1 **Myeloid-specific KDM6B inhibition sensitizes Glioblastoma to PD1 blockade**

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22   **Abstract**

23   Glioblastoma (GBM) tumors are enriched in immune-suppressive myeloid cells and are refractory

24   to immune checkpoint therapy (ICT). Targeting epigenetic pathways to reprogram the functional

25   phenotype of immune-suppressive myeloid cells to overcome resistance to ICT remains

26   unexplored. Single-cell and spatial transcriptomic analyses of human GBM tumors demonstrated

27   high expression of an epigenetic enzyme - histone 3 lysine 27 demethylase (KDM6B) in intra-

28   tumoral immune-suppressive myeloid cell subsets. Importantly, myeloid-cell specific *Kdm6b*

29   deletion enhanced pro-inflammatory pathways and improved survival in GBM tumor-bearing

30   mice. Mechanistic studies elucidated that the absence of *Kdm6b* enhances antigen-presentation,

31   interferon response and phagocytosis in myeloid cells by inhibiting mediators of immune

32   suppression including *Mafb, Socs3* and *Sirpa*. Further, pharmacological inhibition of KDM6B

33   mirrored the functional phenotype of *Kdm6b* deleted myeloid cells and enhanced anti-PD1

34   efficacy. Thus, this study identified KDM6B as an epigenetic regulator of the functional phenotype

35   of myeloid cell subsets and a potential therapeutic target to improve response to ICT.

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44 **Main**

45 Immune cells of the myeloid lineage constitute a dominant portion of the tumor immune

46 microenvironment and demonstrate significant plasticity depending on cues received from the

47 environment [1,2]. While pro-inflammatory myeloid cells are crucial for mounting an effective anti-

48 tumor immune response, immune-suppressive myeloid cells are associated with poor prognosis

49 and therapeutic resistance in multiple cancer types [3-6]. Emerging evidence suggests that immune-

50 suppressive myeloid cells play a critical role in both primary and adaptive resistance to

51 immunotherapy [7-11]. Therefore, developing therapeutic strategies to target immune-suppressive

52 myeloid cells is a critical approach to enhance response to cancer immunotherapy and has been

53 the focus of intense research for many years [12-14] .

54 Most studies have focussed on depleting intratumoral immune-suppressive myeloid cells,

55 blocking their trafficking, and targeting individual immune-suppressive pathways to enhance anti-

56 tumor immunity [15-20]. However, many of these strategies have not been successfully translated to

57 the clinic partly due to the functional heterogeneity and redundancy of pathways in myeloid cell

58 subsets. Newer technologies such as single cell RNA sequencing (scRNA seq) further

59 demonstrated the wide spectrum of functional states attained by each of these subsets based on

60 signals received from the niche they inhabit [21-25]. This significant plasticity of myeloid cells

61 highlights the important role of epigenetic regulation of their cell state [26,27]. However, the impact

62 of epigenetic regulation of intratumoral myeloid cell plasticity on therapeutic resistance remains

63 largely unexplored.

64 Glioblastoma (GBM) is an aggressive form of brain tumor, highly infiltrated with immune-

65 suppressive myeloid cells and demonstrates resistance to ICT [28-31]. We have previously shown

66 the persistence of tumor-associated macrophages (TAMs) in the GBM tumor microenvironment

67 even after treatment with anti-PD1 therapy [32]. In this study, we aimed to identify epigenetic factors

68 regulating the functional phenotype of intratumoral myeloid cell subsets in order to reprogram

69 these cells to a pro-inflammatory state thus enhancing anti-tumor immunity and efficacy of ICT.

70 scRNA seq of GBM tumors resected from patients demonstrated high expression of histone 3

71 lysine 27 demethylase (KDM6B) in myeloid cell subsets including monocytes, macrophages and

72 dendritic cells (DCs). Further, spatial transcriptomic analysis of human GBM tumors showed

73 significant infiltration of *KDM6B* expressing immune-suppressive myeloid cells in the tumor

74 microenvironment (TME). KDM6B is an epigenetic enzyme that demethylates the repressive

75 trimethylation mark at histone 3 lysine 27 (H3K27me3) thereby promoting gene transcription [33].

