24} by identifying predictive biomarkers such as immunologic signatures or profiles for the patients who will most likely benefit from current immunotherapies.\textsuperscript{25,26}

We therefore hypothesized that cells of the tumor microenvironment, as well as the tumor itself, would influence the activity of effector cells of the immune system with antitumor action, such as CD8+ T cells, and reduce the immune system’s ability to eliminate the tumor. The importance of M2 macrophages, neutrophils, dendritic cells and CD8+ T lymphocytes in the biology of different subtypes of gliomas was recently shown, especially for the mesenchymal subtype.\textsuperscript{27} Here, we evaluated the impact of validated immune-related meta-signatures\textsuperscript{28,29} individually and integrated rationally on the overall survival of glioblastoma patients to explore the importance of the immune system in the pathology of this tumor.

**Results**

**Immune meta-signatures in GBM**

The lack of consistent tumor rejection in GBM patients suggests that infiltrated T cells are functionally deficient, inactivated, eliminated, excluded from the tumor milieu or even playing a role in fostering tumor development.\textsuperscript{30} These characteristics of exhaustion can be caused by several factors and cell types, including the presence of some cell types of the immune system. In order to identify which meta-signatures are associated with potential immune response impairment, a heatmap analysis was performed using exhaustion meta-signature as a guide to the analysis, aiming to observe which meta-signatures clustered together. We observed a correlation of meta-signatures of immunosuppressive characteristics with the exhaustion meta-signature program. When clustering the exhaustion meta-signature by Pearson correlation coefficient, four main clusters could be identified. The first cluster is formed by the meta-signatures of NK, NK T, Th1 and exhaustion (Fig. 1A and Supp. Fig. 1). A second cluster is formed by the meta-signatures of MDSC, Macrophages (Mac) and Tregs. A third cluster consists of CD8 and CD4 signatures and a fourth cluster is formed by Th17 and Th2 (Fig. 1A). A correlation rate above 0.8 between a correlation rate above 0.8 between the exhaustion, Th1, NK T and NK meta-signatures and between the MDSC, Treg and macrophage meta-signatures was observed. The CD4 and CD8 meta-signatures presented a correlation >0.71. Additionally, the meta-signatures corresponding to Th17 and Th2 cells did not present significant correlations to any signatures evaluated (Fig. 1A).

In order to evaluate the immune contexture by using an alternative method to the meta-signatures, we estimated the immune population composition by applying the CIBERSORT\textsuperscript{31} in silico deconvolution algorithm on the TCGA cohort. The landscape shows that GBM tumors in this cohort can be clustered in different groups with macrophages (both M0 and M2) representing major contributors to the tumor microenvironment (Fig. 1B). Activated mast cells seem to be highly present in a subgroup of tumor samples. The landscape was confirmed in an independent GBM tumors cohort (Supp. Fig. 2A and B). The absolute contribution of immune populations in the validation cohort indicates that tumors have heterogeneous composition not only in the proportion of immune cells but also of the absolute presence of immune cells, reinforcing the notion that GBMs can be different in immune composition both qualitatively and quantitatively (Supp. Fig. 2B).

**Impact of different meta-signatures on patients overall survival**

Considering that different meta-signatures may correlate with different outcomes in patients, we next evaluated the impact of each meta-signature on patients overall survival. The greatest differences were found in MDSC and NK T meta-signatures, with p values <0.001, and Tregs, NK and Macrophage (p < 0.05), with patients bearing low meta-signatures expression showing longer survival compared to those patients with higher expression of these meta-signatures. Th17, Th2, CD8 and CD4 meta-signatures showed no impact on patient survival when evaluated individually (Fig. 2A). Only MDSC high expression produced a hazard ratio (HR) significantly superior to 1 individually and adjusted to all other meta-signatures (Fig. 2B) or when adjusted to age and sex (data not shown). Surprisingly, we did not identify significant differences when analyzing meta-signatures that were supposed to have antitumor activity, such as CD8, nor classical pro-tumoral meta-signatures, such as exhaustion. Among the individual immune inhibitory genes, LGALS9, PDCD1LG2 (PD-L2) and IL-10 expression levels impacted the HR of tumor evolution in the GBM cohort (Supp. Fig. 3A) with PD-L2 being the only gene confirmed to impact HR in the validation cohort (Supp. Fig. 3B), reinforcing the importance of an integrated analysis of the immune players in glioma biology. We further compared expression levels of inhibitory receptors and deconvolution analysis of cell subpopulations by overlaying clustered inhibitory receptor expression heatmaps and CIBERSORT output analysis (Supp. Fig. 3C and D). We found no obvious correlation of inhibitory receptors expression with any of the cell populations abundance as determined by the deconvolution algorithm, neither ordering data by inhibitory molecules...
clustering (Supp. Fig. 3C) nor by cell population clustering (Supp. Fig. 3D).

