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Allergic airway inflammation delays glioblastoma progression and reinvigorates systemic and local immunity in mice

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Abstract

Background: Numerous patient-based studies have highlighted the protective role of immunoglobulin E-mediated allergic diseases on glioblastoma (GBM) susceptibility and prognosis. However, the mechanisms behind this observation remain elusive. Our objective was to establish a preclinical model able to recapitulate this phenomenon and investigate the role of immunity underlying such protection.

Methods: An immunocompetent mouse model of allergic airway inflammation (AAI) was initiated before intracranial implantation of mouse GBM cells (GL261). RAG1-KO mice served to assess tumor growth in a model deficient for adaptive immunity. Tumor development was monitored by MRI. Microglia were isolated for functional analyses and RNA-sequencing. Peripheral as well as tumor-associated immune cells were characterized by flow cytometry. The impact of allergy-related microglial genes on patient survival was analyzed by Cox regression using publicly available datasets.

Results: We found that allergy establishment in mice delayed tumor engraftment in the brain and reduced tumor growth resulting in increased mouse survival. AAI induced a transcriptional reprogramming of microglia towards a pro-inflammatory-like state, uncovering a microglia gene signature, which correlated with limited local immunosuppression in glioma patients. AAI increased effector memory T-cells in the circulation as well as tumor-infiltrating CD4^{+}T-cells. The survival benefit conferred by AAI was lost in mice devoid of adaptive immunity.

Conclusion: Our results demonstrate that AAI limits both tumor take and progression in mice, providing a preclinical model to study the impact of allergy on GBM susceptibility and prognosis, respectively. We identify a potentiation of local and adaptive systemic immunity, suggesting a reciprocal crosstalk that orchestrates allergy-induced immune protection against GBM.
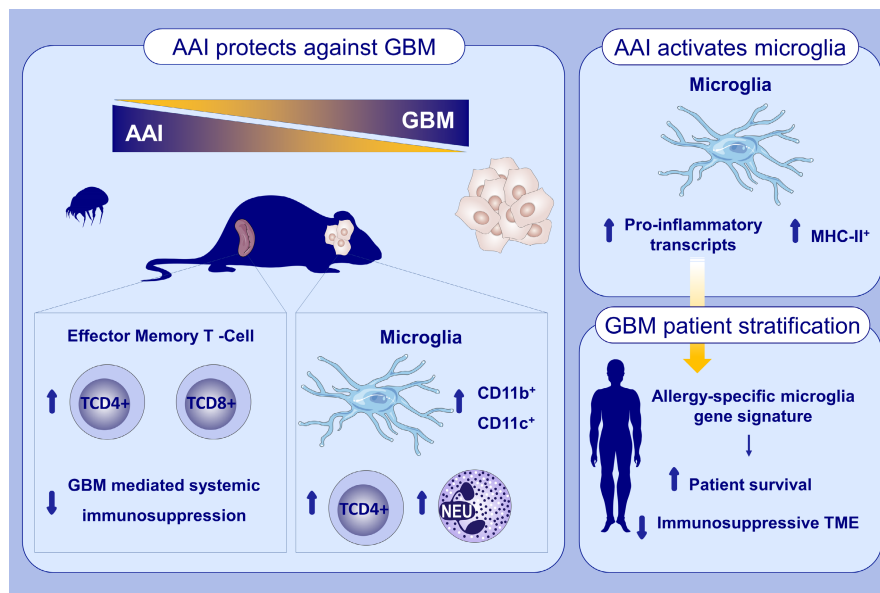
Simone P. Niclou and Markus Ollert equally contributed to this work.

GRAPHICAL ABSTRACT
Allergy-induced protection against GBM is recapitulated in a preclinical syngeneic mouse model. Allergic airway inflammation skews microglia toward pro-inflammatory and antigen-presenting like-states, uncovering a gene-signature in mice associated with improved patient survival and reduced local immunosuppression in glioma patients. Allergic airway inflammation promotes the recruitment of CD4+T-cells within the tumor microenvironment and mitigates glioma-induced systemic immunosuppression.
Abbreviations: AAI, allergic airway inflammation; GBM, glioblastoma; Neu, neutrophil; TME, tumor-microenvironment.

1 | INTRODUCTION

Gliomas represent a heterogeneous group of malignant brain tumors, of which the most frequent subtype, glioblastoma (GBM), remains incurable.[1] Compelling epidemiological[2,3] and clinical[4,5] evidence points to a strong protective effect of immunoglobulin E (IgE)-mediated allergic disease against gliomas.[6] In individuals with IgE-mediated allergy, the risk of developing glioma has been reported to decrease up to 40%.[2,6] Furthermore, GBM patients presenting with high levels of serum IgE survive significantly longer compared with those with a low IgE titer.[3–6] This prominent effect of IgE-mediated allergy on both the risk and the prognosis of GBM is thought provoking, but has remained unexplained over the last decades.[2–6]

The invasiveness and high plasticity of GBM are major obstacles to successful patient care. GBM is known as immunologically "cold,"[7] being characterized by a low level of lymphocyte infiltration and the massive recruitment of immunosuppressive cells, mainly represented by tumor-associated microglia and macrophages (TAM/M).[8,9] In addition, GBM progression is accompanied by severe systemic immunosuppression and peripheral T-cell dysfunction.[10,11] These local and systemic immune dysfunctions are well-recognized barriers to the emergence of effective immunotherapies, which to date have failed to improve the prognosis of GBM patients.[11,12] Thus, new avenues to better understand and exploit the ability of the immune response in unlocking immunosuppression would certainly aid in the design of novel immunotherapeutic approaches.

While the epidemiological data for a protective effect of respiratory allergies in GBM are being consolidated,[2,6] several important questions remain. The main conundrum is how allergy can modulate the outcome of GBM. Despite growing evidence suggesting that mediators of allergic inflammation are involved in anti-cancer immunity, inspiring the field of allergooncology,[13,14] supporting mechanistic insight is currently sparse. A widely accepted hypothesis proposes that atopic conditions may be linked to an elevated state of immune activation[15] and tumor immune-surveillance[2,3]; as recently proposed in a mouse model of NF1-low-grade glioma of the optic pathway.[16]

Here, we show that allergic airway inflammation (AAI) limits tumor progression in mouse brains. We find that AAI induces the activation of microglia toward pro-inflammatory and antigen-presenting-like states. We demonstrate that glioma-induced systemic immunosuppression can be partially reverted through AAI favoring the recruitment of CD4+T-cells in the tumor microenvironment (TME). Lastly, we provide evidence of a key role for adaptive immunity in allergic protection against GBM.

2 | MATERIALS AND METHODS

Additional details are provided in Supplementary Materials.

2.1 | Animals and cell line

C57BL/6JRj mice were purchased from the Janvier Labs (France), and RAG1-KO mice (B6.129S7-Rag1^{tm1Mom}/J) from the Jackson Laboratories. All mouse experiments were performed according to protocols approved by the Ethics Committee for animal experimentation of the LIH and the National Authorities of Luxembourg. The GL261 cell line was generously provided by Prof. Andreas Bikfalvi from the U1029 INSERM-Angiogenesis and Cancer Lab (Bordeaux - Pessac, France).

2.2 | Allergic airway inflammation and orthotopic glioma model

For the induction of AAI, mice were sensitized by repeated nasal instillation with a solution of either 10 µg (HDM_10) or 50 µg (HDM_50) house dust mite (HDM) extract (Stallergenes Greer, USA). Allergic airway responsiveness assessment was performed as previously described.[17] After AAI induction, all mice underwent surgery for intracranial implantation of GL261 tumor cells as previously described.[18] MRI was used to measure the tumor volume.

2.3 | RNA-sequencing (RNA-seq) of microglia and bioinformatics analysis

Microglia were prepared from whole brain of PBS-perfused mice and enriched using a Percoll density gradient as previously described.[19] RNA was isolated from a pure microglia fraction obtained via cell sorting (FACSAria™ cell sorter, BD Biosciences, USA) (RNA-Seq Data accession number: PRJNA695424). Differentially expressed genes (DEGs) were detected using edgeR. Individual prognostic genes were identified out of the list of DEGs using univariable Cox regression analysis, applied to gene expression datasets collected from TCGA and CGGA for glioma stratified by their isocitrate dehydrogenase (IDH) status wild type (WT) (Figure S1A,B).