76 Importantly, in murine models of GBM, LysM[cre]KDM6B^{fl/fl} mice carrying *Kdm6b* deletion in myeloid

77 cells had enhanced pro-inflammatory pathways and improved survival compared with their wild-

78 type counterparts. Single-cell assay for transposase-accessible chromatin sequencing (scATAC

79 seq) and chromatin immunoprecipitation followed by sequencing (ChIP seq) demonstrated that

80 KDM6B directly regulates H3K27me3 enrichment of genes including *Mafb, Socs3* and *Sirpa* which

81 inhibit critical pro-inflammatory pathways such as cytokine production and phagocytosis in

82 macrophages[19,20,34,35], providing mechanistic insight into enhanced pro-inflammatory pathways

83 noted in the absence of *Kdm6b*. Further, pharmacological inhibition of KDM6B could recapitulate

84 the functional phenotype of *Kdm6b* deleted myeloid cells and improve sensitivity to anti-PD1

85 therapy in GBM. Together, these findings have provided critical insight into KDM6B-mediated

86 epigenetic regulation of intratumoral myeloid cell functions. Overall, this study proposes a new

87 paradigm of targeting the epigenetic machinery to regulate intratumoral myeloid cell plasticity thus

88 reprogramming them into a pro-inflammatory phenotype to overcome myeloid cell-mediated

89 resistance to ICT.

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91 **Single-cell and spatial transcriptomic analyses of human GBM tumors demonstrated**

92 **selective expression of *KDM6B* in immune-suppressive myeloid cells**

93   We performed scRNA seq of intratumoral *CD45*+ cells in GBM tumors resected from patients

94   (n=5). The patient characteristics are highlighted in Supplementary Table 1. Unsupervised

95   clustering and uniform manifold approximation and projection (UMAP) analyses revealed distinct

96   *CD3E+CD4*+T cells (C2), *CD3E+CD4+FOXP3*+ regulatory T cells (C14), *CD3E+CD8A*+T cell

97   (C5,7,9,10,11), *KLRK1*+NK cell (C13)*, CD19+CD79*+ B cell (C15), and myeloid cell subsets

98   (C0,1,4,3,6,8,16) (Fig.1A, Extended Data Fig. 1A). Next, to investigate the intratumoral myeloid

99   cells in human GBM samples in greater detail, we re-clustered myeloid cell populations

100   characterized by the expression of *CD68* (Fig. 1B). Annotation of each of these individual clusters

101   (Fig. 1B-C) revealed the presence of three microglial-like clusters (C0,4 and 8), one

102   *STAB1+LYVE1+DAB2*+ brain-associated macrophage cluster (C3), a

103   *CHI3L1+TIMP3+SPARC+COL1/3/4A1*+ population (C9), a *CD1C+CD1A+AREG*+ dendritic cell

104   cluster (C6) and four distinct monocytic/macrophage populations (C1, C2, C5, and C7).

105   Out of the three microglial clusters, C4 has several pro-inflammatory markers including interferon

106   signature genes (ISGs)- *IFI27, IFI16, IFI6* (Fig. 1C). In contrast, C0

107   (*APOE+TREM2+CD81+OLR1+HLA-DPA+C1Q+)* and C8 (*OLFML3+ P2RY13*+) are noted to

108   express suppressive markers such as *VEGFA, CCL4* (C0) and *SPP1, CCL4, ARG2* (C8)

109   respectively (Fig. 1C). Expression of immune-suppressive markers such as *MRC1, CD163,*

110   *TGFBI and SELENOP* was also noted in brain- associated macrophage cluster (C3) (Fig. 1C).