High levels of CD8 meta-signature combined with low levels of exhaustion meta-signature correlates with increased overall survival

Since the CD8 solo meta-signature showed no association with survival, we hypothesize that an effective response of CD8+ lymphocytes could be facing a blockage by immunosuppressive regulatory circuits. We thus evaluated patient overall survival combining CD8 and exhaustion meta-signatures in the Kaplan-Meier analysis. Patients displaying low exhaustion meta-signature and a high CD8 meta-signature showed an increased survival (p = 0.04), when compared to patients with exhaustion high, CD8 low meta-signatures, (Fig. 3A and B).

The same effect was observed in an independent cohort (Supp. Fig. 4A-C). This was not observed in lower grade gliomas (Supp. Fig. 4D), suggesting that the impact of CD8 and Exh occurs only in more aggressive forms of this disease.

**Figure 1.** Meta-signatures clustering based on patients overall survival A) Correlation analysis between overall survival of the meta-signatures. The correlation analysis is presented in a heat-map plot. Hot colors refer to higher correlations and cold colors to lower correlations. B) Immune cell populations frequencies as analyzed by CIBERSORT in the TCGA GBM cohort.
Macrophage display different impact on patients overall survival depending on the CD8/exhaustion ratios

Macrophages have substantial diversity and plasticity and are largely influenced by the tissue microenvironment, being able to play either pro or anti-tumor roles. We thus tested the impact of macrophages on patient prognosis in the tumors CD8 High, Exh Low (HL) and Low, High (LH) group. The largest impact of the macrophage meta-signature was observed in the CD8 Exh HL (Fig. 3A), with macrophages increasing the hazard ratio (HR) in the HL but not LH subgroup (Fig. 3B).

Several immune cell populations such as tumor associated macrophages (M1 and M2) or tumor associated neutrophils (N1 and N2) can play a double role in the tumor microenvironment. To explore whether the impact of the infiltrated macrophages on survival is affected by genes expressed in pro- or anti-tumoral immune states, we added a small set of genes, namely NOS-2 and IL-12a, expressed by anti-tumor cells (such as M1 macrophages and dendritic cells) and a set of genes (ARG-1, IL-10, TGFB1, VEGFA) expressed by potential pro-tumor cell populations such as monocytes, Th-2, Tregs, MDSCs and M2 macrophages to the analysis. When considering only the Mac high group, high expression of these markers represents a risk factor while expression of the anti-tumoral markers did not impact survival chances (Fig. 3B). These results suggest that polarization of macrophages or other immune cells to a pro-tumoral state has a positive impact in tumor progression in glioblastoma.

Extending the CD8-Exh HL meta-signatures analysis, we observed that patients in this group presented a significantly positive HR for the high expression of MDSC (Fig. 3B), NK, NK T, Th1 and Treg (Fig. 3C) meta-signatures. None of these meta-signatures conferred risk in the CD8 Exh LH subgroup whereas high expression of MDSC, NK, NK T, Th1 and Treg meta-signatures conferred a significant risk when the whole group of patients was evaluated (Fig. 3C).

Clustering of Meta-signatures

Finally, we searched for combinations of meta-signatures that best predict prognosis of patients. As shown in Fig. 1A, we identified four distinct clusters. We defined clusters 1 and 2 as immunosuppressives and clusters 3 and 4 as anti-tumoral, based on our previous results on overall survival. High expression of clusters 1 and 2 significantly increased the risk, while clusters 3 and 4, individually, did not significantly affect the risk of patients (Fig. 4A and Supp. Fig. 5A).

We then tested the impact of different clusters on patient overall survival by combining clusters. When we combined the clusters 2 by 2 we observed that the largest impact on survival was obtained by reduced levels of C1 or C2. If one of these clusters was highly expressed, survival was reduced in relation to patients with low expression for both clusters (Fig. 4B and Supp. Fig. 5B). Combining C2 with C3 indicated that the dominant cluster is C2, as only the levels of C2 separate patients in

Figure 2. Impact of the individual meta-signatures on survival. A) Kaplan-Meier survival plots of the individual meta-signatures. Significant log-rank (Mantel-Cox) p values are indicated. B) Multivariate Cox proportional Hazards Ratio (HR) for the individual meta-signatures adjusted to all other meta-signatures (blue) or not adjusted (Red).
groups with significantly different survival (Fig. 4C). Other combinations 2 by 2 also indicated that the dominant groups were C1 and C2 (Supp. Fig. 5B).