2.4 | Immuno-profiling of the tumor microenvironment by flow cytometry staining

All animals were perfused with ice-cold PBS to wash out brain tissue from circulating blood cells. The right hemispheres, where GL261 were implanted, were dissected and dissociated with MACS Neural Tissue Dissociation Kit (P) (Miltenyi) following the manufacturers' instructions. Single cells were washed and recovered in PBS 0.2% BSA to perform flow cytometry staining. Cells were incubated 5 min at 4°C with Fc Receptor block CD16/32 Ab (Biolegend, USA) followed by 30 min incubation at 4°C with antibodies and dead cell stain kit as specified in Table S1. Cells were extensively washed with PBS 0.2% BSA before data acquisition using FACS Novocyte Quanteon (Agilent).

2.5 | Functional analysis of microglia

Cytokine measurements from enriched microglia culture supernatants were performed using CBA (Enhanced Sensitivity Flex Sets, BD Biosciences). Cytotoxicity assays were assessed as previously described.[19] The phagocytic capacity of microglia was tested using FITC–dextran (FD4, Sigma) uptake analysis by fluorescence-activated cell sorting (FACS) for 1 h at 37°C.

2.6 | Statistical analysis

All data are presented as median. Numerical data were analyzed using Mann–Whitney t-test or using one-way ANOVA (Tukey's multiple comparisons test), as noted within the corresponding figure and/or table legends. The number of biological replicates per groups as well as the p-values are also reported in figures or in legends. Survival was analyzed using the Kaplan–Meier and the Log-rank (Mantel–Cox) test or the Gehan test as indicated in figure legends. The standard statistical analyses (ANOVA, Wilcoxon, Gehan, correlation test) were conducted in GraphPad Prism 8 software (La Jolla, CA). Genomics related data analysis, such as multidimensional scaling (MDS), detection of DEGs, Gene set enrichment analysis (GSEA) and single sample GSEA (ssGSEA), gene-level Cox regression were performed in R/Bioconductor. Benjamini–Hochberg's false discovery rate (FDR) was used for p-value adjustment in case of multiple hypothesis testing.

3 | RESULTS

3.1 | Allergic airway inflammation limits tumor take and progression in an experimental GBM model

Our first goal was to establish a mouse model mimicking the allergy-driven glioma protection observed in humans.[2,3,5,6] Since the most robust epidemiological correlations with an impact on GBM risk were shown for respiratory allergies,[2] we used a mouse model of AAI induced by HDM extract.[17] We evaluated the efficiency of the AAI protocol in inducing a typical allergic reaction after sensitization with either low (group HDM_10) or high (group HDM_50) concentration of HDM extract compared to non-allergic control mice (CTR), receiving saline solution. To this aim, we sacrificed mice 14 days after AAI induction (Figure 1A). As expected, we observed an increase of the airway responsiveness following a bronchial provocation test, as demonstrated by an elevation of the lung resistance concomitantly to the decrease of dynamic compliance (Figures S2A,B), in HDM groups compared with CTR. In parallel, we found an infiltration of
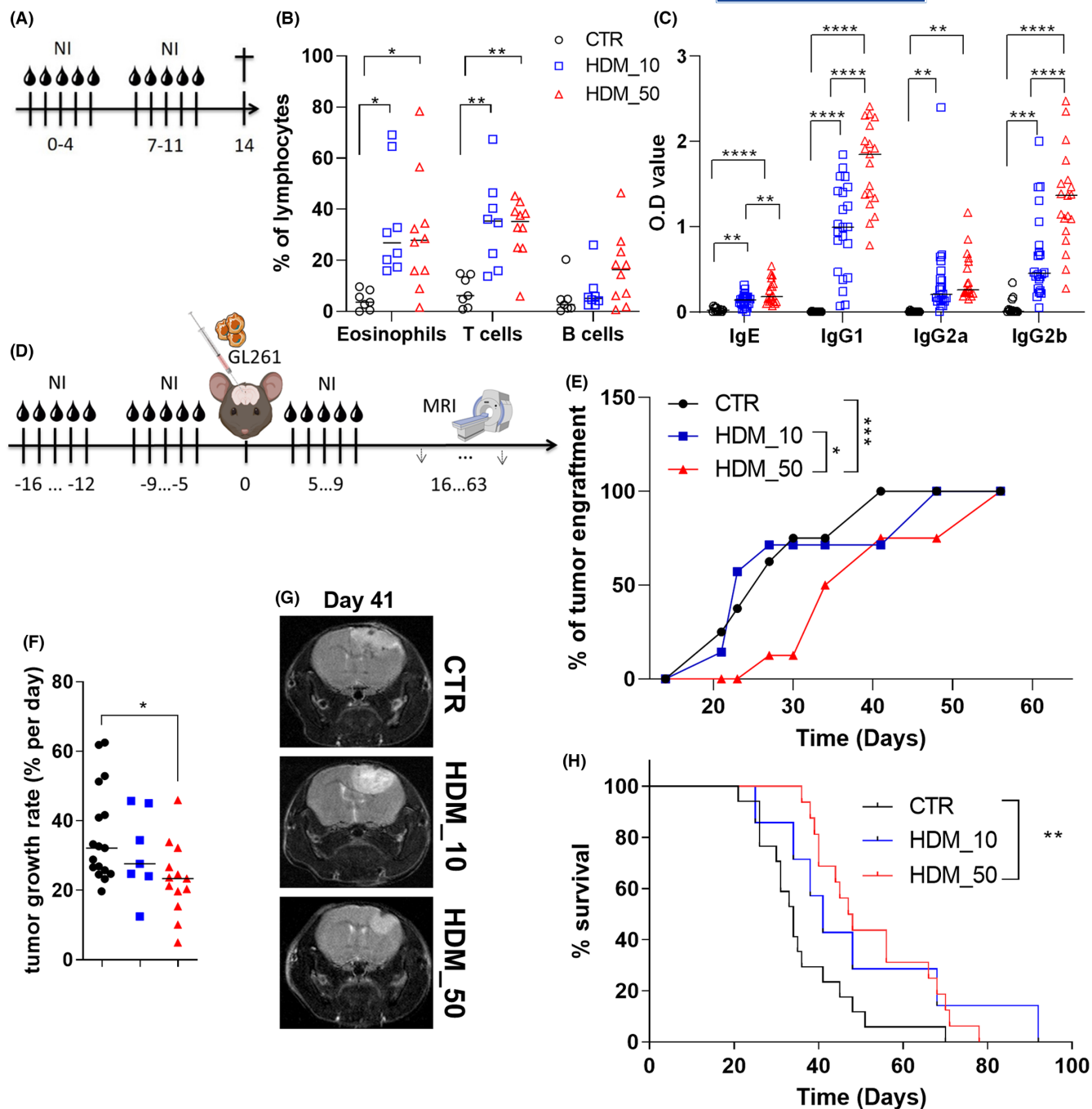
FIGURE 1 Severe AAI delays tumor engraftment, reduces tumor growth, and increases animal survival. (A) Experimental design of the in vivo approach for AAI induction. (B) Cellular composition of BALF for eosinophils, T and B cells. Data are derived from CTR ($n = 7$), HDM_10 ($n = 8$), and HDM_50 ($n = 10$) groups. (C) HDM-specific Igs in the serum. Serum titers for HDM-specific Ab in CTR ($n = 15$), HDM_10 ($n = 22$), and HDM_50 ($n = 19$) were determined. (D) Experimental design of the in vivo approach for AAI induction combined with intracranial implantation of GL261 cells. (E) Percentage of tumor take by groups over time. The percentage of tumor engraftment was defined as the percentage of mice presenting a tumor mass at each MRI time point. The survival analysis of the groups was performed applying a Gehan–Breslow–Wilcoxon test, defining the median time where 50% of mice had no tumors yet. Comparison using Log-rank test is presented in Table S2A. (F) Tumor growth rate monitored on sensitized HDM_10 ($n = 7$), HDM_50 ($n = 13$) and on CTR ($n = 17$) mice through MRI between two time points. (G) Longitudinal axial post-contrast T2-weighted images of mice bearing GL261 showing one representative animal per group at 41 days post-sensitization. (B, C and F) Data are plotted as median. Statistical significance was determined using one-way ANOVA (Tukey's multiple comparisons test). (H) Kaplan–Meier survival curve of GL261-implanted mice without (CTR) ($n = 17$) or with allergic sensitization with 50 µg/NI of HDM (HDM_50) ($n = 13$) or 10 µg/NI of HDM (HDM_10) ($n = 7$). Comparison of survival curves, using Log-rank (Mantel–Cox) test. *$p < .05$, **$p < .01$, ***$p < .001$ and ****$p < .0001$. Open symbols indicate samples collected before tumor implantation, full symbols indicate samples collected after establishment of the tumor, where data-points from CTR are represented by black circle, HDM_10 in blue squares and HDM_50 in red triangles.

eosinophils and T-lymphocytes in the broncho-alveolar lavage fluid (BALF) (Figure 1B) and elevated Th2 (IL-4; IL-5; IL-13), Th1 (TNF-α), and Th17 (IL-17) cytokines after restimulation of draining lymph node cells with the allergen (Figure S2C). These results demonstrate that both doses of HDM significantly induced the characteristic features of AAI compared with CTR.[17] No significant differences were seen between HDM_10 and HDM_50 for these allergy-specific parameters in the effector organ of the lung (Figures 1B and S2A–C). However, HDM-specific IgE, IgG1, IgG2a, and IgG2b were significantly higher in the serum of HDM_50 compared with HDM_10 mice (Figure 1C).