111   Additionally, all the monocytic macrophage clusters display a predominantly suppressive

112   phenotype. We noted the presence of a pro-angiogenic/hypoxic

113   *FN1+SPP1+MIF+BNIP3+HMOX1+CXCL8+ANXA2*+ cluster (C2), a *KLF2+KLF6+IL10+NR4A1*+

114   cluster (C1), a *S100A9+MARCO+CXCL1+CXCL2+CXCL8+CRIP1+ANXA2*+ cluster (C5) and a

115   *CCL4+CXCL2+CXCL8+IL10+AREG+NLRP3*+ cluster (C7) (Fig.1C). Overall, this analysis

116   highlighted the enrichment of suppressive myeloid cell subsets in GBM tumors resected from

117   patients.

118 To identify epigenetic factors which are critical for the regulation of immune-suppressive

119 phenotype and function in the tumor microenvironment (TME), we investigated gene expression

120 of several canonical epigenetic modifiers previously implicated in the regulation of myeloid cell

121 polarization and function (Fig. 1D)[26,27]. Interestingly, amongst the selected epigenetic enzymes,

122 we noted high levels of expression and selective enrichment of *KDM6B/JMJD3* in the myeloid

123 cells, specifically in the subsets expressing immune-suppressive markers such as *CSF1R*, *KLF2,*

124 *KLF6, CXCL8 and SPP1* (Extended Data Fig.1B-C). H3K27 methylation is an important

125 epigenetic determinant of myeloid cell phenotype and function [36]. *KDM6B* works in tandem with

126 other epigenetic modifiers such as *KDM6A* and *EZH2* in the regulation of H3K27 mediated gene

127 expression . Unlike *KDM6B, KDM6A* and *EZH2* showed minimal expression in the intratumoral

128 myeloid cell subsets (Fig.1D). Next, to confirm our findings, we used two independent scRNA seq

129 datasets with n=4[32] and n=20 [37] GBM patients respectively. Analyses of these two datasets

130 demonstrated distinct *CD3E+* T cell and *CD68+* myeloid cell clusters (Extended Data Fig. 2A-D).

131 Importantly, similar to our primary cohort of GBM patients, *KDM6B* enrichment was observed in

132 intratumoral immune-suppressive myeloid cells (Extended Data Fig. 2A-D).

133 Next, we performed spatial transcriptomic analysis of GBM tumors (n=3) resected from patients

134 (charcteristics enlisted in Supplementary Table 1) using the 10X-Genomics Visium platform.

135 Hematoxylin and Eosin (H&E) staining was done to determine the overall architecture of each

136 tumor section (Fig S3A). Immunofluorescence microscopy demonstrated the presence of

137 intratumoral CD3+, CD8+ T cells and CD68+CD163+ myeloid cells (inset) and confirmed that

138 human GBM tumors are diffusely infiltrated with myeloid cells (Fig. 1E). Further,

139 immunohistochemical (IHC) staining confirmed the expression of KDM6B protein in all the human

140 GBM sections studied (Extended Data Fig.3B). To visualize the spatial localization of *KDM6B+*

141 immune-suppressive myeloid cells in the GBM TME, we used matched scRNA seq data to embed

142 single cells to their spatial coordinates in tissue sections by applying CellTrek[38]. Multiple myeloid

143 cell, T cell, B cell, and NK cell clusters could be spatially delineated based on characteristic gene

144 signatures (Fig.1F,G, Extended Data Fig.4A-D and Extended Data Fig.5A-C). As expected, we

145 noted inter-tumoral qualitative and quantitative heterogeneity in the spatial landscape of immune

146 cell subsets.   However, enrichment of *KDM6B* expressing myeloid clusters co-expressing

147 immune-suppressive markers such as *SPP1, CXCL8, MARCO* and *MAFB* was observed across

148 all the tumor sections studied (Fig. 1F-H, Extended Data Fig. 4A-F and Extended Data Fig. 5A-

149 C).

150   Together, single-cell and spatial transcriptomic analyses of human GBM tumor samples

151 demonstrated the expression of KDM6B in the immune-suppressive myeloid cell subsets in

152 patients with GBM.

