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Macrophage ontogeny underlies differences in tumor-specific education in brain malignancies

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SUMMARY

Extensive transcriptional and ontogenetic diversity exists among normal tissue-resident macrophages, with unique transcriptional profiles endowing the cells with tissue-specific functions. However, it is unknown whether the origins of different macrophage populations affect their roles in malignancy. Given potential artifacts associated with irradiation-based lineage tracing, it remains unclear if bone marrow-derived macrophages (BMDM) are present in tumors of the brain, a tissue with no homeostatic involvement of BMDM. Here, we employed multiple models of murine brain malignancy and genetic lineage tracing to demonstrate that BMDM are abundant in primary and metastatic brain tumors. Our data indicate that distinct transcriptional networks in brain-resident microglia and recruited BMDM are associated with tumor-mediated education, yet are also influenced by chromatin landscapes established before tumor initiation. Furthermore, we demonstrate that microglia specifically repress *Itga4* (CD49D), enabling its utility as a discriminatory marker between microglia and BMDM in primary and metastatic disease in mouse and human.

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AUTHOR CONTRIBUTIONS

R.L.B. and J.A.J. conceived the study, designed and interpreted experiments, and wrote the manuscript. R.L.B., F.K., L.A., S.M.P., L.S., D.F.Q., S.D. and K.S. performed experiments and analyzed results. R.L.B. performed all computational analyses. E.E.G., C.I.D., C.W.B., V.T., P.H.G. provided patient samples. J.A.J. supervised the study. All authors commented on the manuscript.

Keywords

Macrophage; microglia; tumor-associated macrophages; CD49D; glioma; brain metastasis

INTRODUCTION

Macrophages are a terminally differentiated cell of the myeloid lineage, with critical functions in tissue development and homeostasis (Okabe and Medzhitov, 2015). These cells serve as a nexus between adaptive and innate immunity, regulating responses to inflammation and wound healing (Mosser and Edwards, 2008). To facilitate these diverse functions, macrophages employ considerable plasticity in response to a range of cytokines. These responses fall within a spectrum of different phenotypes ranging from classically activated pro-inflammatory macrophages to alternatively activated anti-inflammatory macrophages (Xue et al., 2014). Macrophages also possess substantial diversity and plasticity, with recent studies revealing important insights into the developmental origins of tissue-resident macrophages and uncovering tissue-specific gene expression patterns and enhancer landscapes (Gautier et al., 2012; Ginhoux et al., 2010; Gomez Perdiguero et al., 2015; Lavin et al., 2014; Mass et al., 2016).

While the local tissue environment sculpts macrophage transcriptional profiles and epigenetic states in homeostasis (Lavin et al., 2014), it is unknown whether an inflammatory tissue environment may promote differences between macrophage populations of distinct ontogenies. This is particularly relevant in cancer, where tumor-associated macrophages (TAMs) are derived from monocytes and also potentially from tissue-resident macrophages (Du et al., 2008; Pyonteck et al., 2013; Solga et al., 2015).

Brain-resident macrophages, microglia (MG), develop from erythromyeloid precursors in the yolk sac (Gomez Perdiguero et al., 2015; Kierdorf et al., 2013a; Schulz et al., 2012). Unlike other tissue-resident macrophages, during homeostasis MG undergo self-renewal and their pool is not replenished by monocytes (Ajami et al., 2007). Microglia are also resistant to myeloablative irradiation (Kennedy and Abkowitz, 1997). Indeed this property has been used extensively in bone marrow transplantation (BMT) models to distinguish radio-resistant MG from BM-derived macrophages (BMDM) (Huang et al., 2014; Sedgwick et al., 1991). However, only under conditions of blood-brain barrier (BBB) disruption, e.g. via irradiation (IR) or chemical manipulation, does there appear to be a significant contribution of BMDM to the brain macrophage pool in a non-pathological context (Bruttger et al., 2015; Mildner et al., 2007). This is relevant to brain tumors such as gliomas where there is also disruption of the BBB with disease progression (Dubois et al., 2014). IR-BMT has shown BMDM abundance in murine CNS cancers (Biffi et al., 2004; De Palma et al., 2005; Huang et al., 2014; Muller et al., 2015; Pyonteck et al., 2013); however given the current lack of markers definitively distinguishing MG and BMDM, it remains unclear if BMDM recruitment indeed occurs in brain tumors in the absence of irradiation. The need for markers distinguishing these cells is especially critical in human disease, where lineage tracing is not possible.

Here, we utilize multiple genetic lineage tracing models to demonstrate that BMDM are indeed present in murine brain tumors. Gene expression profiling showed that while BMDM

and MG share features of tumor education, they also exhibit distinct activation modes. Our data suggest these faculties are a result of inherent transcriptional networks poised before the onset of tumorigenesis, where ontogeny pre-biases cells to engage in distinct macrophage activation states. Lastly, we identify markers that distinguish MG and peripherally-derived macrophages under homeostasis, as well as in glioma and brain metastasis in both mice and humans.

RESULTS

Tumor-associated BMDM are present in mouse glioma models

To track the ontogeny of myeloid cells in murine gliomas we utilized a hematopoietic lineage tracing system, Flt3:Cre; Rosa26:mTmG, which has been used to show that peripheral myeloid cells develop from Flt3⁺ short term hematopoietic stem cells (ST-HSC), and are GFP⁺, while parenchymal MG develop independently of ST-HSC precursors, and are thus negative for the GFP reporter, remaining TdTomato⁺ (Boyer et al., 2011; Gomez Perdiguero et al., 2015). In non-tumor bearing mice, >98% of blood monocytes were GFP⁺, and <1% of MG showed recombination for the mTmG reporter (Figure 1A). The spleen was composed of GFP⁺ lymphocyte-rich follicles, surrounded by TdTomato⁺ stromal cells, while the brain parenchyma did not contain any detectable GFP⁺ cells (Figure S1A).

We next bred this line to the nestin:Tva (nTva) line to trace myeloid cell ontogeny in a genetically engineered mouse model (GEMM) of glioma. We induced gliomas by intracranial injection of DF1 cells transfected with RCAS vectors encoding PDGFB and a short hairpin against P53 (Ozawa et al., 2014) (Figure 1A), termed GEMM-shP53 herein. Flow cytometry of end-stage gliomas demonstrated that all monocytes (Cd45⁺Cd11b⁺Ly6C^{high}Ly6G⁻) and granulocytes (Cd45⁺Cd11b⁺Ly6C^{low}Ly6G^{high}) in the tumor were GFP⁺ (Figure 1B), while the bulk TAM compartment (Cd45⁺Cd11b⁺Ly6C⁻Ly6G⁻) was composed of both GFP⁺ TAM BMDM and GFP⁻ TAM MG (Figure 1A, B, Figure S1B), confirmed by tissue immunofluorescence (IF) co-staining with the pan-macrophage marker Iba1 (Figure 1C). By contrast, the contralateral, non-malignant brain contained only GFP⁻ MG, demonstrating the specific abundance of TAM BMDM only within the tumor mass (Figure 1B).

We and others have utilized IR-BMT to show that TAM BMDM are recruited to murine gliomas (Huang et al., 2014; Pyonteck et al., 2013). However, IR can lead to ectopic recruitment of BMDM to the brain and thereby increase their relative abundance (Muller et al., 2015). We verified these findings in the orthotopic, syngeneic GL261 glioma model, and found the TAM compartment was composed of both TAM MG and TAM BMDM using both IR-BMT lineage tracing and IR-independent Flt3:Cre lineage tracing (Figure S1C, D). TAM BMDM abundance was significantly increased in the IR-BMT model compared to the Flt3:Cre model (Figure S1D), reinforcing previous reports that IR-BMT can skew the ratio of MG and BMDM. Critically, however, using Flt3:Cre lineage tracing we found that BMDM composed >35% of the bulk TAM population in gliomas without IR-preconditioning, demonstrating that BMDM infiltration into tumors is not solely an artifact of IR (Figure S1D).

To exclude the possibility that this finding was due to a subset of TAM MG spontaneously upregulating *Flt3* expression, we utilized a complementary lineage tracing approach previously indicated to be specific for MG in the normal brain: *Cx3cr1:CreER-IRES YFP*; *Rosa26:lsI-TdTomato* (see Supplemental Experimental Procedures for details) (Parkhurst et al., 2013). At 3 days post tamoxifen-induced labeling, >99% of MG and circulating monocytes were TdTomato⁺ (Figure S1E). However, after 3 weeks blood monocytes no longer retained the TdTomato⁺ reporter, indicating their turnover and replenishment by tamoxifen ‘naïve’ monocytes (Figure S1E). By contrast, >99% of MG remained TdTomato⁺ (Figure S1E). We induced GL261 tumors in these mice, at 7 weeks of age, and observed both TdTomato⁺ TAM MG and TdTomato⁻ TAM BMDM (Figure 1D, E). Meanwhile, all monocytes and granulocytes were TdTomato⁻ in the tumor and periphery (Figure 1E). These findings were substantiated by IF co-staining of tissue sections with Iba1 (Figure 1F). Importantly, there was a gradient of eYFP reporter expression levels, with highest expression in TdTomato⁺ TAM MG, slightly lower levels in TdTomato⁻ TAM BMDM, and lowest levels in monocytes (Figure S1F), demonstrating the capacity of TAM BMDM to express *Cx3cr1* in brain tumors. Thus, *Cx3cr1* expression alone cannot be used to strictly identify MG in gliomas. Together, these complementary genetic lineage-tracing models show that BMDM contribute to the TAM pool in several murine models of glioma, in the absence of IR.