Next, AAI was induced with both doses of HDM extract before the intracranial implantation of the mouse GBM cell line, GL261 (Figure 1D). This syngeneic orthotopic GBM mouse model is commonly used for experimental GBM research requiring an immunocompetent background.[20] We observed a significant delay of tumor engraftment in mice from the HDM_50 group in comparison with mice from HDM_10 and CTR groups (Figures 1E and S3A; Table S2A). Furthermore, the HDM_50 group presented a significant reduction in the tumor growth rate over time compared with CTR mice (Figure 1F,G; Table S2B). This combined effect on tumor take and growth in the HDM_50 group was associated with a significant increase in survival of the animals, with a median survival time of 47.5 days compared with 34 and 41 days in the CTR and HDM_10 groups, respectively (Figure 1H; Table S2A). Of note, tumor growth rate and mouse survival in the HDM_10 group were not significantly different to the CTR group (Figure 1F,H; Tables S2A,B), indicating that the effect mediated by AAI on GBM engraftment, progression, and survival is dependent on the severity of AAI. Furthermore, to rule out the possible influence of endotoxins present in HDM extract, we included a group of mice receiving NI with an equivalent concentration of lipopolysaccharide (LPS) in saline solution. These animals did not present any change in terms of tumor engraftment, tumor growth rate or survival compared with the CTR group (Tables S2A,B). These observations indicate that the beneficial effect observed in animals having received 50 μg of HDM cannot be attributed to its endotoxin content. Taken together, we present a novel mouse model that recapitulates previous epidemiological observations made in humans, where the level of circulating IgE (reflecting allergic severity[21]) was associated to lower GBM incidence and to prolonged patient survival.[3,5] Thus, we took advantage of this model to further decipher the protective impact of AAI on GBM by investigating its effect on local and systemic cellular immunity.

3.2 | Allergic airway inflammation induces the activation of microglia toward pro-inflammatory- and antigen-presenting-like states before tumor implantation
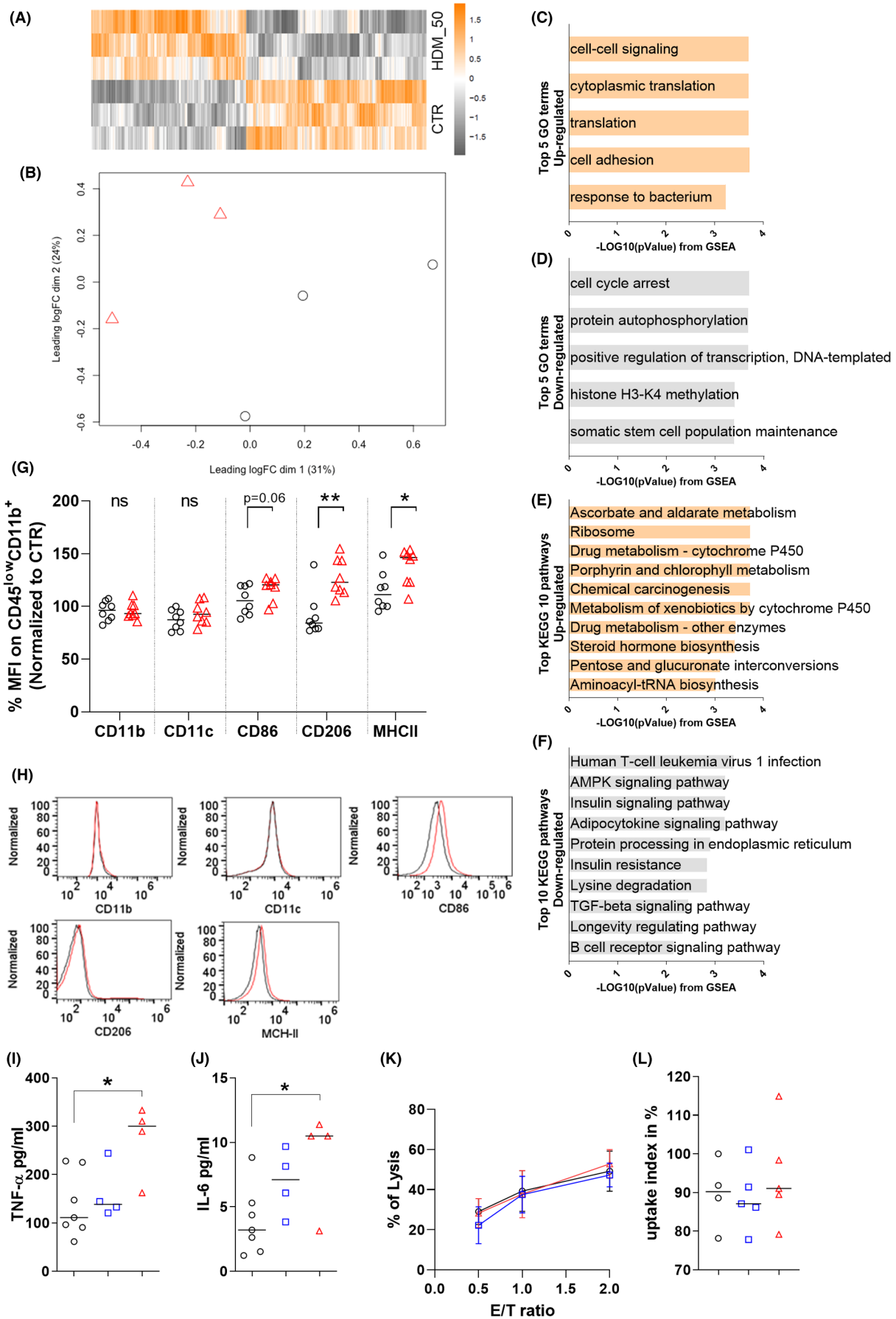
The critical determinant supporting GBM establishment is the development of a permissive microenvironment driven by the local immune system where microglia play a central role.[8,9,22] Since microglia can switch from a homeostatic state to a reactive state as a consequence of systemic inflammation,[23,24] we hypothesized that AAI might improve microglia-based immuno-surveillance leading to glioma elimination at early stages. Therefore, we explored the impact of AAI on the microglia transcriptome before tumor implantation (Figure S4A).

We identified 511 DEGs, of which 276 were up- and 235 down-regulated in HDM_50 mice compared with CTR (with FDR < 0.05; Figure 2A, Table S3A; Supplementary Methods). In contrast, we did not detect any significantly dysregulated genes in the HDM_10 group (Table S3B) compared with CTR, indicating a concentration-dependent effect of peripheral allergen exposure on microglia (Table S3C). This is in line with our data on tumorigenicity, where engraftment and progression were only affected in the high HDM allergen group (Figure 1). A MDS plot confirmed the transcriptomic reprogramming of microglia during AAI induced by high dose of HDM (HDM_50) (Figure 2B).

When we compared our list of allergic-specific microglia DEGs (ASM-DEGs) for transcripts typically assigned to the microglia with pro-(M1-like) or anti-(M2-like) inflammatory polarization, we noted a down-regulation of the expression of typical anti-inflammatory genes such as *Nfe2l2* (Nrf2), *Chil3* (Ym1), *Tgfb1*, *Stat6*, or *Stat3*. On the opposite, genes classified as pro-inflammatory, such as *Stat1*, *Cd68*, *COX2*, *Hvcn1*, *Il6*, and *Tnf* were up-regulated. Nonetheless, we also observed the down-regulation of certain M1-like genes (*Nfkb*) and the up-regulation of M2-associated genes (*Il4*) (Figure S4B). Interestingly, we observed that AAI up-regulated 3 genes coding for cytokines-related molecules (*Ifit2*; *Il1rl1*; *Tnfrsf13b*), 2 genes coding for chemokines (*Ccl9*; *Ccl6*), 7 genes coding for CD molecules (*Icos*; *Cd46*; *Itgae*; *Cd180*; *Slc7a5*; *Cd276*; *Ms4a6c*), and 2 coding for complement related molecules (*Cd46* and *C1rl*) (Table S3A).