Interestingly, immune status affected the impact of therapy, as the use of alkylating agent therapy only impacted patients with immunosuppressed tumors, i.e. C3 C1 and C3 C2 LH subgroup (Fig. 4 D and E). Thus, we can conclude that low expression of C1 and C2 or potential reduction of these components through therapy lead to high survival in relation to high expression of these clusters, mainly due to a dominant block that C1 or C2 impinge on C3 (Fig. 4F).

**Discussion**

Tumor genome expression patterns are a representation of tumor biology and their diversity also reflects the diversity of tumor microenvironment among patients. Thus, linking gene expression patterns with clinical outcomes is a key issue in understanding differences in tumor microenvironments and their effects on the outcome of the disease. In this sense, the identification of elements that activate or inhibit effective immune responses against tumors may find great utility in tumor therapy. Identification of cells that may block the antitumor immune response through T cell inhibition and the characterization of tumor supporting activities by those cells such as the secretion of tumor promoting molecules is essential to identify novel targets and/or biomarkers for cancer treatment.

We herein demonstrate that several meta-signatures showed significant correlation with the exhaustion program, suggesting an abrasive tumor microenvironment to effector cells. In this context, exhaustion-promoting cells might play an essential role in blocking anti-tumor cells populations by different
mechanisms. Exhausted T cells have several characteristics, such as significant loss of their effector functions, positive regulation of inhibitory receptors, impaired proliferation and changes in their metabolic profile. MDSCs, for instance, can suppress T cell function by producing arginase, decreasing the levels of L-arginine, which is an essential amino acid for normal T-cell function. Reduced levels of arginine appears to reduce T-cell receptor chain expression, promoting T cell dysfunction. These cells also secrete nitric oxide and reactive oxygen species (ROS), which are also capable of inducing T cell suppression. Otherwise, regulatory T cells can suppress the proliferation of any cytokine secreting effector T cells by negative regulation of IL-2 and/or interferon-gamma (IFN-γ) production. Macrophages polarized to the M2 phenotype acquire the ability to inhibit phagocytosis and induce secretion of immunosuppressive cytokines, resulting in inhibition of T cell proliferation. In addition, the number of infiltrated macrophages is correlated with vascular density, a factor correlated to metastasis, and M2 macrophages are enrolled as potential players in pathophysiological processes in the mesenchymal subgroup of gliomas, especially when tumors carry the deletion of NF1. Therefore, these cell populations, largely abundant in GBMs as shown by the cell population prediction algorithm, contribute to an immunosuppressive and tumor-supportive microenvironment. We further confirmed the contribution of the meta-signatures of these cells to an aggressive and highly immunosuppressive tumor microenvironment by correlating the expression of meta-signatures with patient overall survival: meta-signatures associated to the exhaustion program were linked to a poor prognosis when highly expressed. Interestingly, the meta-signatures representing NK cells, NK T cells and Th1 cells also showed strong correlations with the exhaustion program, forming cluster 1 and indicating a possible blockage of Th1 cell effector functions. Having characterized that immune-suppressing circuits are probably playing major roles in tumor microenvironment, we evaluated the expression levels of available immune inhibitory molecules in tumor microenvironments. By doing so, we demonstrate that PD-L2 (PDCD1LG2) was the only evaluated inhibitory gene to impact HRs in the test and the validation cohorts analyzed. PD-L2 is one of the PD-1 ligands, being expressed by DCs, macrophages and a plethora of tumor microenvironment cells, including non immune cells such as fibroblasts and endothelial cells. PD-L1 and PD-L2 often present overlapped expression patterns in tumors and these molecules are being considered as targets for immunotherapy. Unfortunately, the cohort evaluated here has no PD-L1 expression levels data available for analysis, impairing any correlation of this relevant
marker with patients outcome, although this marker, when expressed, has been correlated to immune impairment and poorer outcomes in glioma patients.

Surprisingly, we did not find a correlation of CD8, CD4, Th2 and Th17 meta-signatures with the survival of patients when evaluated individually, indicating that the effector mechanisms of these cells may be affected by the presence of other components in the tumor microenvironment such as several components of the immune tumor microenvironment described here through the meta-signatures (MDSCs, Tregs, macrophages etc) or CIBERSORT analysis (macrophages and Tregs).