RNA-sequencing reveals multimodal patterns of TAM education

We next analyzed the transcriptional profiles of TAM MG and TAM BMDM in gliomas. We performed RNA-sequencing on sorted populations of TAM MG and TAM BMDM from GEMM-shP53 and GL261 tumors using the *Flt3*-based and *Cx3cr1*-based lineage tracing systems respectively. We also collected MG and Ly6C^{high} blood monocytes from non-tumor bearing *Flt3:Cre Rosa26:mTmG* mice. Global correlation analyses revealed distinct clustering of all TAM populations from normal MG and monocytes, with further cell type-specific and tumor-specific clustering (Figure 1G). As expected, monocytes were enriched for *Ly6c2* expression, while both TAM MG and TAM BMDM expressed higher levels of macrophage differentiation markers (e.g. *Aif1*, *Mertk*) compared to monocytes (Figure S1G). Normal MG and TAM MG expressed higher levels of MG-enriched genes (e.g. *Cx3cr1*, *P2ry12*, *Tmem119*) compared to monocytes and TAM BMDM (Figure S1G).

We next delineated cell type-specific, tumor-specific, and conserved patterns of tumor education among TAMs (Figure 1H). We identified differentially expressed genes between each TAM population from GEMM-shP53 and GL261 tumors compared to normal MG and monocytes (Figure 1I, Figure S1H, Table S1A). Using normal MG as the reference, we found 91 genes specifically upregulated in TAM MG from both GEMM-shP53 and GL261 models (Figure 1I, red bar), and 342 genes upregulated in TAM BMDM from both GEMM-shP53 and GL261 models (Figure 1I, green bar). We also identified genes that were specifically upregulated in TAM MG and TAM BMDM from either the GEMM-shP53 (n=102) or GL261 (n=778) models. The largest gene set (n=1383) was significantly upregulated in all TAM populations compared to normal MG (Figure 1I, orange bar). Similar patterns of expression were observed when monocytes were used as the reference population (Figure S1H, Table S1B).

Many cell cycle-related genes were upregulated, suggesting increased TAM proliferation compared to normal MG and monocytes (Table S1A,B). Indeed, we found Ki67⁺ cells in both Iba1⁺GFP⁺ TAM BMDM and Iba1⁺GFP⁻ TAM MG in the Flt3-based lineage-tracing model (Figure 1J). Conserved upregulation of complement-related factors, extracellular matrix components, proteases, lipid metabolism mediators, and clotting factors were also evident in both TAM populations (Table S1A). In addition to these programmatic changes, compared to normal MG there was upregulation of growth factors (*Igf1*, *Areg*, *Osm*), chemokines and cytokines (*Spp1*, *Ccl5*, *Cxcl9*, *Cxcl10*), and other immune modulators including *Cd274*/PD-L1 and MHC class I molecules (*H2-K1*, *H2-D1*, *B2m*) (Table S1A). A similar distribution of differentially expressed genes was evident in comparing the TAM populations from both glioma models to blood monocytes (Figure S1H, Table S1B). Interestingly, we found several MG-enriched genes (e.g. *Tmem119*, *Olfml3*, *Lag3*, *Jam2*, *Sparc*) (Gautier et al., 2012) enriched in TAM BMDM in both GL261 and GEMM-shP53 models compared to monocytes (Table S1B). Despite this difference, there was still higher expression of MG-related genes in normal MG and TAM MG than in TAM BMDM. Meanwhile, other MG-enriched genes showed no such induction in TAM BMDM (*P2ry12*, *Sall1*, *Mef2c*). Collectively, these data are consistent with Cx3cr1 upregulation specifically in gliomas (Figure S1F), and the notion that macrophages acquire tissue-resident gene expression upon infiltration into a foreign tissue (Gosselin et al., 2014; Lavin et al., 2014).

TAM BMDM and TAM MG possess distinct education patterns

We investigated transcriptional differences between TAMs derived from BMDM versus MG and identified 378 differentially expressed genes enriched in TAM MG compared to TAM BMDM in both GEMM-shP53 and GL261 models and 485 genes enriched in TAM BMDM compared to TAM MG (Figure 2A, Table S2). As expected, among the 378 TAM MG genes, we found markers previously shown to be enriched in MG compared to other macrophage populations including *P2ry12*, *Tmem119*, *Slc2a5*, *Pros1*, and *Sall1* (Figure 2A) (Gautier et al., 2012). Consistent with their tissue-specific functions, we found that normal MG and TAM MG were enriched for *Jam2* and *Ocln* (Figure S2A, Table S2), integral components of the blood-brain barrier (Liu et al., 2012). Similarly, TAM MG expressed higher levels of classical complement factors *C4b*, *C2*, and *Cfh* (Figure S2A), a pathway important for MG function in synaptic pruning and host defense (Stephan et al., 2012).

Meanwhile, TAM BMDM expressed high levels of alternative complement cascade components *Cfb* and *Cfp* (Figure S2A), and enrichment of many immune effectors including *Cd40*, *Jak2*, *Ifitm1*, *Ifitm2*, *Tlr11*, *Tlr5*, *Tlr8*, *Mefv*, and *Fas* (Figure S2A). In the GEMM-shP53 model IL-1 pathway ligands were differentially expressed, with *Il1a* enriched in TAM MG and *Il1b* in TAM BMDM, and similar trends observed in the GL261 model (Figure S2B). While *Il1r1* levels did not significantly differ, TAM BMDM expressed higher levels of the IL-1 signaling antagonist *Il1rn*, and the IL-1 decoy receptor *Il1r2* (Figure S2B). These results complement reports in non-cancer contexts demonstrating *Il1a* enrichment in MG compared to BMDM, where IL-1 signaling played a critical role in MG repopulation and maintenance (Bruttger et al., 2015).

We next interrogated chemokines, growth factors and immune modulators associated with different macrophage activation states. In addition to model-specific gene expression changes (Figure S2C, Table S3), we found in both GEMM and GL261 models that TAM BMDM were enriched for chemokines involved in wound healing including *Ccl22*, *Ccl17*, *Cxcl2*, *Cxcl3*, and *Cxcl16* (Figure 2B) (Xue et al., 2014). Interestingly, TAM MG were enriched for expression of *Ccl4* and *Tnf*, chemokines associated with a pro-inflammatory response (Xue et al., 2014). This difference in activation states was supported by a programmatic increase in antigen presentation centered around increased expression of the MHC-II master regulator *Ciita* (Reith et al., 2005), and its transcriptional targets *H2-Aa*, *H2-Dmb1*, *H2-Eb1* and *Cd74* in TAM BMDM (Figure 2C). IF staining in Flt3:Cre; GEMM-shP53 tumors revealed a marked increase in MHC-II in tumors, compared to adjacent brain, restricted to GFP⁺ TAM BMDM (Figure 2D). In addition to this antigen presentation program, costimulatory molecules such as *Cd80*, *Cd40*, and *Cd200r4* were increased (Figure S2A). These findings were further complemented by TAM BMDM-enriched expression of the Aryl-hydrocarbon receptor (*Ahr*), a transcription factor previously shown to mediate immune suppression (Murray et al., 2014; Opitz et al., 2011) (Figure S2A). Critically, we also found that the immunosuppressive cytokine *Il10* was enriched in TAM BMDM compared to TAM MG (Figure 2B). Collectively, these results suggest that TAM BMDM engage in a chronic wound healing-like state reminiscent of an alternatively-activated macrophage (Mosser and Edwards, 2008). Similar phenotypes have been shown in models of oligodendrocyte cell death, where, despite high MHC-II expression, myeloid cells did not activate a robust T cell response (Locatelli et al., 2012), suggestive of a tolerogenic program.

We next asked if the differences in inflammatory mediators were an inherent feature of BMDM upon entry into the brain or rather a consequence of tumor education. Previous studies demonstrated that when MG are depleted and the brain preconditioned by IR, BMDM can seed the brain and contribute significantly to the brain macrophage pool (hereafter termed “ectopic BMDM” in a normal “repopulated brain”) (Bruttger et al., 2015). We used this dataset for comparative analyses with our TAM BMDM and TAM MG RNA-seq data to discriminate tumor education differences from ontogenetic, non-tumor associated differences. This juxtaposition allowed us to identify genes enriched in TAM MG vs TAM BMDM as well as normal MG vs “ectopic BMDM”. These “Core MG” genes included known MG markers such as *Jam2*, *Siglech* and *P2ry12*, but also complement factors *C2*, *C4b*, and *Cfh*, as well as the pro-inflammatory cytokines *Ccl4* and *Tnf* (Figure S2D, n=245 genes, Table S4). We identified genes enriched in TAM BMDM (n=294) specifically in the context of a tumor, including *Il10*, *Cxcl2*, *Cxcl3*, *Ccl17*, *Ccl22*, and *H2-Dmb1* (Figure 2E). In contrast to this tumor-specific expression profile, there were also 164 “Core BMDM” genes enriched in BMDM compared to MG, regardless of the presence or absence of a tumor, including *Ciita*, *Ahr*, *Runx2*, *Runx3*, *Vav3* and *Vdr* (Figure 2E, Table S4). These data indicate some features distinguishing TAM BMDM and TAM MG are inherent to their differential ontogenies, while others are only acquired upon interaction with, and education by, the tumor microenvironment.