To further define the nature of this unique transcriptional conversion induced by AAI, we performed a GSEA interrogating the

FIGURE 2 Severe AAI leads to a transcriptional reprogramming as well as phenotypic and functional modifications of microglia before tumor implantation. (A) Heat map of DEGs, where columns indicate DEGs and rows represent individual samples from the two groups HDM_50 (n = 3) and CTR (n = 3). Color scale represents normalized gene counts. (B) Multidimensional scaling (MDS) plot from HDM_50 group compared with CTR groups. (C–F) Representative pictures of the GSEA analysis interrogating (C, D) GO and (E, F) KEGG databases. (G) Phenotyping by FACS and percentage of mean fluorescence intensity (%MFI) on CD45^low CD11b+ microglia for CD11b, CD11c, CD86, CD206, and MHC-II from CTR (n = 8) and HDM_50 (n = 8) mice, data normalized to CTR. (H) Representative histogram depicting increased expression levels of CD86, CD206, and MHC-II on CD45^low CD11b+ microglia from HDM_50 compared with CTR. (I, J) CBA quantification of (I) TNF-α and (J) IL-6 cytokines released from microglia extracted from CTR (n = 7), HDM_10 (n = 4) and HDM_50 (n = 4) after AAI. Cell supernatants were collected after 18 h of culture. (K) Effect of HDM sensitization on the lytic capacity of microglia against the glioma cell line GL261 ex vivo according to the effector/target ratio (E/T) following 18 h of co-culture. (L) Effect of HDM sensitization on the phagocytic capacity of microglia ex vivo. Data are plotted and report median, *p < .05, **p < .01. Statistical significance was determined using (G) Mann–Whitney *t*-test and (I–L) one-way ANOVA (Tukey's multiple comparisons test). Representation of the experimental in vivo approach depicted in Figure S4A. Open symbols indicate samples collected before tumor implantation, where data-points from CTR are represented by black circle, HDM_10 in blue squares and HDM_50 in red triangles.

(A)

(B)

(C) Top 5 GO terms Up-regulated
- cell-cell signaling
- cytoplasmic translation
- translation
- cell adhesion
- response to bacterium

-LOG10(pValue) from GSEA

(D) Top 5 GO terms Down-regulated
- cell cycle arrest
- protein autophosphorylation
- positive regulation of transcription, DNA-templated
- histone H3-K4 methylation
- somatic stem cell population maintenance

-LOG10(pValue) from GSEA

(E) Top KEGG 10 pathways Up-regulated
- Ascorbate and aldarate metabolism
- Ribosome
- Drug metabolism - cytochrome P450
- Porphyrin and chlorophyll metabolism
- Chemical carcinogenesis
- Metabolism of xenobiotics by cytochrome P450
- Drug metabolism - other enzymes
- Steroid hormone biosynthesis
- Pentose and glucuronate interconversions
- Aminoacyl-tRNA biosynthesis

-LOG10(pValue) from GSEA

(F) Top 10 KEGG pathways Down-regulated
- Human T-cell leukemia virus 1 infection
- AMPK signaling pathway
- Insulin signaling pathway
- Adipocytokine signaling pathway
- Protein processing in endoplasmic reticulum
- Insulin resistance
- Lysine degradation
- TGF-beta signaling pathway
- Longevity regulating pathway
- B cell receptor signaling pathway

-LOG10(pValue) from GSEA

(G)

(H)

(I)

(J)

(K)

(L)

scored list of genes (Table S3D) for GO terms and KEGG pathways (Figure 2C–F; Table S3E,F; Supplementary Methods). The exploration on GO functions demonstrated an enrichment of genes involved in biological processes regulating cell interaction, that is, "*cell–cell signaling*," and pro-inflammatory response, that is, "*response to bacterium*" (Figure 2C; Table S3E). In parallel, we noted a negative enrichment score for numerous GO terms implicated in the regulation of gene transcription and epigenetic processes, that is, "*regulation of transcription, DNA-templated*"; "*histone H3-K4 methylation*" (Figure 2D; Table S3E). Epigenetic mechanisms were demonstrated to be a determinant in controlling the balance between the resting state and the cellular polarization of reactive microglia, in part through the fine-tuning of genes involved in metabolic pathways.[25] Accordingly, we found the enrichment for KEGG terms related to metabolic pathways involved in the metabolism of carbohydrates, such as, "*ascorbate and aldarate metabolism*", of lipids, such as, "*steroid biosynthesis*" or of ATP, such as, "*oxidative phosphorylation*"(Figure 2E; Table S3F). Interestingly, using KEGG analysis, we observed the down-regulation of TGF-β, AMPK, and Insulin pathways (Figure 2F; Table S3F), known to support anti-inflammatory properties of TAM/M, thus contributing to an immunosuppressive GBM microenvironment.[9,26–29] Altogether, these transcriptomic data demonstrate that microglia exhibit a unique transcriptomic profile in the context of AAI, with a higher resemblance to a pro-inflammatory-like state.

To explore the microglia phenotype upon AAI, we performed multicolor flow cytometry analyses of surface antigens typically expressed on microglial cells with antigen presentation capacities, including CD11b, CD11c, co-stimulatory molecule CD86 and major histocompatibility complex class II (MHC-II), and inflammatory polarization determinants, such as CD206. We found that AAI induces a significant increase of MHC-II, CD206 as well as a trend for elevated CD86 expression, without modulation of CD11b nor CD11c expression on microglia compared with control (Figure 2G,H).

To define the activation of microglia during AAI at the functional level, we investigated their secretory capacity ex vivo. We observed a significant increase in the secretion of the pro-inflammatory cytokines TNF-α and IL-6 in the HDM_50 microglia, confirming their activation profile (Figure 2I,J). However, we did not observe an altered capacity neither in the killing of GL261 cells nor in the phagocytic potential (Figure 2K,L).

In summary, we find that microglia acquire features of pro-inflammatory and antigen-presenting cells accompanied by increased cytokine secretion ex vivo, which might play an indirect role in reducing tumor take in the brain.

3.3 | Allergic airway inflammation increases the fraction of CD1c⁺ microglia and CD4⁺T-cells in the tumor microenvironment

Given the impact of AAI on microglia behavior before tumor implantation, we sought to determine the nature of tumor-infiltrating immune cells in the TME upon allergic sensitization. To investigate this, we applied multicolor flow cytometry on brain cell suspensions harvested from tumor-bearing mice with and without AAI to distinguish and characterize myeloid and lymphocytic subsets (Figure S5A).

We first discriminated between the overall percentage of CD45^{high} and of CD45^{low} infiltrating immune cells, the latter of which were mainly composed of resident microglia. No statistical difference between non-allergic and allergic groups was observed (Figure S5B). Similarly, the fraction of myeloid subsets, such as microglia (CD45^{low}CD11b$^+$), border-associated macrophages (BAMs) (CD45^{high}CD11b$^+$CD206$^+$Ly6C$^-$), blood-derived monocytes/macrophages (BDM/M) (CD45^{high}CD11b$^+$CD206$^-$Ly6C$^+$), and T-cells remained similar regardless of the allergy status (Figure 3A). We detected a small but significant increase in the percentage of neutrophils (CD45^{high}Ly6G$^+$) in the TME following the induction of AAI compared with controls (Figure 3A,B).

Interestingly, while BAMs, BDM/M, and neutrophils did not exhibit variations in the expression level of CD11b, CD11c, CD86, and MHC-II (Figure S5C–E), tumor-associated microglia showed significant increased expression of CD11b, CD11c with no discernible modulation of CD86, CD206, or MHC-II expression after AAI compared with saline conditions (Figure 3C,D).

The analysis of T-cell subsets revealed a significant elevation of the proportion of CD4$^+$T-cells with a concomitant reduction of the percentage of CD8$^+$T-cells in the tumor of allergic compared with non-allergic mice (Figure 3E,F). However, the proportion of naive (CD62L$^+$CD44$^-$), central memory (CD62L$^+$CD44^{high}, CM) and effector memory (CD62L$^-$CD44^{high}, EM) CD4$^+$T-cell subsets was not modulated in the TME upon AAI (Figure 3G).

These results indicate that AAI skews microglia towards CD11c$^+$ antigen-presenting-like cells and induces the preferential recruitment of neutrophils and CD4$^+$T-cell subsets in the TME.