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154 **Myeloid cell-specific deletion of *Kdm6b* improves survival in preclinical models of GBM**

155 **and results in a pro-inflammatory milieu in murine GBM tumors**

156 To interrogate the impact of KDM6B-mediated regulation of the functional phenotype of myeloid

157 cell subsets on anti-tumor immunity, we generated a murine model bearing myeloid cell-specific

158 deletion of the *Kdm6b* gene (LysM^{cre}KDM6B$^{fl/fl}$) (Extended Data Fig. 6A,B). Mass cytometry

159 (CyTOF) based immunophenotyping studies showed no significant differences in the relative

160 abundance of the major immune cell subsets including myeloid cells in the immune cell repertoire

161 of bone marrow, spleen, and lymph-node from wild type (control) and LysM^{cre}KDM6B$^{fl/fl}$ mice

162 (Extended Data Fig.6C), indicating that myeloid-specific deletion of *Kdm6b* does not alter the

163 development of immune cell populations. Next, we orthotopically inoculated murine GBM cell line

164 -GL261 into the brain of control and LysM^{cre}KDM6B$^{fl/fl}$ mice. Magnetic Resonance Imaging (MRI)

165 studies done on day 14 post tumor inoculation revealed a lower tumor burden in mice having

166 myeloid-cell specific *Kdm6b* deletion as compared with control (Fig. 2A). We also observed a

167   similar reduction in tumor volumes in LysM^creKDM6B^fl/fl mice bearing another murine GBM cell

168   line- CT-2A (Fig. 2A). In addition, survival studies showed improvement in survival of both GL261

169   and CT-2A GBM tumor-bearing LysM^creKDM6B^fl/fl mice compared with control (Fig. 2B). Thus,

170   *Kdm6b* expression in myeloid cell subsets potentially aids in maintaining the suppressive

171   phenotype as constitutive deletion of *Kdm6b* in myeloid cells attenuated tumor growth and

172   provided a survival advantage in two pre-clinical models of GBM tumor bearing mice.

173   To determine the impact of myeloid cell-specific *Kdm6b* deletion on the GBM tumor immune

174   microenvironment, we performed scRNA sequencing of the murine GBM (GL261) tumors from

175   control and LysM^creKDM6B^fl/fl mice. UMAP analyses of *Cd45+* immune cell subsets revealed

176   distinct clusters of immune cell subsets including NK cells (C2, C18), CD8 T cells (C5,11,21),

177   CD4 T cells (C8), regulatory T cells (C6, 22), *Il17+Rorc+* T cells (C20), B cells (C13), neutrophils

178   (C24), mast cells (C25), microglial cells (C0 and C23), conventional dendritic cells

179   (C3,4,7,9,17,19), plasmacytoid dendritic cells (C16) and multiple subsets of monocytic

180   macrophages (C1,10,12,14,15) (Fig. 2C, Extended Data Fig. 7A). We noted an increase in the

181   abundance of cytotoxic G*zmb+Ifnγ+Cd8+* T cells (C11), NK cells (C2) with a concomitant

182   decrease in the abundance of immunosuppressive *Cd4+Foxp3+* regulatory T cells- Tregs (C6)

183   (Extended Data Fig. 7A,B). Further, the frequency of *Il17+* T cells (C20) was lower in the TME of

184   GBM tumors derived from LysM^creKDM6B^fl/fl mice compared to the control mice (Extended Data

185   Fig. 7A,B). Overall, a high cytotoxic T lymphocyte (CTL) to Treg ratio in LysM^creKDM6B^fl/fl mice

186   (Extended Data Fig.7C) indicated a pro-inflammatory skewing of the intratumoral milieu

187   characterized by a heightened anti-tumor T cell response in LysM^creKDM6B^fl/fl mice.