As many of the “Core BMDM” genes are central players in innate immunity, we queried the immunological genome project database to determine if these genes were overrepresented in any particular myeloid cell population. Interestingly, we found that these genes were actually

repressed in MG compared to tissue-resident macrophages of the BM, spleen, lung, peritoneum, small intestine, and monocyte progenitors (Figure 2F). Meanwhile “Core MG” genes were indeed enriched in MG compared to other myeloid cells (Figure 2F). These data suggest that “Core BMDM” genes are neither specifically enriched in TAM BMDM nor macrophages in general, but rather are specifically repressed in MG.

Recent studies have highlighted extensive epigenetic diversity amongst tissue-resident macrophages (Lavin et al., 2014); thus we hypothesized that the MG-repressed genes may be epigenetically altered in MG compared to even the distantly related monocytes. Indeed, when we analyzed these published datasets, we observed increased H3K27 acetylation in the promoters of normal monocytes compared to normal MG for the “Core BMDM” genes (Figure S2E). Similarly, there was increased H3K27 acetylation in the promoters of “Core MG” genes in MG compared to monocytes (Figure S2E). Enhancer specification and epigenetic states in MG and other macrophage populations have been associated with differential PU.1 occupancy. Interrogating previously published data (Gosselin et al., 2014), we observed that several macrophage subsets (including BMDM) all showed increased PU.1 binding at the promoters of our “Core BMDM” genes compared to normal MG (Figure S2F). Meanwhile, variability in PU.1 occupancy was minimal at the promoters of “Core MG” genes (Figure S2F). Similar binding dynamics were evident in enhancer elements, where PU.1 occupancy in enhancer regions of “Core BMDM” genes was higher in BMDM than MG, with less pronounced differences present in “Core MG” genes (Figure S2G). Thus, epigenetic landscapes established before the development of a tumor may play a role in regulating differential activation patterns subsequently observed in malignancy.

Identification of transcription factor networks underlying TAM activation

Given the epigenetic differences in the non-malignant setting, we next determined if chromatin states also differed between TAM BMDM and TAM MG. We performed Assay for Transposase-Accessible Chromatin (ATAC)-sequencing (Buenrostro et al., 2013) to assess chromatin accessibility in TAM BMDM and TAM MG sorted from the GL261 model (Figure 1D). We found the ATAC-seq signal was associated with cell type-specific gene expression. In TAM BMDMs, the promoters of Core BMDM and TAM BMDM genes had higher ATAC-seq signal than Core MG and TAM MG genes, while TAM MG promoters of Core MG and TAM MG genes had a higher ATAC-seq signal than Core BMDM and TAM BMDM genes (Figure S3A). Within enhancers and intronic elements of these gene sets we identified 120 BMDM-specific peaks in TAM BMDM genes including *Vav3*, and 704 MG-specific peaks in TAM MG genes including *P2ry12* and *Sall1* (Figure 3A, Figure S3B,C, Table S5A).

We analyzed the transcription factor (TF) landscape underlying these different peaks, and performed *de novo* motif analysis (motifs are shown in all capital letters). Motif analysis of these peaks revealed an enrichment of FOS/JUN and PU.1 binding sites in both TAM BMDM and TAM MG peaks (Figure 3B, Table S5B), reinforcing previous analyses demonstrating the critical role of PU.1 in establishing specific enhancer landscapes in tissue-resident macrophages (Gosselin et al., 2014). Besides these shared enrichments, we found

TAM BMDM peaks enriched for RUNX and CREB/bZIP motifs, while TAM MG peaks were enriched for SMAD3 and MEF2A motifs (Figure 3B).

To determine if these motifs reflected pathway activation of particular TFs we modeled the expression of their predicted downstream targets (see Supplemental Experimental Procedures). We identified TF families with enriched activity in TAM BMDM relative to TAM MG in the GEMM-shP53 and GL261 models (and vice versa) (Figure 3C, Figure S3D). Among a panel of different TFs, EGR1 and MEF2A were enriched in TAM MG (Figure 3C, Figure S3D, Table S5C). Interestingly, MEF2 is associated with MG identity (Lavin et al., 2014). In TAM BMDM, TF motifs involved in monocyte to macrophage differentiation were enriched including RUNX, CEBP, and PU.1 (Figure 3C, Figure S3D) (Alder et al., 2008). STAT3 and IRF4 were also enriched (Figure 3C), both of which have been associated with differential functions in macrophage activation (Mosser and Edwards, 2008; Ostuni and Natoli, 2011). We complemented these genome-wide TF activity analyses with motif enrichment analysis on the promoters of TAM BMDM-specific and TAM MG-specific genes using HOMER (Heinz et al., 2010) (Figure S3E). This also revealed an enrichment of MEF2 motifs in TAM MG, demonstrating the consistent role of tissue-specific transcriptional programs in TAM MG education. Meanwhile, TAM BMDM-specific genes were again enriched in PU.1, RUNX and CEBP motifs (Figure S3E). These findings were further corroborated by increased expression of brain-specific TFs (*Mef2c*, *Sall1*, *Sall3*) in TAM MG, while TAM BMDM were enriched for *Ciita*, *Vdr*, *Ahr*, and *Runx* family members (Figure 3D, Table S2A).

Given the consistent enrichment of RUNX activity in TAM BMDM, we next focused on examining the expression and chromatin state of *Runx* family members. *Runx2* and *Runx3* were enriched in TAM BMDM compared to TAM MG (Figure 3D). While no differences were found in the chromatin state of *Runx1* or *Runx2*, in the first intron of *Runx3* (Figure 3E-ii) we observed a peak present in TAM MG but reduced in TAM BMDM (Figure 3E). Meanwhile, the *Runx3* promoter showed little open chromatin in TAM MG, and a distinct peak in TAM BMDM near the transcription start site (Figure 3E-i). Interestingly, both peaks have been shown to be TGF β -responsive PU.1 binding sites associated with *Runx3* expression (Chopin et al., 2013), indicating the same signal transduction pathway can produce distinct outputs in TAM BMDM and TAM MG.

We also identified enrichment of the epigenetic modifiers *Hdac7* and *Hdac9* in TAM BMDM, while *Hdac11* was enriched in TAM MG (Figure S3F), the latter of which has been shown to repress *I110* expression in macrophages (Villagra et al., 2009). Interestingly, an upstream enhancer element in *Hdac11* was significantly enriched in TAM MG compared to TAM BMDM, a peak that contained a SMAD-responsive element (Figure 3F-i). Collectively, these results suggest that differential genomic PU.1 occupancy underlies distinct open chromatin states in BMDM and MG, whereupon additional factors such as TGF β /SMAD signaling and RUNX family members cooperate with PU.1 to enforce distinct transcriptional networks. Subsequent regulation of TFs and chromatin modifying factors, such as *Hdac11*, may explain the distinct cytokine expression patterns observed such as TAM BMDM expression of *I110*, and TAM MG expression of *Tnf*.

***Itga4*/Cd49d distinguishes microglia and peripherally-derived macrophages in murine models of brain malignancy**

We next sought to identify tools capable of distinguishing TAM BMDM and TAM MG in human disease, where genetic lineage tracing is not possible. Given that TAM BMDM in gliomas upregulated *Cx3cr1* (Figure S1F), a proposed MG marker, we sought to identify TAM BMDM-specific markers that instead remained silent in TAM MG. From the 164 “Core BMDM” genes, we identified 40 candidate transmembrane proteins that might serve as useful markers for flow cytometry. Amongst these, the integrin subunit alpha 4, *Itga4*/Cd49d, emerged as a promising candidate, particularly given previous reports that it, along with the integrin subunit alpha L, *Itgal*/Cd11a, is regulated by RUNX family members, including *Runx1* and *Runx3* (Dominguez-Soto et al., 2005). Consistently, we found that *Itga4* and *Itgal* were specifically repressed in MG compared to other macrophage populations (Figure S4A). This was confirmed by flow cytometry, where Cd49d expression in MG was negligible or absent compared to macrophages of the spleen, liver, lung, bone marrow and blood Ly6C⁺ monocytes (Figure 4A). Ly6G⁺ granulocytes were also Cd49d⁻, which, along with Cd49d⁺ lymphocytes in the CD45⁺Cd11b⁻ gate, served as useful gating controls in subsequent experiments (Figure 4A).