3.4 | Allergic airway Inflammation reduces glioma-induced systemic immune suppression

Systemic adaptive immunity is recognized as an essential element of brain tumor immune surveillance and a dysfunctional T-cell immunity has been reported in GBM patients.[10,11,30] We therefore investigated the status of systemic immunity in tumor-bearing mice in the context of AAI. Blood, spleen, and bone marrow were collected at the moribund stage (Figure 1D; Supplementary Methods). At this time point, the levels of circulating lymphocytes, monocytes, and granulocytes were not significantly modified compared with control mice (Figure 4A). However, AAI resulted in a significant expansion of the spleen in tumor-bearing mice (Figure 4B). Spleen shrinkage was previously reported in both GBM patients and murine models, reflecting systemic immune suppression mediated by brain tumors and impacting mainly T-cell subpopulations.[10] Our observation suggested that severe AAI could revert such detrimental effects. T-cell
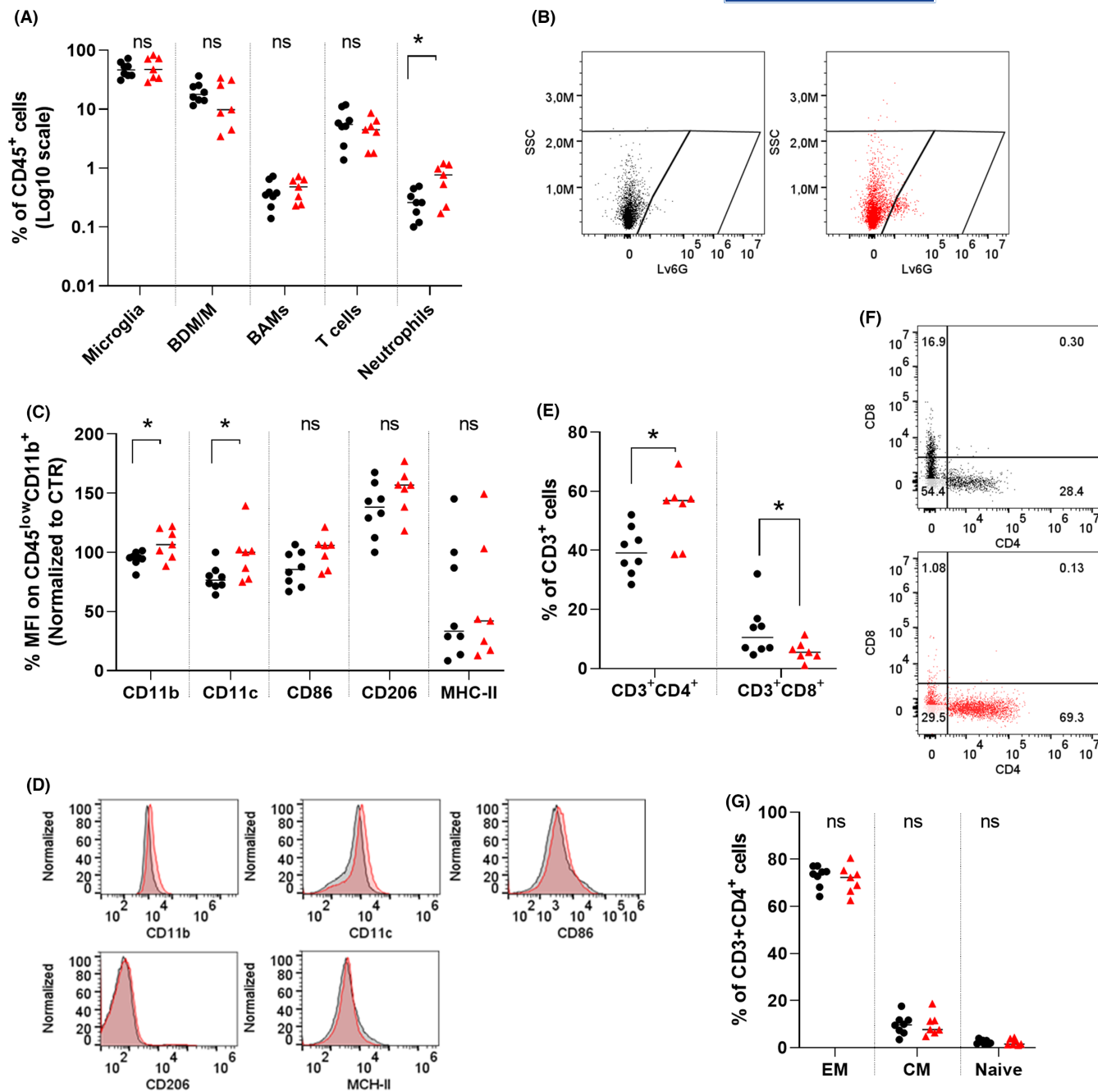
FIGURE 3 Severe AAI elevates surface expression of CD11b and CD11c on tumor-associated microglia and favors the recruitment of CD4$^+$T-cells and neutrophils in the tumor microenvironment. Brain cell suspensions from tumor-bearing mice were incubated with a multicolor panel of specific mAbs (as indicated in Table S1) for the determination of the TME's immune composition in CTR ($n = 8$) compared with HDM_50 ($n = 7$) conditions, as indicated in the supplementary materials. The gating strategy is presented in Figure S5. (A) The figure depicts the percentage of microglia, BDM/M, BAMs, T-cells, and neutrophils out of the CD45$^+$ cells in the TME of allergic mice compared with CTR. We did not observed any variation between groups, except a significant increase of TME-infiltrated neutrophils. (B) Representative picture of the dot plot for CTR (left, black panel) and HDM_50 (right, red panel) conditions, depicting gating strategy on SSC-H versus Ly6G for the detection of tumor-infiltrating Ly6G$^+$ neutrophils. (C) Phenotyping by FACS and percentage of mean fluorescence intensity (%MFI) on CD45^{low}CD11b$^+$ microglia for CD11b, CD11c, CD86, CD206, and MHC-II from CTR and HDM_50 mice, data normalized to CTR. (D) Representative histogram depicting increased expression levels of CD11b, CD11c on CD45^{low}CD11b$^+$ microglia from HDM_50 compared with CTR. (E) Modulation of CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$T-cells percentages in the TME upon AAI. (F) Representative picture of the dot plot for CTR (left, black panel) and HDM_50 (right, red panel) conditions, depicting gating strategy on CD8 versus CD4 for the detection of tumor-infiltrating CD3$^+$T-cell subsets. (G) No modulation of CD3$^+$CD4$^+$ subsets, effector memory (EM), central memory (CM), and naive in the TME upon AAI. Full symbols indicate samples collected after establishment of the tumor, where data-points from CTR are represented by black circle, and HDM_50 in red triangles.

subsets among splenocytes and bone marrow were analyzed by flow cytometry (Figure S6A–G). Following CD3⁺CD4⁺ and CD3⁺CD8⁺ gating, we determined naive, CM, and EM T-cell subsets (Figure 4C). AAI induction did not modulate the frequency (Figure 4D,E) and absolute cell counts (Figure 6H–M) of total CD4⁺ and CD8⁺T-cells (Figure 4D,E) as well as their naive and CM subsets (Figure 4F–I) in both the spleen and bone marrow of tumor-bearing mice. However, we detected a significant increase in percentage of EM CD4⁺T-cells in the spleen and bone marrow (Figure 4F,H), as well as in percentage and cell numbers of EM CD8⁺T-cells in the spleen only (Figures 4G,I and S6J–M). The increase of this T-cell subset showed a trend toward positive correlation with animal survival (Figure S6L–Q) that was significant for EM CD4⁺T-cells from bone marrow considering all groups together. Nonetheless, this association was not significant when separated by groups (Figure S6P). Our results indicate that AAI impacts glioma-mediated peripheral immunosuppression and potentially favors the circulation of EM T-cells in tumor-bearing mice.

3.5 | Adaptive immunity is required for tumor growth inhibition during allergic airway inflammation

To address the functional contribution of adaptive immunity in improving mouse survival in experimental GBM (Figure 1H), we performed experiments using RAG1⁻/⁻ mice, lacking mature T- and B-cells. Interestingly, using the same experimental procedure described in Figure 1D, the benefit of severe AAI on tumor engraftment, growth rate, and animal survival was lost in these mice (Figure 5A–C; Table S2A,B). This data implies that adaptive immunity is a necessary determinant in reducing brain tumor growth and promoting animal survival following the induction of AAI (Figure 1E–H). Overall, our results indicate that AAI limits glioma-induced systemic immunosuppression, potentially favoring tumor immune surveillance via modulation of microglia's functional phenotype toward antigen-presenting-like cells that may subsequently favor the recruitment of adaptive immune cells from the periphery.