188   The LysM-cre model used in this study harbors genetic deletion of *Kdm6b* specifically in myeloid

189   cells [39,40]. Hence, the changes observed in non-myeloid immune subsets including increased T

190   cell mediated anti-tumor immunity in the LysM^creKDM6B^fl/fl GBM (GL261) tumor-bearing mice

191   could be secondary to the changes in *Lyz2* expressing myeloid subsets following *Kdm6b* deletion.

192   In our scRNA seq analysis, we noted *Lyz2* expression in intratumoral monocytic macrophages,

193   neutrophils, certain clusters of DCs and microglial cells (Extended Data Fig.7D). To investigate

194   the functional changes in these myeloid cell subsets, we studied the differentially expressed

195   genes (DEGs) in these subsets from control and LysM[cre]KDM6B^{fl/fl} mice. This analysis revealed

196   that depletion of *Kdm6b* significantly altered the transcriptomic landscape of intratumoral myeloid

197   cells in GBM tumor-bearing mice. Expression of several pro-inflammatory genes such as those

198   involved in phagocytosis (*Fcgr3, Lgals3, Clta, Arpc3, Arf1),* antigen presentation (*H2-Ab1, H2-*

199   *Eb1*) as well as several ISGs (*Oas1a, Isg15, Irf7, Cxcl9* and *Cxcl10)* were upregulated whereas

200   genes associated with immune-suppression such as *Zeb2, Klf2* and *Klf6* were downregulated in

201   monocytic macrophages (Fig. 2D) and DCs (Fig. 2E). Next, we performed Gene Set Enrichment

202   Analysis (GSEA) with the DEGs which revealed the differences in the major functional pathways

203   in the intratumoral monocytes, macrophages and DCs derived from control and LysM[cre]KDM6B^{fl/fl}

204   GBM (GL261) tumor-bearing mice. Prominent enrichment of Fc Gamma Receptor mediated

205   phagocytosis, antigen presentation pathway as well as type I and type II interferon response was

206   observed upon *Kdm6b* deletion (Fig.2F). In addition to the transcriptomic changes, we noted a

207   decrease in the abundance of tumor-infiltrating neutrophils and mast cells (Extended Data

208   Fig.7A,B). Further, we observed a concomitant increase in the abundance of pDCs (C16), a major

209   type I interferon producing cells [41] and migratory cDC1s (C7) which are known to be efficient

210   antigen-presenters [42,43] in LysM[cre]KDM6B^{fl/fl} mice compared to the control (Extended Data Fig.

211   7A,B). Of note, migratory cDC1s express *Lyz2* while pDCs lack expression of *Lyz2* (Extended

212   Data Fig. 7D). Hence, the changes in migratory DCs could be directly attributed to *Kdm6b*

213   deletion, while the effect on pDCs could be due to changes in the TME. Cumulatively, the findings

214   from scRNA seq demonstrated a global transcriptomic change in intratumoral myeloid cell subsets

215   in the absence of *Kdm6b,* leading to a pro-inflammatory milieu in the GBM TME. Additionally,

216   mass cytometry analysis (Extended Data Fig. 8A,B) of GBM tumors from CT-2A tumor bearing

217   control and LysM[cre]KDM6B^{fl/fl} mice showed a decrease in the abundance of PDL1+TGFβ+ (C26)

218   and CD115+TGFβ+ (C15) suppressive myeloid cell clusters (Extended Data Fig. 8B,C) in the

219   absence of *Kdm6b* with a concurrent increase in the abundance of GZMB+ CD8 T cells (C22)

220   (Extended Data Fig. 8B,C) and the CTL to Treg ratio (Extended Data Fig. 8D). Thus scRNA seq

221   and CyTOF analysis of tumors derived from two pre-clinical GBM models demonstrated that the

222   absence of *Kdm6b* in myeloid cells results in a pro-inflammatory milieu in murine GBM tumors.