We examined Cd49d and Cd11a expression in TAM BMDM and TAM MG using Flt3:Cre-based lineage tracing in the GEMM-shP53 model. After gating on Cd45⁺Cd11b⁺Ly6C⁻Ly6G⁻ cells, the normal brain only contained Cd45^{low}Cd49d⁻ cells, and all peripheral monocytes were Cd45^{high}Cd49d⁺ (Figure 4B). In tumors we found two cell populations; Cd45^{low}Cd49d⁻ and Cd45^{high}Cd49d⁺, which contained GFP⁻ TdTomato⁺ MG and GFP⁺ TdTomato⁻ BMDM respectively (Figure 4B). Similar results were found for Cd11a (Figure 4B), and were replicated in the GL261 model using both *Cx3cr1*-based and Flt3:Cre lineage tracing strategies (Figure S4B, C). Lastly, we evaluated Cd49d expression in a *Pten* loss of function-PDGFB-driven glioma model (GEMM-*Pten*^{Fl^{ox}}) where *Pten*^{Fl^{ox}/Fl^{ox}}; nTva⁺ mice were injected with RCAS vectors encoding PDGFB and Cre (Huse et al., 2009). Using IR-BMT for lineage tracing, we found that Cd49d distinguishes donor and host-derived cells, including in glioma models with extended latency (~12 weeks for the GEMM-*Pten*^{Fl^{ox}} model) (Figure S4D).

To evaluate other models of brain malignancy, we utilized an intracardiac injection model of brain metastasis (BrM) colonization using a tumor cell line (99LN-BrM). 99LN-BrM cells were originally derived from the lymph node of a MMTV:PyMT breast cancer GEMM and subjected to *in vivo* selection. We used this syngeneic, immunocompetent BrM model in conjunction with *Cx3cr1*-based lineage tracing and found that BrM lesions contained both TdTomato⁺ Iba1⁺ and TdTomato⁻ Iba1⁺ cells indicating recruitment of both TAM MG and TAM BMDM respectively (Figure 4C, D). We validated these findings by flow cytometry, where Cd49d and Cd11a served as reliable markers of BMDM as in the glioma models described above (Figure 4E). We again found that eYFP levels, a direct readout of *Cx3cr1* expression, were similar between TAM BMDM and TAM MG in BrM, reinforcing the necessity of the *Cx3cr1*:CreER lineage tracing approach over that of the *Cx3cr1* reporter (Figure S4E). Lastly, we confirmed these data in a well-established xenograft BrM model using brain homing MDA-MB-231 cells (Bos et al., 2009), in conjunction with IR-BMT

lineage tracing using mRFP⁺ donor cells. In this model, we identified two cell populations; Cd45^{low}Cd49d⁻ MG and Cd45^{high}Cd49d⁺ BMDM. The mRFP⁺ donor cells were exclusively found within the Cd45^{high}Cd49d⁺ BMDM gate (Figure S4F).

Together, our results obtained in multiple models of brain malignancy with distinct lineage tracing approaches demonstrate that TAM BMDM accumulation is independent of BBB preconditioning by IR or intracranial injection. These data also thoroughly establish Cd49d as an efficient marker to distinguish resident MG and peripherally-derived macrophages in homeostasis as well as in primary and metastatic brain malignancies.

CD49D identifies microglia and macrophages in human brain malignancies

We next investigated whether CD49D could be used to discriminate MG and peripherally-derived macrophages in human brain tumors. We assessed CD49D expression by flow cytometry across a panel of surgical samples composed of nonmalignant normal brain (n=3), untreated high-grade glioma (GBM) (n=3), lung adenocarcinomas (n=6), and peripheral blood mononuclear cells (PBMCs) (n=6). Consistent with our data in mice, granulocytes (CD45⁺CD11B⁺CD66B⁺CD14^{low}CD16⁺) did not express CD49D, and were used as a reference guide for gating CD49D⁺ and CD49D⁻ TAMs (Figure S5A). Importantly, we never identified CD49D⁻ TAMs in primary lung tumors, or CD49D⁻ monocytes in healthy donor PBMCs, indicating that, as predicted, low expression of CD49D is restricted to MG, and not a general phenotype of tissue-resident macrophages (Figure 5A). By contrast, the CD45⁺CD11B⁺CD66B⁻CD14⁺CD16⁻ compartment in non-malignant brain was predominantly composed of CD49D⁻ MG (Figure 5A). Critically, in each GBM sample we identified both CD49D⁺ and CD49D⁻ TAMs, presumably representing BMDM and brain-resident MG respectively (Figure 5A).

Interestingly, in human samples we found no difference in CD45 expression between CD49D⁻ and CD49D⁺ TAMs (Figure 5B), a marker previously suggested to be informative for distinguishing BMDM and MG in brain malignancy (Hussain et al., 2006; Parney et al., 2009; Sedgwick et al., 1991). Indeed, CD45 expression differed most prominently between granulocytes and TAMs, as opposed to MG and BMDM (Figure 5B). However, this lack of differential CD45 expression is not the case in mouse, where Cd45 adequately discriminates MG and BMDM in the models tested (Figure 5B). We next sorted paired CD49D⁻ and CD49D⁺ TAMs from GBM patients to verify these populations indeed reflected TAM MG and TAM BMDM respectively. Using genes specific for TAM MG and TAM BMDM from our mouse models (Figure 2A), we found that CD49D⁻ TAMs were indeed enriched for TAM MG genes ($p = 7.78 \times 10^{-3}$), while CD49D⁺ TAMs were enriched for TAM BMDM genes ($p = 5.01 \times 10^{-3}$) (Figure 5C).

Previous analyses of TAM expression in human gliomas have utilized bulk CD11B⁺ cells, a population likely composed of both TAM BMDM and TAM MG, as well as other myeloid populations. We queried one available RNA-seq dataset from bulk CD11B⁺ cells (Szulzewsky et al., 2016), which showed increased *ITGA4*/CD49D expression in purified CD11B⁺ cells in GBM compared to normal MG from either post-mortem samples, or resections from epileptic patients (Figure S5B). This was complemented by a relative decrease in the MG-enriched transcript *P2RY12* in GBM compared to nonmalignant brain

(Figure S5B). In querying an additional microarray-based dataset of purified CD11B⁺ cells (Gabusiewicz et al., 2016), we observed that peripheral blood CD11B⁺ cells from GBM patients expressed similar levels of *ITGA4* compared to GBM tumor samples, while there was higher *P2RY12* expression in GBM samples compared to peripheral blood (Figure S5C), as we would have expected. We extended these analyses to whole tissue RNA-seq data from the TCGA-GBM cohort (Brennan et al., 2013), and observed that *ITGA4* expression was significantly increased in GBM compared to normal brain (Figure S5D). Collectively, these analyses suggest that TAMs in GBM represent a heterogeneous population composed of both BMDM and MG, reinforcing the necessity of refined sorting strategies for accurate discrimination between these cells, and highlighting the utility of a CD49D-based gating approach.

We next assessed TAM BMDM and TAM MG gene set expression in the TCGA cohort as a whole. TAM BMDM genes and TAM MG genes showed high intra-gene set correlation, where TAM BMDM genes such as *RUNX2*, *IL10*, *RUNX3*, *ITGA4* and *VDR*, showed significant pairwise correlations, and TAM MG genes such as *MEF2C*, *P2RY12*, *RXRG*, *SALL1*, *KLF12*, and *SALL3* similarly showed significant pairwise correlations (Figure S5E). Moreover, *ITGA4* showed a high correlation with a TAM BMDM gene signature score ($p = 2.2 \times 10^{-16}$), but not with a TAM MG signature score (Figure 5D), showing increased *ITGA4* expression is specific to TAM BMDM abundance, and not TAMs as a whole.

Previous transcriptional and epigenetic analyses have identified distinct GBM subtypes (Noushmehr et al., 2010; Verhaak et al., 2010), where the Mesenchymal subtype was enriched for tumor stroma and inflammatory molecules. Here, we find TAM BMDM signature scores are significantly different among molecular subtypes of GBM ($p = 2.2 \times 10^{-16}$), with the highest scores in the Mesenchymal GBM subtype, and lowest in G-CIMP patients (Figure 5E). Correspondingly, TAM BMDM signature scores were lowest in patients with *IDH1* mutations (Figure 5F, $p = 5.93 \times 10^{-3}$). By comparison, TAM MG signature scores displayed a blunted association with tumor subtype ($p = 0.041$) and no association with *IDH1* mutation status ($p = 0.153$) (Figure S5F, G). These analyses reinforce our findings that TAM BMDM are a distinguishable immune cell population from TAM MG with distinct abundance and characteristics in specific subtypes of human GBM.

DISCUSSION

IR-BMT has been used widely in animal models to perform lineage tracing of TAMs in brain malignancy (Ajami et al., 2007; De Palma et al., 2005; Huang et al., 2014; Mildner et al., 2007; Muller et al., 2015), albeit with concerns regarding potential artifacts due to effects of IR on BBB disruption. Alternative chemical BMT approaches have been suggested, though similar effects on BBB permeability cannot be ignored (Alder et al., 2008; Kierdorf et al., 2013b). Here, we confirm that IR-BMT leads to increased TAM BMDM content in the GL261 glioma model, a finding that has been recently reported by juxtaposing IR-BMT with and without head-shielding (Muller et al., 2015). While IR-BMT may confound lineage-tracing studies, it remains to be seen if IR preconditioning before the onset of tumorigenesis

significantly alters TAM activity in tumor development, or if the inflammatory environment of the tumor supersedes any antecedent effects of the IR-BMT protocol.