3.6 | Identification of a microglia gene signature associated with limited immunosuppression in GBM patients

In order to determine the translational value of our findings for human GBM, we screened our ASM-DEGs for those with prognostic relevance in GBM patients taking advantage of publicly available databases. We collected clinical and transcriptomic data from patient-derived GBM tumor biopsies within the TCGA and CGGA databases (Table S4A–C). Mouse ASM-DEGs were converted to their human homologues, before carrying out Cox regression analyses (Figure S1A; Table S5A–C; Supplementary Methods). Focusing on the most robust prognostic genes shared by both databases (Table S5D) and up-regulated upon AAI, we defined 9 candidate genes (DNAJC27; ARHGEF9; NR1D2; KIAA1671; FAM169A; RALGPS1; VAMP1; DGKE; and CUX2), associated with improved prognosis of GBM patients (Figures 6A; Table S5E). Their respective biological functions as well as their reported implication in glioma biology are listed in Table S5F.

Given the potential of these specific ASM-DEGs to link allergy with the prognosis of GBM patients, we further combined them to form a gene signature (thereafter called ASM-Sign) for subsequent in silico analysis on TCGA GBM biopsy samples. GBM patients were divided into ASM-Sign^low and ASM-Sign^high groups with the enrichment score (ES) calculated for our gene signature using ssGSEA (Table S6; Figure S1B). Kaplan–Meier survival analysis confirmed that the signatures were able to stratify GBM patients based on survival, with ASM-Sign^high patients displaying improved survival (Figure 6B). The allergic status of GBM patients is not exhaustively documented in TCGA;[4,31] nonetheless, we found that 15.25% of ASM-Sign^high patients reported a history of allergy while none of the ASM-Sign^low patients did (Figure 6C).

In the next step, we analyzed the impact of the ASM-Sign^low/high signature on the molecular characteristics of GBM via DEG analysis comparing samples of GBM biopsies classified as either ASM-Sign^high or ASM-Sign^low (Figure S1B). We retrieved 817 DEGs out of which 602 were up-regulated and 215 were down-regulated (Figure 6D; Table S7A). Most top GO terms related to biological processes regulating immune cell functions, with the majority of the associated DEGs being down-regulated in GBM samples with ASM-Sign^high (Figure 6E; Table S7B). Interestingly, many of these down-regulated genes code for chemokines (CCL8; CCL13; CCL17; CXCL8; CXCL10; CSF2), secreted proteins (S100A12; S100A9), and receptors (CCR2; CD70) well known to be expressed by anti-inflammatory TAM/M favoring local immune suppression[32] (Figure S1C). Interestingly, we also found enrichment of genes promoting "positive regulation of ERK1 and ERK2 cascade" (Figure 6E), recently demonstrated to be predictive of a positive response to PD-1 blockade with TAM/M presenting elevated expression of MHC class II in GBM patients.[33]

FIGURE 4 Severe AAI reduces glioma-induced systemic immunosuppression. (A) The hemocytometer showed that white blood cells, lymphocytes, monocytes, and granulocytes were not modulated by severe AAI in whole blood of mice from CTR (n = 15) and HDM_50 (n = 14). (B) The spleen weight (in g) were compared following the experimental procedure between CTR (n = 15) and HDM_50 (n = 14) groups. (C) Splenocyte and bone marrow cells suspensions were incubated with a multicolor panel of specific mAbs for the characterization of the percentage of immune cells comparing CTR (n = 8) with HDM_50 (n = 6) groups. Dead cells, doublets, and unstable events were gated out for the determination of the percentage of total cells, naive, central memory (CM), and effector memory (EM) T-cell subtypes for both CD3⁺CD4⁺ and CD3⁺CD8⁺ subsets from (D, F, G) spleen and (E, H, I) bone marrow. Gating strategy presented in Figure S6. Data are plotted and report median. Statistical significance was determined using Mann–Whitney t-test.
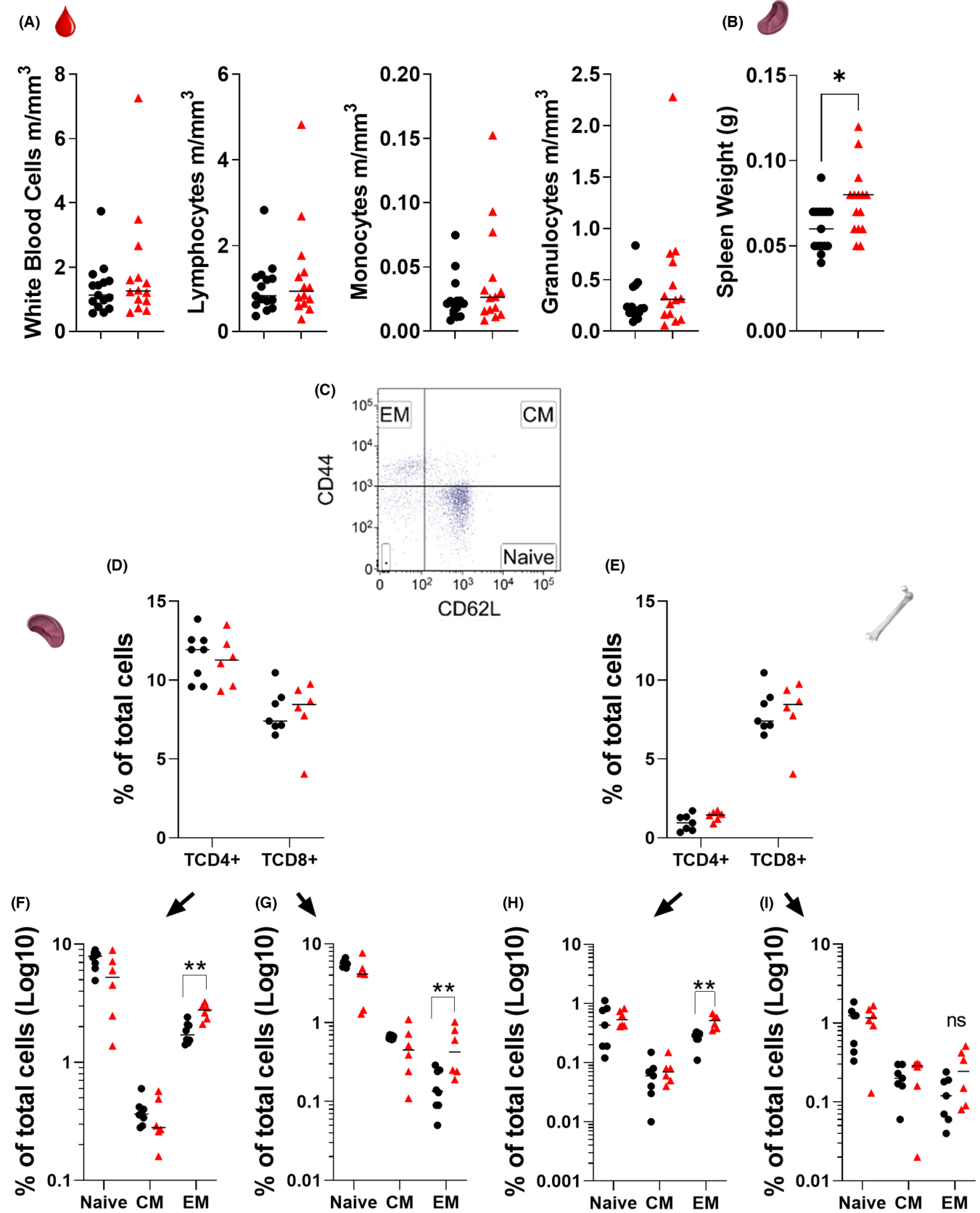
Given this inverse association between ASM-Sign and transcriptomic features, indicating local immunosuppression mediated by TAM/M, we subsequently explored its relationship with immune cell infiltration using ssGSEA (Table S7C). In silico analysis suggests that ASM-Sign[high] GBM displays lower infiltration of immune cells with known immunosuppressive functions, such as monocyte-derived macrophages (MDM), myeloid-derived suppressor cells (MDSC), mast cells and T-regulatory cells (Treg), compared with ASM-Sign[low]

tumors (Figure 6F). Accordingly, genes associated with immuno-suppression, TAM chemotactic and skewing molecules were also reduced (Figure 6F). Similarly, T-lymphocytes, natural killer (NK) cells, and dendritic cells (DC) were significantly reduced in the ASM-Sign[high] tumors (Table S7C), in accordance with previous findings showing different proportions of immune cells according to GBM subtype.[34] Interestingly, we found an increase of eosinophils in ASM-Sign-WT[high] patients (Figure 6F), in line with recent data showing eosinophil enrichment in "immunologically active," compared to "immunologically inert" GBM mouse models.[35] In summary, our in silico findings suggest that high expression of an allergy-specific gene signature is linked to reduced local immunosuppression in human GBM.