223

224   **Kdm6b deletion alters the abundance and transcriptomic landscape of intratumoral**

225   **monocytes and macrophages**

226   To gain a deeper understanding of the transcriptomic changes in the highly abundant

227   monocytes/macrophages in the GBM TME following *Kdm6b* deletion, we performed reclustering

228   of *Itgam*+ clusters C0,1,10,12,14,15,23 (Fig. 3A, Extended Data Fig. 9A). Annotation of all the

229   distinct cell subsets demonstrated several monocytic macrophage clusters (C1-9, 11), brain-

230   associated macrophages (C0) as well as a microglial cluster (C10) in the GBM TME (Fig. 3A,

231   Extended Data Fig. 9A,B). Overall, we noted a decrease in the infiltration of *Chil3+Ccr2+S100a4*+

232   classical monocytic macrophages (C7) in the LysM^cre^KDM6B^fl/fl^ tumor-bearing mice (Fig. 3B,C).

233   Clusters expressing antigen presenting molecules (*H2-Eb1+H2-Ab1+H2-Aa+Ciita*+)(C2),

234   interferon signature genes (*Isg15+Ifit+Irf7+Rsad2+Isg20+Tlr2+Cxcl9+Cxcl10*+)(C3) and

235   phagocytic genes (*Lgals3+Gpnmb+Fabp5*+)(C9) were more abundant in LysM^cre^KDM6B^fl/fl^ GBM

236   tumor-bearing mice whereas cluster expressing immunosuppressive genes

237   (*Klf2+Klf4+Zeb2+Atf4+Mafb+Klf6*+)(C6) were more abundant in GBM tumors derived from control

238   mice (Fig. 3B,C). In addition to cellular abundance, comparison of gene expression patterns of

239   these five individual clusters between GBM tumors derived from control and LysM^cre^KDM6B^fl/fl^

240   mice showed an increase in the expression of several MHC molecules in C2 indicating

241   enhancement of their ability to present antigens as well as increased *Myd88, Irf5, Isg20, Ifitm3,*

242   *Oas1a, Isg15* in C3, indicating stronger interferon signaling in response to *Kdm6b* deletion (Fig.

243   3C). Further, immune-suppressive clusters C6 and C7 also showed significant upregulation of

244   pro-inflammatory genes following *Kdm6b* deletion (Fig. 3C).

245   Together, these findings revealed that the absence of *Kdm6b* in monocytes/macrophages leads

246   to upregulation of pro-inflammatory gene expression, thus regulating the phenotypic plasticity of

247   intratumoral myeloid cells.

248

249   Myeloid cell-specific deletion of *Kdm6b* alters the chromatin landscape of key genes

250   regulating the functional phenotype of intratumoral myeloid cells

251   To determine the chromatin landscape responsible for the changes in the gene expression

252   observed in response to *Kdm6b* deletion, we performed scATAC seq of CD45+ cells sorted from

253   the GBM (GL261) tumors of control (n=5, pooled) and LysM^{cre}KDM6B$^{fl/fl}$ mice (n=5, pooled).

254   Single-cell ATAC profiling of the immune cell compartment of GL261 tumors followed by UMAP

255   analysis showed distinct immune cell subsets (Fig. 4A,B, Fig. S10A). Genes proximal to cluster-

256   specific cis-elements were also used to annotate individual cell types (Fig. 4C). Briefly, C(0-2,6)

257   showed accessibility at cis-elements neighboring macrophage associated genes, including *Mafb,*

258   *Cebpb* and *F10*; C(10,13) demonstrated accessible cis-elements proximal to DC associated

259   genes such as *Mreg, Nr4a3* and *Anxa3*, while C(5,14) harbored accessible cis-elements

260   neighboring B cell associated genes, including *Fam43a, Cd19* and *Ms4a1* (Fig. 4C). Additionally

261   CD4 T cells (*Cd4, Tcf7, Zap70*), CD8 T cells (*Cd8a, Cd8b, Ifng*), regulatory T cells (*FoxP3*), NK

262   cells (*Eomes, Prf1*) and even rare cell subsets such as mast cells (*Homer2, Tbc1d8*) could be

263   identified from accessibility profiles of cell type specific cis-regulatory elements (Fig. 4C). We also

264   noted a prominent increase in the abundance of CTLs (C12) and a concomitant decrease in the

265   abundance of Tregs (C21) (Extended Data Fig.10 A,B), mirroring the findings from scRNA seq

266   and confirming the pro-inflammatory skewing of the intratumoral milieu in LysM^{cre}KDM6B$^{fl/fl}$ mice.