Other than IR-BMT, the most widely employed approach to discriminate MG and peripherally-derived macrophages relies upon Cd45 expression, with Cd45^{high} cells considered BMDM and Cd45^{low} cells considered MG (Gabrusiewicz et al., 2011; Sedgwick et al., 1991). While this marker seems adequate in the murine models we have employed here, cell type-specific CD45 expression appears to be different between mouse and human. Our data indicate that CD45 does not accurately discriminate MG and BMDM in patient samples, emphasizing the need for extensive flow cytometry panels to clearly distinguish these cells in both species. Additionally, our genetic lineage tracing models also show that expression of *Cx3cr1*, which is commonly used to trace normal MG, is subject to upregulation in BMDMs upon tumor education (Figure 2A, Figure S1F, Figure S4E) and thus cannot be used to discriminate MG versus BMDM in brain tumors.

Instead, we present *Itga4* (Cd49d) as an effective, consistent marker that works in both mice and humans to distinguish MG and peripherally-derived macrophages in multiple brain malignancies. Cd49d may also prove a useful tool in determining the precise origin and kinetics of peripherally-derived macrophages in brain tumors. Recent efforts to understand the heterogeneity and origins of non-parenchymal myeloid cells in the brain (including perivascular, meningeal and choroid plexus macrophages) revealed that a subset of these cells are labeled using similar Flt3-Cre and Cx3cr1-CreER based lineage tracing systems as employed here (Goldmann et al., 2016). Thus it will be of interest to determine if any of these populations, in addition to monocytes, contribute to the TAM pool.

Our data supports the hypothesis that epigenetic states influence stimulus-dependent transcriptional induction, thus leading to differential TAM education between MG and BMDM. Differential genomic occupancy of PU.1 between MG and other macrophage populations in non-cancer contexts has been shown to dictate differential enhancer selection (Gosselin et al., 2014). Indeed, within this dataset we found that PU.1 binding sites at enhancers and promoters were already different between MG and BMDM for the genes we identified to be specific to their respective TAM populations. This suggests that TAM BMDM and TAM MG are poised to engage in different transcriptional networks based on initial enhancer selection. It is likely that differential expression of binding partners influences PU.1 genomic occupation. Cooperative binding is evident between PU.1 and CEBP β to promote macrophage differentiation, and in B cell development where PU.1 occupancy is influenced by E2A expression (Heinz et al., 2010). Such a hypothesis has also been shown to account for MG-specific PU.1 binding in cooperation with TGF β -induced SMAD activity (Gosselin et al., 2014). Similar dynamics may be at play in brain tumors, where binding partners that are absent in MG and expressed in BMDM can sculpt genomic PU.1 occupancy. For example, the RUNX family member *Runx3* is one such candidate, which is enriched in TAM BMDM versus TAM MG and shows motif enrichment in promoters where PU.1 binds in BMDM, but not MG.

While our studies here focus predominantly on identifying recurrent signatures distinguishing TAM MG and TAM BMDM across multiple mouse models and patient

samples, there were also tumor-specific gene expression patterns in TAM education (Figure 2E, Figure S2C,D), which may provide insights into how tumor-derived signals can generate inter-tumoral heterogeneity in TAM activation profiles. In addition, analysis of TCGA data showed that gene signatures associated with TAM BMDM were differentially enriched in the distinct tumor subtypes of GBM. Recent reports have identified mixed activation states in bulk TAM populations in glioma patients (Gabrusiewicz et al., 2016; Szulzewsky et al., 2016), and our data now shows that TAM MG and TAM BMDM possess distinct activation states, potentially resolving this mixed phenotype. Importantly, the identification of CD49D as a cell surface marker to discriminate between TAM MG and TAM BMDM in human disease will permit extensive interrogation of these cell populations in patient samples.

Collectively, the studies presented here definitively demonstrate that peripherally-derived macrophages are indeed present in multiple mouse and human brain malignancies, and have distinct transcriptional profiles from their brain-resident counterparts. We posit that while macrophages can acquire tissue-resident macrophage-like traits upon entry into a tissue (Lavin et al., 2014), an inflammatory microenvironment, such as in the context of cancer or neuroinflammation, may further amplify differences between the cells leading to diverse functional outcomes for tissue-resident and peripherally-derived macrophage populations.

EXPERIMENTAL PROCEDURES

Tumor and lineage tracing models

Mouse models of gliomagenesis and brain metastasis, cell line generation, and the use of lineage tracing models have been previously reported (Boyer et al., 2011; Parkhurst et al., 2013; Pyonteck et al., 2013; Sevenich et al., 2014) and are described in full in Supplemental Experimental Procedures.

Institutional Review Board (IRB) approval and patient information

All human specimens were collected from patients consented to MSKCC IRB protocols #06-107, #14-230. Glioma patients that presented with contrast-enhancing brain lesions and no prior history of brain malignancy or therapy were included. Tumor specimens were collected from the operating room and processed as described below. Pathological analyses confirmed grade IV GBM. Non-malignant normal brain samples were collected from two sources: non-malignant distant sites from low-grade disease, and post-mortem samples with no history of brain malignancy. Pathological analysis confirmed the absence of tumor. Samples from patients with primary lung tumors were included based on pathological analysis of lung adenocarcinoma, with no screening based on prior malignancy or therapy.

Flow cytometry and cell sorting

For blood analysis, mice were bled via either retro-orbital or submandibular routes under isoflurane anesthesia. For all other tissue analyses, mice were anesthetized with 1.25% avertin, and transcardially perfused with PBS. Single cell suspensions from spleen and BM were isolated by macrodissection and mechanical tissue dissociation. Liver, kidney, and lung were macrodissected and dissociated using the Mouse Tumor Dissociation Kit (mTDK, Miltenyi) and the OctoMACS dissociator. Mouse and human brain specimens were

macrodissected and dissociated using the Brain Tumor Dissociation Kit (BTDK Miltenyi) and a single cell suspension generated using the OctoMACS dissociator. Human lung tumors were dissociated with the Human Tumor Dissociation Kit (hTDK Miltenyi.) All tissue suspensions were filtered through a 40 μ M mesh filter and underwent red blood cell lysis (PharmLyse BD). Brain and brain tumor tissues were incubated with Myelin Removal Beads (Miltenyi). Single cell suspensions were FC blocked (BD #553141) for 15 mins at 4°C, then incubated with directly-conjugated antibody panels for 15 mins at 4°C. Cell suspensions were washed (PBS +2% fetal bovine serum) and resuspended in a DAPI solution. All flow cytometry analysis was completed on a BD Fortessa device and all sorting was performed on an Aria III. Cells were sorted directly into Trizol LS and snap frozen in liquid nitrogen. Antibodies and methods for immunohistochemistry can be found in Supplemental Experimental Procedures.

Statistical methods

RNA-sequencing, ATAC-sequencing and bioinformatics—RNA was isolated by chloroform extraction and isopropanol precipitation. RNA-sequencing libraries were generated with the SMART-Seq preparation kit (CloneTech). Single end, 100 base pair, sequencing was performed by GeneWiz (New Jersey, USA) on an Illumina HiSeq 2500. FASTQ files were mapped to the mouse genome (mm10) or the human genome (hg19) using STAR (version 2.5.0e) with default parameters (Dobin et al., 2013). Transcript abundance was quantified using STAR with a GTF file from iGenomes (Illumina). A count matrix was produced in R and differential gene expression was assessed with DESeq2 using a fold change cutoff of ± 2 and a false discovery rate of 5% (Love et al., 2014). Gene ontology analysis was performed using DAVID with default parameters (Dennis et al., 2003). ATAC-Seq was performed as previously described (Buenrostro et al., 2013). Paired end, 50 base pair sequencing was performed on an Illumina HiSeq 2500 with an average read depth of ~35,000,000 reads per sample. Reads were mapped to mm10 using STAR (version 2.5.0e) using (`-alignIntronMax 1 -alignEndsType EndToEnd`). Peak calling, annotation, and differential peak identification was performed using HOMER.