NK and NK T cells were also associated with a negative impact on survival for the CD8/exhausted HL sub-population. NK cells are considered classical tumor protective cell components, although NK cell exhaustion has been observed in several human tumors and animal tumor models. Continuous activation of NK cells has been shown to result in in situ differentiation followed by a state of anergy, high expression of inhibitory receptors, including Tim-3, and upregulation of activation receptors and transcription factors such as EOMES and T-bet. This suppressor effect of Tim-3 is mediated by interaction with the galectin-9 ligand, followed by the promotion of Th1 cell apoptosis. Furthermore, continuous exposure of NK cells in presence of tumors appears to reduce cytotoxic activity and its excessive proliferation also contributes to NK cell dysfunction. The clinical relevance of decreased NK cell function is also evident in disease progression. In breast cancer, NK cells with reduced function and high inhibitory receptor expression are associated with more aggressive and invasive tumors.

These results suggest that even meta-signatures of classical anti tumor cells such as Th1 can be correlated to worst survival when they are tightly correlated to immune suppressive populations, as is the case for the signatures grouped in cluster 1. The context of immune suppressive meta-signatures present in C1 or C2 clusters prevail over anti-tumor signatures such as CD4 and CD8 present in cluster 3 (Fig. 4F) suggesting that several immune-suppressive cell populations can be acting in dampening the antitumor response, as has been largely documented for melanoma breast cancer, and ovarian cancer amongst others. Since not all the populations identified by the meta-signatures applied in our analysis are amongst those that can be identified by CIBERSORT, limited correlations on the landscape provided by these two approaches could be established. For instance, MDSC, and exhaustion signatures are not present in CIBERSORT. Nonetheless, from the significant meta-signatures correlated with poor prognosis in our work, at least Treg and macrophages (mainly M2) are largely represented in the CIBERSORT analysis, reinforcing the model of immune-suppressive cluster populations (C1 in this case) performing a veto effect on C3 cluster cell populations (mainly CD4 cells, also abundant in the CIBERSORT analysis).

Some of the immune-suppressive cell populations can be highly impacted by cancer therapies such as chemo or radiotherapy or even targeted therapy.

Several alkylating and alkylating-like agents such as cyclophosphamide, carboplatin, oxalaplatin and others have been largely linked to the elimination of immune-suppressive immune populations such as MDSCs, Tregs (cyclophosphamide) and to the promotion of immunogenic cell death (Oxalaplatin). Besides the largely explored role of cyclophosphamide as an immunomodulating drug, the impact of other alkylating agents used for glioma treatment, such as lomustine, in immune-suppressive cell components of the tumors is still largely unknown. The reported effects for other alkylating agents support the potential of this class of drugs in modulating immune-suppressive cells. The results presented herein of the effects of alkylating agents on immune-suppressive cells composing the tumor microenvironment probably account for the effect of alkylating agents in patients survival on the sub-population of patients with CD8/Exhaustion LH but not in patients with less immune suppressed microenvironments.

One of the main impediments to the development of effective immunotherapies is the complexity of the already addressed GBM microenvironment. Tumor-associated macrophages and MDSCs levels have been shown to be high in patients with glioblastoma and provide a supportive environment for tumor cell growth by inhibiting the expansion of CD8+ and CD4+ T cells and supporting the presence of Tregs. We also observed that cluster 2, containing macrophage, MDSC and Treg meta-signatures, seems to contribute not only to the suppression of immune system, but also supporting tumor growth, due the fact that cluster 2 contributed to the worst prognosis when other immunological meta-signatures were lowly expressed. Thus, clusters 1 and 2 have prognostic roles and clusters 3 and 4 appear to have an anti-tumoral activity. An interesting approach would be the blockage of tumor-associated macrophages in order to moderate antitumor responses, promoting an increased CTL infiltration, and diminished tumor progression. That strategy was previously shown through the blockage of CSF1/CSF1R signaling combined with immune checkpoint blockers, which improved the response of the therapy and led to tumor regressions. Moreover, drugs that can deplete Tregs or MDSCs in the tumor microenvironment could represent important tools for combined therapies.