267   To define the chromatin landscape of myeloid cells, we analyzed *Lyz2+* population which showed

268   an increase in abundance of antigen presenting cluster (C0), cluster expressing IFN-related

269   genes (C2) as well as the phagocytic cluster (C4) (Fig. 4D-F, Extended Data Fig.11A). In addition

270   to the quantitative changes observed in the myeloid cell populations, we aimed to address the

271   qualitative changes occurring in the different myeloid subsets in response to *Kdm6b* deletion.

272   Interrogation of chromatin accessibility of individual genes of interest via coverage plots revealed

273   greater accessibility of genes associated with antigen presentation such as *H2-Eb2, H2-Ab1* (C0),

274   genes encoding positive regulators of phagocytosis such as *Fcgr1* and *Fcgr4* (C0) (Extended

275   Data Fig.11B), and genes involved in interferon signaling and response such as *Ifnar1, Ifngr1,*

276   *Isg15, Ifitm6* (C2) in LysM[cre]KDM6B^{fl/fl} mice (Extended Data Fig.11C).

277

278   Thus, the findings from scATAC seq showed open chromatin landscape of pro-inflammatory

279   genes in intratumoral myeloid cells in the absence of *Kdm6b.*

280

281   **Kdm6b regulates H3K27me3 enrichment of genes regulating phagocytosis, antigen**

282   **presentation, and interferon response in myeloid cells**

283   As we previously mentioned, KDM6B promotes gene expression by demethylation of

284   H3K27me3[33]. Therefore, to test whether the observed changes in the chromatin accessibility and

285   gene expression leading to pro-inflammatory skewing of myeloid cells were regulated by KDM6B-

286   mediated H3K27me3 demethylation, we performed ChIP seq assays on bone marrow derived

287   macrophages (BMDMs) from control and LysM[cre]KDM6B^{fl/fl} mice. ChIP using anti-KDM6B

288   antibody allowed identification of genes directly bound by KDM6B. We identified *Socs3* and *Mafb*

289   as direct targets of KDM6B in control BMDMs (Fig. 5A,D). Further, in BMDMs harbouring *Kdm6b*

290   deletion there was a drastic reduction in KDM6B occupancy of these genes as expected (Fig.

291 5A,D), and a concurrent enrichment of H3K27me3 (Fig. 5B,E). We also used quantitative PCR to

292 confirm the reduction in expression of these genes in LysM[cre]KDM6B^{fl/fl} BMDMs (Fig. 5C,F). These

293 findings demonstrated that KDM6B directly binds to *Socs3* and *Mafb* encoding genetic regions

294 and demethylates H3K27me3 to induce gene expression. Hence in the absence of KDM6B, these

295 genes are enriched for the repressive H3K27me3 marks thus inhibiting gene expression. SOCS3

296 is a known suppressor of cytokine signalling[34] and MAFB has been established as a suppressor

297 of type I IFN signalling[35]. Therefore, reduced expression of these immune-suppressive genes in

298 the absence of KDM6B provides a strong rationale for the pro-inflammatory skewing of myeloid

299 cells observed in response to *Kdm6b* deletion. Further, ChIP seq identified *Sirpa* as a direct target

300 of KDM6B, with reduction in KDM6B occupancy (Fig. 5G) and H3K27me3 enrichment (Fig. 5H)

301 in *Kdm6b* deleted BMDMs. SIRPA acts as a negative regulator of phagocytosis by generating

302 "don't-eat-me" signals[19]. Overall, the findings from the ChIP-sequencing study provided

303 mechanistic insight into KDM6B mediated regulation of macrophage phenotype and function.