Methods for analyzing external datasets, TF activity analysis, and additional statistical methods are described in Supplemental Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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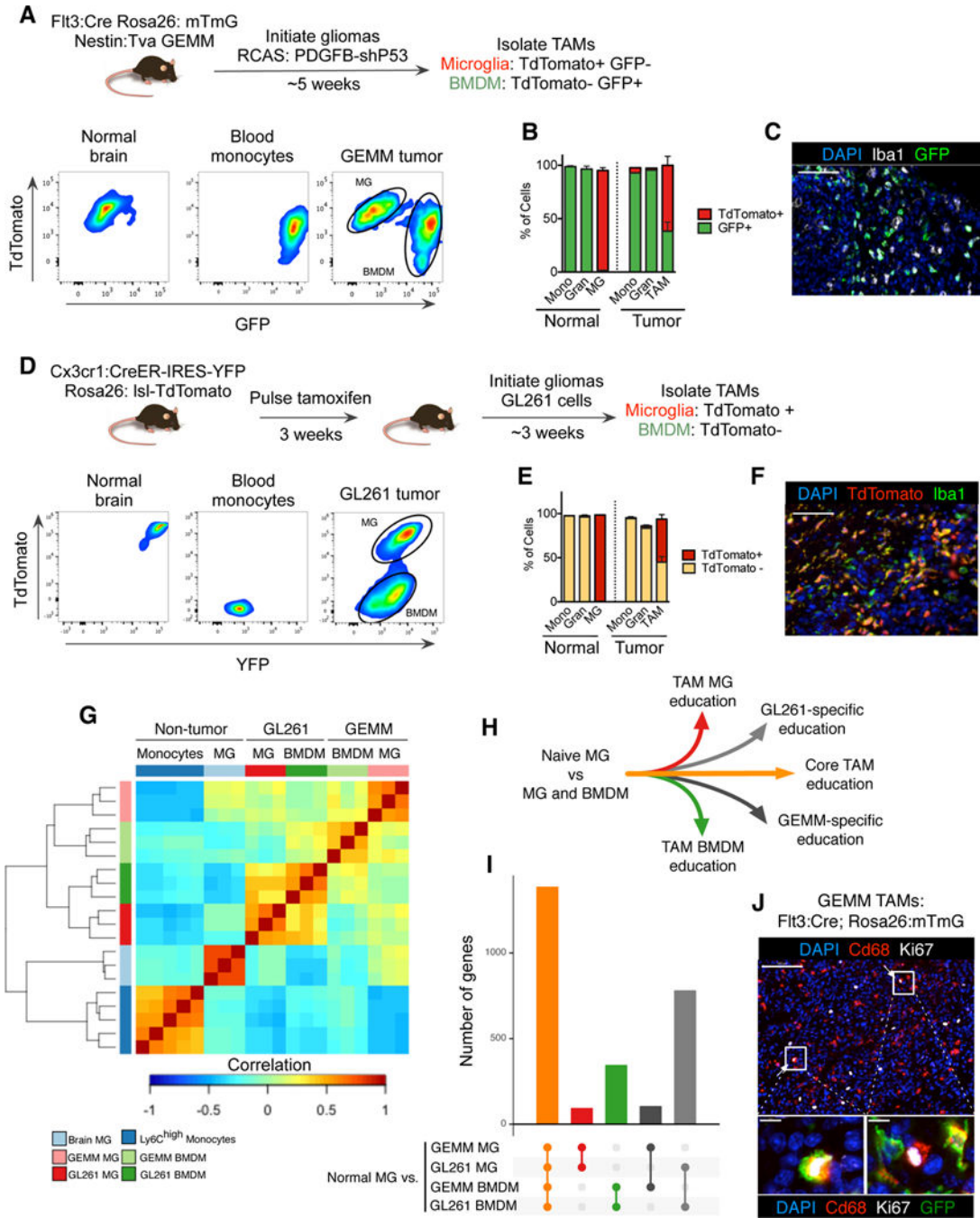


Figure 1. Lineage tracing systems demonstrate heterogeneity in TAM ontogeny in multiple models of glioma

(A) Experimental scheme for the GEMM-shP53 model, see Supplemental Experimental Procedures for details. Representative flow cytometry panels for TdTomato and GFP are shown for Cd45⁺Cd11b⁺Ly6G⁻Ly6C⁻ microglia (MG), Cd45⁺Cd11b⁺Ly6G⁻Ly6C⁺ monocytes, and Cd45⁺Cd11b⁺Ly6G⁻Ly6C⁻TAMs from GEMM-shP53 gliomas. (B) Quantitation of TdTomato⁺ and GFP⁺ monocytes (Mono) and granulocytes (Gran) in peripheral blood, MG in non-tumor bearing brain, and monocytes, granulocytes and TAMs

in GEMM-shP53 gliomas as depicted in (A). Bars represent mean and s.e.m. n=3–5 for each group. (C) Representative immunofluorescence (IF) staining of Iba1 (white), GFP (green) and DAPI (blue) in a GEMM-shP53 tumor as depicted in (A). Scale bar: 50 μ m. Representative of n=5 tumors. (D) Experimental design for Cx3cr1 lineage tracing model, see methods for details. Monocytes, MG and TAMs were isolated as described in (A), and evaluated for TdTomato and YFP reporter expression. Representative of n=3 mice. (E) Flow cytometry quantitation of TdTomato⁺ and TdTomato⁻ monocytes and granulocytes in peripheral blood, MG in non-tumor bearing brain, and monocytes, granulocytes and TAMs in GL261 gliomas as depicted in (D). Bars represent mean and s.e.m. n=3 for each group. (F) Representative IF staining for Iba1 (green), TdTomato (red) and DAPI (blue) in a GL261 tumor. Scale bar: 50 μ m. (G) Pairwise correlation matrix of normalized RNA-seq counts from monocytes (n=5), normal MG (n=3), and the four TAM populations from the different models: GEMM-shP53 TAM MG, GEMM-shP53 TAM BMDM, GL261 TAM MG, and GL261 TAM BMDM (n=3 for each group). (H) Diagram depicting different modules of TAM education compared to normal MG. (I) Differentially expressed genes between normal MG and the four TAM populations were tabulated. Barchart depicts the number of differentially expressed genes shared between the different groups, (J) Representative IF staining of Ki67⁺ TAM BMDM and TAM MG in the GEMM-shP53 model as depicted in (A). Ki67 (white), CD68 (red), DAPI (blue), GFP (green) (omitted from top panel). Scale bars: 100 μ m on upper panel, 10 μ m on lower panels. Representative of n=5 tumors.

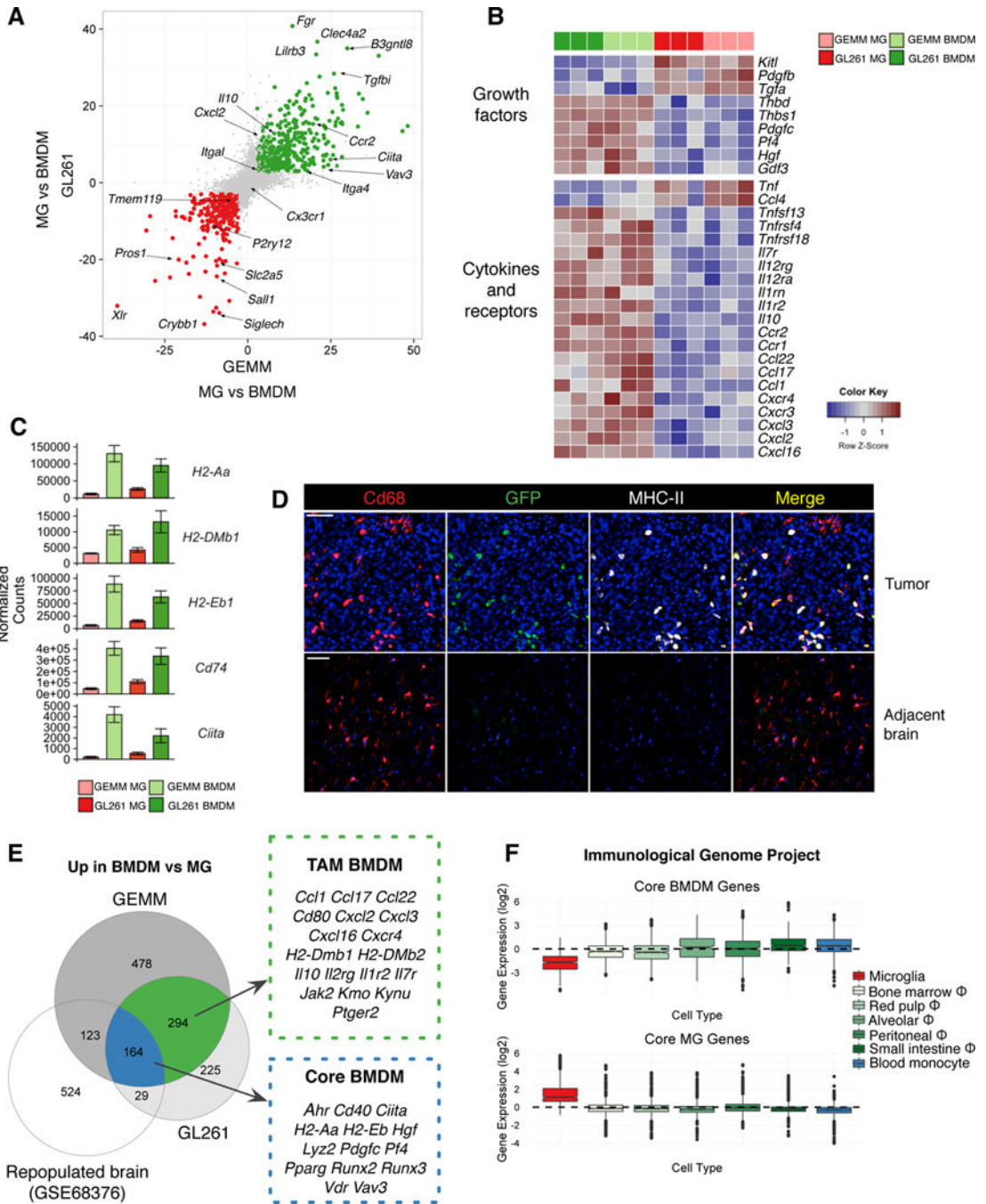


Figure 2. TAM BMDM and TAM MG possess distinct gene expression patterns
(A) Scatterplot depicting $-\log_{10}(\text{pvalue}) \cdot \text{sign}(\text{fold change})$ between TAM BMDM and TAM MG in GEMM-shP53 gliomas (x-axis) and GL261 gliomas (y-axis). Significantly upregulated genes (\log_2 fold change $> \pm 1$ and $\text{FDR} < 1\%$) are in green for BMDM and red for MG. **(B)** Heatmap depicting row-normalized \log_2 gene expression values for indicated genes in GL261 TAM BMDM (dark green), GEMM-shP53 TAM BMDM (light green), GL261 TAM MG (dark red), and GEMM-shP53 TAM MG (light red). **(C)** Barplots depicting normalized gene expression values for indicated genes in these four different TAM