4 | DISCUSSION

We find that epidemiological and clinical data linking IgE-mediated allergy to the risk and a favorable prognosis in GBM patients can be experimentally reproduced in immunocompetent mice. To date, little experimental insight regarding the mechanisms by which AAI impacts GBM risk and outcome exists. A common hypothesis is

that allergy may favor immune surveillance.[2,3,15,16] In the present study, we provide evidence supporting this hypothesis through a parallel effect on three axes: (i) stimulation of microglial activation with (ii) subsequent increased infiltration of CD4+T-cells in the TME and (iii) limitation of glioma-induced systemic immune suppression. Moreover, we find that the impact of allergic inflammation is dose-dependent, analogous to clinical findings.[3]

Our analysis revealed a unique transcriptomic microglial repro-gramming through AAI that is related to a pro-inflammatory state, in line with previous studies, where a switch of microglia towards a pro-inflammatory state was induced by timothy grass pollen,[36] ov-albumin,[37] and fungal allergens.[38] Our data suggests that epigenetic mechanisms are relevant in the context of AAI, in analogy to a report on microglia isolated from the offspring of dams with allergic asthma.[39] The potential of microglia being activated in vivo to either a pro-inflammatory, M1-like, or anti-inflammatory, M2-like state is debated,[40] as these cells are highly plastic and were found to exhibit a large spec-trum of activation states, especially in brain tumors.[8,9,41] Several recent findings,[42–46] including ours,[19,47,48] indicate that pro-inflammatory and antigen-presentation properties of microglia contribute to improved survival in experimental models of brain tumors by favoring the es-tablishment of an anti-tumoral microenvironment. We observed that
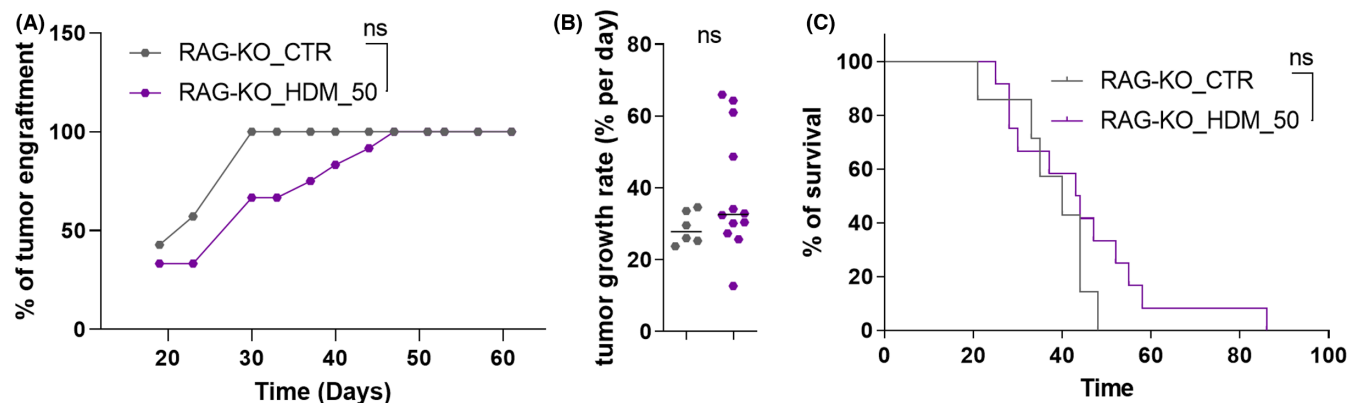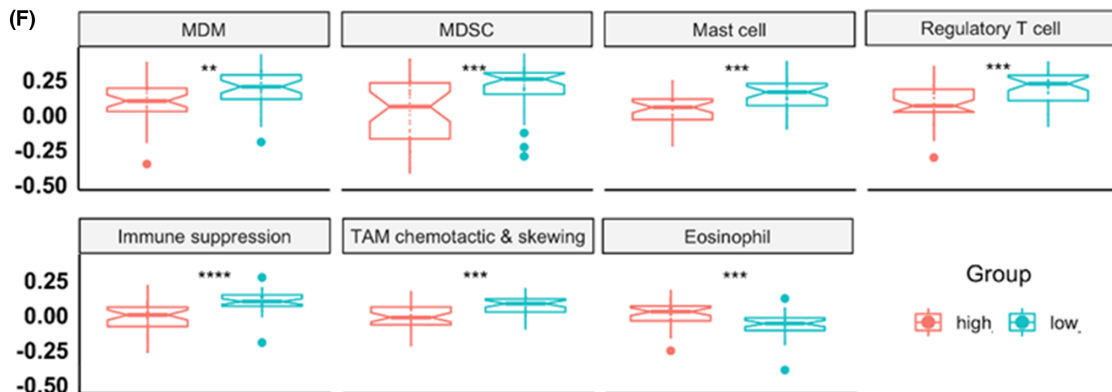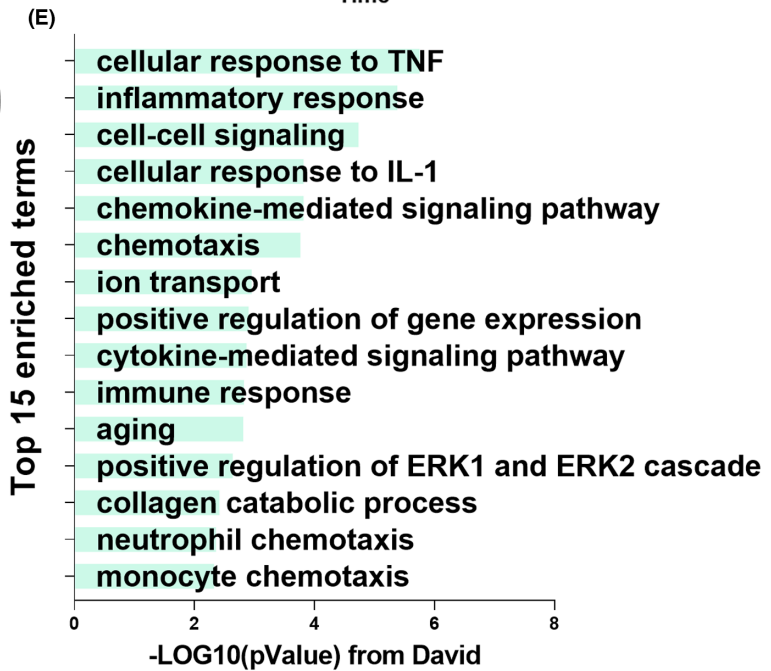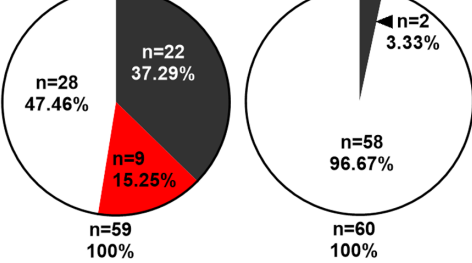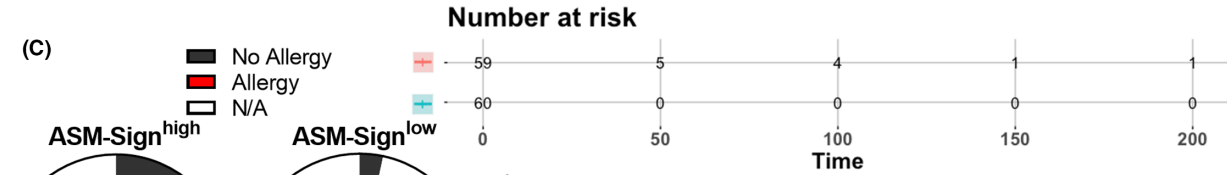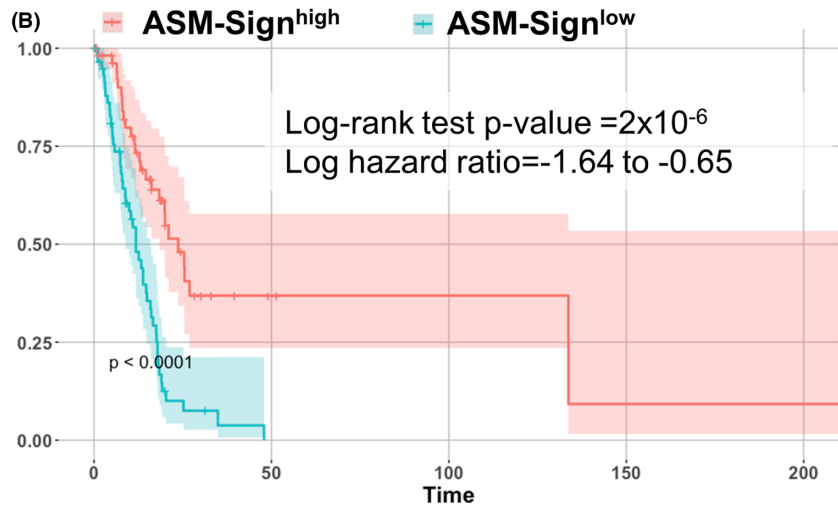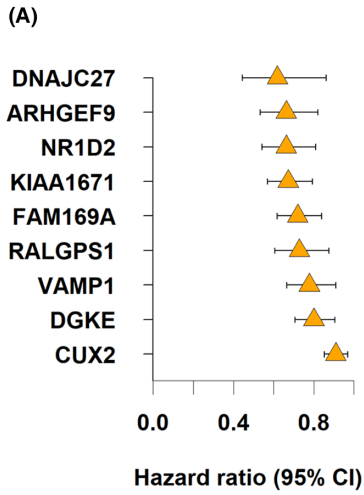