Our study, while being comprehensive at the level of meta-analysis of the expression of different components, has certain limitations. The meta-analysis was performed on public data sets that did not clearly describe the levels of tumor heterogeneity. Although the number of glioblastoma patients included in this study may partially offset tumor heterogeneity from patient to patient, emerging evidence suggests that immunological intra-tumor heterogeneity (e.g., tumor nuclei versus invasive margins) needs to be taken into account for the analysis. In this sense, it would also be important to differentiate the expression profiles of tumor cells versus cells of the immune system – a point that could not be reached in the present study. Even more important, the differential expression of certain cell types per se may not be representative of their signaling context. For example, many epigenetic, post-transcriptional or post-translational modifications play an important role in regulating the outcome of immunological signaling. Thus, differential gene expression levels do not always reflect the presence of cells or cellular activity. In this sense, the meta-signature will be more stable and less susceptible to variations in subsets of the samples compared to the use of single markers. As a result, predictive genes in a
meta-signature may lead to more reliable information about tumor progression and patient survival.

Understanding the various regulatory factors and stimulators of the patient’s immune system as well as the tumor microenvironment is essential to delineate an effective strategy to increase the anti-tumor immune response and generate real clinical benefits. Moreover, as immunotherapy evolves, prognostic and predictive biomarkers are of paramount importance in determining which patients will be the best candidates for different immunotherapy strategies. In this context, mutational burden, immune checkpoint expression and mismatch repair genes may be used to predict patients that are likely to benefit from immune checkpoint inhibition. Analyzing the composition of immune infiltrates and their biological functions based on their prognostic values constitutes an important approach to characterize the immunological microenvironment of glioblastomas at transcriptional levels and contribute to the selection of patients more likely to benefit from immunotherapy.

**Methods**

**Data origin**

Data from The Cancer Genome Atlas (TCGA) was obtained through the UCSC Cancer Genome Browser platform (www.genome-cancer.ucsc.edu, accessed on November 18th, 2016). The glioblastoma data “GBM gene expression (AffyU133a)” was used. Tumor gene expression data that did not contain clinical information of the patients were excluded, leading to a final number of 524 cases used in the present study. Clinical data of the patients are shown in Supp. Table 1.

**Meta-signatures**

Meta-signatures representing different cells of the immune system were previously described by Charoentong et al. (2017) and the exhaustion meta-signature was described by Tirosh et al. (2016). The genes corresponding to each cell type are described in Supplementary Table 2 and the meta-signatures were obtained by the average expression of the genes corresponding to each cell type.

**Heatmap-based Hierarchal Clustering**

The unsupervised hierarchical clustering was performed using the package “pheatmap” of R software (www.r-project.org). Meta-signatures were normalized individually, obtaining all values between 0 and 1. The heatmap was organized based on the exhaustion meta-signature expression and clustered by the expression among the different meta-signatures using Euclidean distance method and single linkage criterion.

**Pearson’s correlation analysis**

The pearson’s correlation analysis between meta-signatures was performed using the package “stats” of R software and the results were plotted as a heatmap.

**Kaplan-meier analysis**

Patients were followed until death or censure at 1500 days. Kaplan-meier analysis was done individually for each meta-signature, using the median as cutoff between patients with high and low expressed meta-signatures. Kaplan-meier analysis using two meta-signatures were performed dividing patients into 4 groups: patients showing both meta-signatures above the median (High/High-HH), patients showing meta-signatures below the median (Low/Low-LL) and two other groups of patients showing one meta-signature above the median and the other meta-signature below the median (High/Low-HL and Low/High-LH). For meta-signatures with high correlation, a cut-off point was plotted parallel to the correlation line, and a second cut-off point was plotted perpendicular to that line, dividing the patient groups into 4 groups.

Likewise, three different meta-signatures were compared in a Kaplan-meier analysis, using the same analysis between the first two meta-signatures and adding a third meta-signature to that analysis by using the median expression as cut-off point between patients with high and low expression levels of the additional meta-signature.

**Cox Regression Analysis**

For all meta signatures, unadjusted cox regression models were done to calculate the hazard ratio (HR) of death comparing high with low expression, using the median as cutoff between patients with high and low expressed meta-signature. To investigate if the effect of each meta signature was independent, adjusted models incorporating all of the meta signatures were performed. Those analysis were done for the total sample and for the subgroups CD8/Exh HL and LH. Diagnosis analysis based on weighted Schoenfeld residuals were used to test the proportional hazard assumption. Cox models were carried out using SAS Studio through SAS OnDemand for Academics (SAS Institute, Cary, NC).

**Integrated analysis of meta-signatures**

In order to integrate all analyzed meta-signatures, meta-signatures were clustered according to the observed separations in the Heatmap-based hierarchal unsupervised clustering. The mean of each cluster was obtained for each patient. Overall survival of patients was evaluated by using one cluster or by integrating two or three different clusters in the analysis.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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