populations. Bars represent mean \pm s.e.m. **(D)** Representative IF staining in GEMM-shP53 Flt3:Cre Rosa26:mTmG gliomas and adjacent normal brain for Cd68 (red, AlexaFluor-594), GFP (green) and MHC-II (white). DAPI is shown in blue and TdTomato fluorescence is not shown. Scale bar: 100 μ m. Representative of n=5 tumors. **(E)** Venn diagram depicting significantly upregulated genes in BMDM vs MG in GL261 model, GEMM-shP53, and non-malignant brain (GSE68376 dataset). Select genes are listed. **(F)** Boxplot of Core BMDM genes (Figure 2E) and Core MG genes (Figure S2D) where each data point represents the z-scored expression of a gene across the indicated cell populations using available datasets from the Immunological Genome Project.

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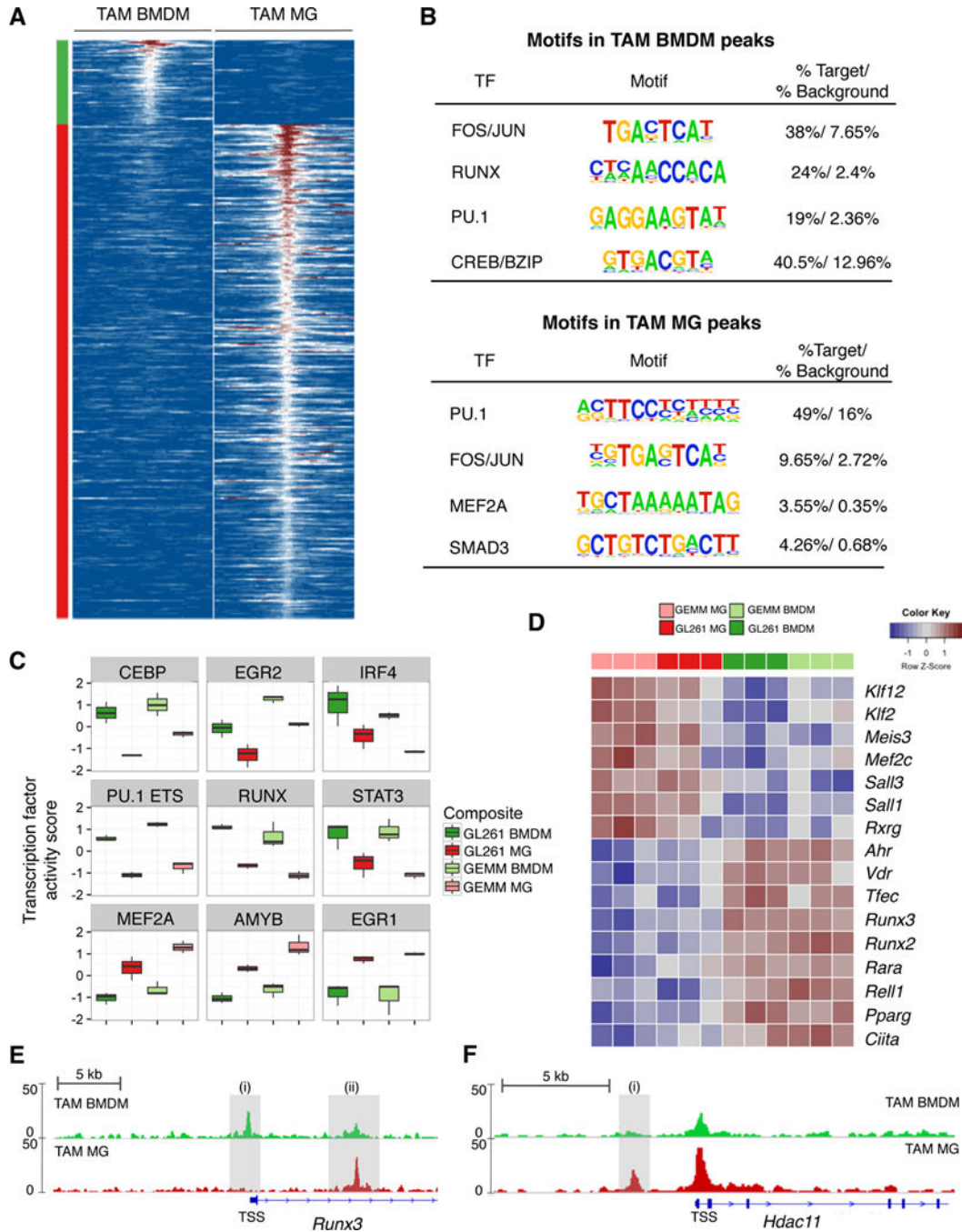


Figure 3. Cell-specific transcription factor activities underlie differences between TAM BMDM and TAM MG

(A) Heatmap depicting ATAC-seq signal 1kb upstream and downstream of peaks specifically enriched in GL261 TAM BMDM (left panel) and GL261 TAM MG (right). Peaks were selected based on association with differentially expressed genes between TAM BMDM (top, green) and TAM MG (bottom, red). (B) Motifs identified by HOMER to be enriched in TAM BMDM and TAM MG peaks shown in (A). (C) Boxplots depicting normalized TF activity scores for indicated motifs across TAM BMDM and TAM MG from GL261 and

GEMM-shP53 gliomas. **(D)** Heatmap depicting row-normalized log₂ gene expression values for indicated genes in four different TAM populations. **(E-F)** ATAC-sequencing tracks from TAM BMDM (top, green) and TAM MG (bottom, red) from GL261 gliomas for **(E)** *Runx3* and **(F)** *Hdac11*. Shaded grey regions indicate peaks specifically referenced in text. Y-axis values indicate tags per 10,000,000 with a range of 0–50. TSS denotes transcription start site.