FIGURE 5 Adaptive immunity is required to favor animal survival following AAI. (A) Percentage of tumor take by groups over time. Comparison using Gehan-Breslow-Wilcoxon test. (B) Tumor growth rate monitored on RAG-KO_CTR mice ($n = 6$) and sensitized RAG-KO_HDM_50 ($n = 12$) through MRI between two time points. Data are plotted as mean ± SD, using unpaired t-test as comparison test. (C) Kaplan–Meier survival curve depicting time to a moribund state of GL261-implanted mice without (RAG-KO_CTR) ($n = 7$) or with allergic sensitization with 50 μg/NI of HDM (RAG-KO_HDM_50) ($n = 12$). Comparison of survival curves, using Log-rank (Mantel–Cox) test.

FIGURE 6 Identification of predictive signature (ASN-Sign) associated with glioma patient survival and reduced immunosuppressive microenvironment in GBM. (A) The prognostic genes were selected by significance (FDR < 0.05) and ranked by hazard ratio (orange triangle, 95% confidence intervals shown with black lines) for GBM (IDH-WT glioma). (B) Kaplan–Meier survival curves for glioma patients partitioned by Sign abundance for IDH-WT glioma. Survival curve differences were tested using a Kaplan–Meier log-rank test and significant differences are indicated. (C) Pie-charts showing the number of patients (n) and the percentage of them with reporting no allergy, allergy or with no information about history of allergy available (N/A) within ASM-Sign[high] ($n = 59$) vs. ASM-Sign[low] ($n = 60$) groups. (D) Volcano plot of DEGs between GBM samples classified as ASM-Sign[high] vs ASM-Sign-[low] using TCGA-GBM-IDH-WT dataset. Green dots represent DEGs with absolute log fold-change > 1, considering significance when adjusted FDR is <0.01. Gray dots represent genes not selected for subsequent analysis. (E) Bar plot representing the top 15 enriched GO terms for biological processes plotted in order of −log10(p value), starting with the largest. (F) Comparison of relative immune cell abundance based on ssGSEA ES in ASM-Sign[high] vs ASM-Sign[low] groups using TCGA-GBM-IDH-WT dataset. Data are plotted as Notched Box Whisker. Statistical significances were determined using unpaired t-test, and adjusted p-values are depicted for each population when significant.

(A)

DNAJC27
ARHGEF9
NR1D2
KIAA1671
FAM169A
RALGPS1
VAMP1
DGKE
CUX2

0.0   0.4   0.8

Hazard ratio (95% CI)

(B)

ASM-Sign^{high} ASM-Sign^{low}

Log-rank test p-value =2×10^{-6}
Log hazard ratio=-1.64 to -0.65

p < 0.0001

Time

Number at risk

59	5	4	1	1
60	0	0	0	0

0   50   100   150   200

Time

(C)

■ No Allergy
■ Allergy
□ N/A

ASM-Sign^{high} ASM-Sign^{low}

n=22 37.29%
n=28 47.46%
n=9 15.25%
n=59 100%

n=2 3.33%
n=58 96.67%
n=60 100%

(D)

-log10(padj)

10.0
7.5
5.0
2.5
0.0

-6 -4 -2 0 2 4 6

log2FoldChange

(E)

Top 15 enriched terms

cellular response to TNF
inflammatory response
cell-cell signaling
cellular response to IL-1
chemokine-mediated signaling pathway
chemotaxis
ion transport
positive regulation of gene expression
cytokine-mediated signaling pathway
immune response
aging
positive regulation of ERK1 and ERK2 cascade
collagen catabolic process
neutrophil chemotaxis
monocyte chemotaxis

0   2   4   6   8

-LOG10(pValue) from David

(F)

MDM   MDSC   Mast cell   Regulatory T cell

** *** *** ***

Immune suppression   TAM chemotactic & skewing   Eosinophil

**** *** ***

Group

high   low

allergy up-regulated determinants of antigen presentation on microglia before (MHC-II) and after (CD11b/CD11c) tumor engraftment, respectively, paralleled by an enrichment of CD4^{+}T-cells in the TME. Future studies will have to delineate if atopy can effectively promote microglia-mediated antigen presentation and activation to T-cells and subsequently favor specific recruitment of anti-tumor immunity. Our results suggest that the modulation of microglia before tumor onset may have the potential to limit tumor take and/or the establishment of an immunosuppressive microenvironment during tumor progression favoring specific adaptive anti-tumor immunity by effector T-cells. This is supported by our results from RAG1-KO mice, implying the adaptive immune system as a necessary determinant in promoting animal survival following the induction of severe AAI in immunocompetent mice. Further studies should delineate the relative contribution of T- versus B-cell-mediated immunity in our model. In glioma patients, systemic lymphopenia is associated with poor prognosis[10] and the pre-operative immune cell count correlates with disease progression.[30] Here, we observed an increase in circulating EM T-cells in association with animal survival. The expansion of EM T-cells has also been associated with increased survival in GBM patients treated with dendritic cell immunotherapy.[49] This T-cell subset is known as a positive prognostic marker for prolonged protective immunity against GBM.[11] Although little is known about the B-cell immunity in GBM, anti-tumor antibodies were reported to be associated with patient survival.[3-6,50]

It remains to be defined how AAI can modulate microglia physiology and whether this is a causal factor for protection in our model of highly malignant GBM, similar to the inhibition of the pro-tumoral capacity of microglia by T-cell-derived decorin in a model of experimental low-grade glioma after allergic asthma.[16] Notably, future work will have to focus on the contribution of allergy-related molecules such as IgE and IgG4/IgG1 isotypes, since antigen-specific antibodies are known for their potential to modulate macrophage polarization towards either pro-inflammatory[51-53] or anti-inflammatory[54,55] states. Similarly, microglia express all classes of Fc gamma receptors (FcγR),[56] which, following their binding with immunoglobulins, lead to inflammatory cascades of activation. We previously demonstrated that the pre-incubation with a tumor-specific mouse IgG1 of TAM/M isolated from GBM human biopsies abolished their capacity to promote tumor survival ex vivo.[19] On the contrary, it has been shown that the administration of tumor-antigen-specific IgE favors the cytolytic function of macrophages via antibody-dependent cellular cytotoxicity/phagocytosis and stimulation of TNF/MCP-1 signaling.[52,57] In addition, IgE support the re-education of alternatively activated human monocytes/macrophages.[51,53] Although microglia do not express conventional high (FcεRI) or low (CD23) affinity receptor for IgE, antigen-IgE complexes may induce their activation via their FcγRIV, as previously documented for macrophages.[58,59] On a side note, immune cells as well as epithelial cells can be directly activated by both the HDM allergens themselves as well as danger signals present in the HDM extract.[14] Although we have analyzed the implication of endotoxin/LPS, we cannot exclude that other danger molecules may have a direct or indirect role in the observed phenotypes.

Lastly, we find that 9 up-regulated ASM-DEGs have prognostic value in GBM patients, and this signature can identify a group of GBM cases with limited local immunosuppression. It will be interesting to determine if our signature might identify patients that better respond to immunotherapeutic treatments.

In conclusion, our study provides novel insight into the intricate link between IgE-mediated allergy and high-grade glioma pathogenesis and establishes a much-needed preclinical model to address the crosstalk of the brain-immune axis in the context of allergy-induced protection against GBM.

CONFLICT OF INTEREST

No conflict of interest regarding the publication of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.