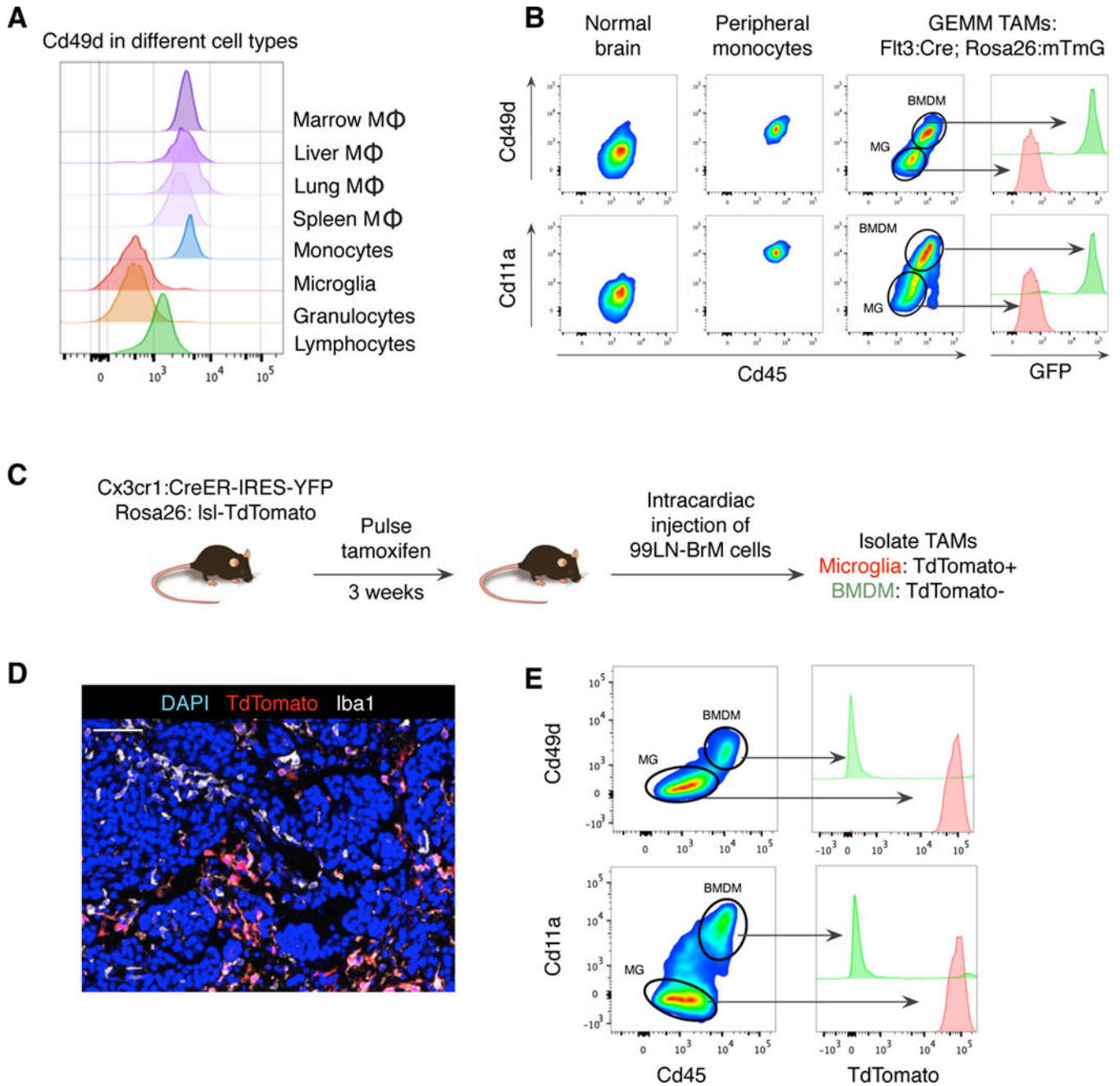


Figure 4. *Itga4*/Cd49d distinguishes TAM BMDM and TAM MG in murine brain malignancy (A) Histogram of Cd49d expression for indicated populations from non-tumor bearing mice. (B) Flow cytometry for Cd45 and either Cd49d (top) or Cd11a (bottom) in normal blood monocytes, normal MG (from adjacent normal brain), or TAMs isolated from Flt3:Cre Rosa26:mTmG mice with GEMM-shP53 tumors. Adjacent histograms indicate GFP expression in indicated populations. (C) Experimental schematic for 99LN-BrM model in Cx3cr1-lineage tracing mice. (D) Representative IF staining of TdTomato (red), Iba1 (white) and DAPI (blue) 99LN-BrM tumors as depicted in (C). Scale bar: 50 μ m. (E) Flow cytometry

as in (B) for 99LN-BrM model, with TdTomato expression indicated in the adjacent histogram. Flow plots are representative of n=5–8 mice.

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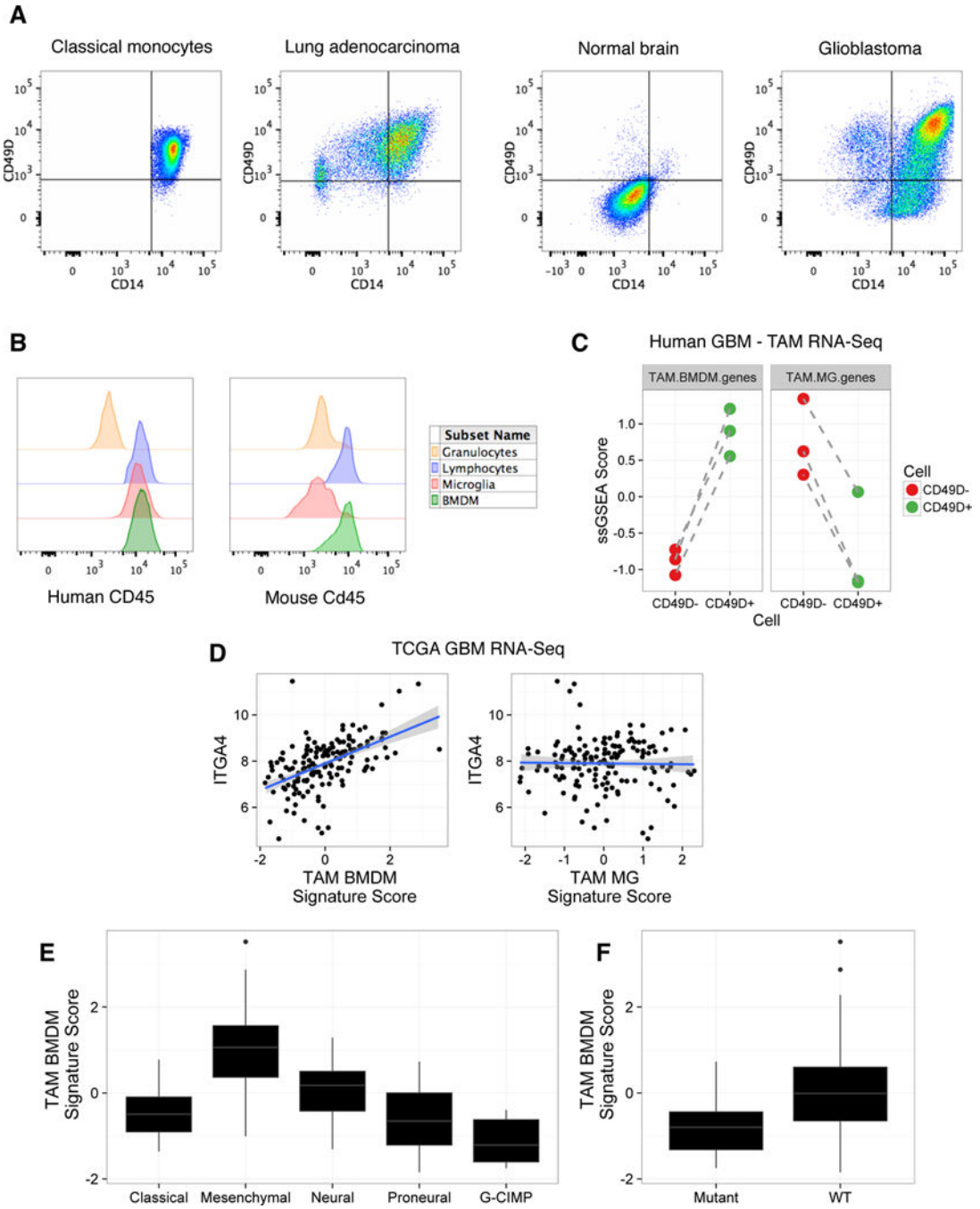


Figure 5. CD49D discriminates TAM BMDM and TAM MG in human brain malignancy
 (A) Classical monocytes, MG, and TAMs were defined as CD45⁺CD11B⁺CD66B⁻CD14⁺CD16⁻. Gated cells are then shown for CD14 and CD49D in representative samples of human classical monocytes from peripheral blood (n=6), TAMs from a lung adenocarcinoma patient (n=6), MG from a non-malignant brain (n=3), and TAMs from a GBM patient (n=3). (B) Histogram of CD45 expression by flow cytometry in human (left) and mouse (right) samples. In human GBM, CD45 expression is shown for granulocytes (CD45⁺CD11B⁺CD66B⁺CD16⁺CD14^{low}), lymphocytes (CD45⁺CD11B⁻),

TAM MG (CD45⁺CD11B⁺CD66B⁻CD16⁻CD14⁺CD49D⁻) and TAM BMDM (CD45⁺CD11B⁺CD66B⁻CD16⁻CD14⁺CD49D⁺). In mouse, Cd45 expression is shown for granulocytes (Cd45⁺Cd11b⁺Ly6C^{low}Ly6G⁺), lymphocytes (Cd45⁺Cd11b⁻), TAM MG (Cd45⁺Cd11b⁺Ly6C⁻Ly6G⁻Tomato⁺GFP⁻) and TAM BMDM (Cd45⁺Cd11b⁺Ly6C⁻Ly6G⁻Tomato⁻GFP⁺) from a Flt3:Cre Rosa26:mTmG GEMM-shP53 glioma. Representative of n=3 patients and n=5 mice. **(C)** Z-scored single sample gene set enrichment analysis scores for TAM BMDM genes (left, paired t-test, $p = 5.01 \times 10^{-3}$) and TAM MG genes (right, paired t-test, $p = 7.78 \times 10^{-3}$) in matched CD49D⁻ and CD49D⁺ TAMs from GBM patients. Dashed lines indicate matched samples (n=3 patients). **(D)** Scatterplot of TAM BMDM signature score (x-axis, left, Spearman rho=0.564, $p = 2.2 \times 10^{-16}$) and TAM MG signature score (x-axis, right, Spearman rho=0.067, $p = 0.411$) and *ITGA4* expression (y-axis) from TCGA-GBM RNA-seq data. Solid blue line indicates line of best fit with shaded areas depicting standard deviation confidence intervals. **(E-F)** Z-scored TAM BMDM signature scores across (E) GBM subtype (ANOVA $p = 2.2 \times 10^{-16}$), and (F) *IDH1* mutation status (Student's t-test $p = 5.93 \times 10^{-3}$).