304 KDM6B binds to negative regulators of interferon response and phagocytosis including *Mafb,*

305 *Socs3* and *Sirpa*. Thus, these findings implicate KDM6B as an upstream regulator of multiple

306 critical functional pathways in macrophages including cytokine production/response, antigen

307 presentation and phagocytosis.

308 In order to investigate the impact of KDM6B depletion on phagocytosis and antigen-presentation

309 functions of myeloid cells, we performed in-vitro phagocytosis and antigen-presentation assays

310 using bone marrow derived macrophages (BMDMs) from control and LysM[cre]KDM6B^{fl/fl} mice. We

311 found that following stimulation with lipopolysaccharide, *Kdm6b* deficient BMDMs demonstrated

312 enhanced phagocytosis compared to control as evident from a higher percentage of fluorescent

313 non-coated latex bead positive BMDM cells (Fig. 5I). Additionally, phagocytosis of fluorescently

314 labelled GL261 cells was higher by BMDMs deficient in *Kdm6b* compared to control (Fig. 5J,

315 Extended Data Fig.11D). For antigen presentation assay we co-cultured gp100-pulsed BMDMs

316   with cell trace violet (CTV) labelled cognate T cell receptor-bearing CD8 T cells isolated from pmel

317   mice[44] (Fig. 5K). Based on differences in dilution of the CTV dye, we observed that co-culture with

318   BMDMs derived from LysM[cre]KDM6B^{fl/fl} mice led to significantly higher T cell proliferation as

319   compared to BMDMs derived from control mice (Fig. 5L). BMDMs pulsed with the non-cognate

320   LCMV peptide, showed minimal proliferation thus confirming the antigen-specificity of the

321   observed proliferation in T cells (Extended Data Fig.11E). Overall, the T cell proliferation assay

322   indicated that *Kdm6b* deficient BMDMs are more efficient antigen presenters as compared to

323   control BMDMs. These findings provided evidence of enhanced phagocytic and antigen

324   presentation function of myeloid cells following *Kdm6b* deletion.

325   Cumulatively, we found that KDM6B regulates H3K27me3 enrichment of genes regulating critical

326   pathways modulating myeloid cell functions such as phagocytosis, antigen presentation, and

327   interferon signaling/response.

328

329   **Pharmacological inhibition of KDM6B improves the efficacy of immune checkpoint therapy**

330   **in GBM**

331   Since KDM6B functions upstream to several critical functional pathways, therapeutic targeting of

332   KDM6B could revert myeloid-derived immune suppression. To determine the translational

333   relevance of our findings from the genetic model, we compared murine GBM tumor growth and

334   the tumor immune microenvironment in the presence and absence of a pharmacological inhibitor

335   of KDM6B (GSK-J4)[45,46]. MRI studies revealed lower tumor burden in GSK-J4 treated GL261

336   tumor bearing mice as compared with control (Fig. 6A). Additionally, we observed improvement

337   in the overall survival of GL261 tumor-bearing mice treated with GSK-J4 (Fig. 6B). Of note, GSK-

338   J4 which targets KDM6A/B, has been shown to inhibit proliferation of glioma cell lines in-vitro[47].

339   Hence, the observed improvement in survival could be due to the direct effect of GSK-J4 on tumor

340   cells as well as its effects on the tumor immune microenvironment.

341   Hence, to investigate the effect of GSK-J4 on the GBM tumor immune microenvironment, we

342   performed scRNA seq. UMAP analyses performed on scRNA-seq data from GSK-J4 treated and

343   control GL261 tumors showed distinct lymphoid (C9-12,15,17) and myeloid cell clusters (C0-

344   8,13,14,16) (Fig. 6C,D, Extended Data Fig. 12). We noted a significant decrease in the abundance

345   of *Cd14+Ly6c2+Plac8+Cxcl2+Vegfa+* monocytic macrophage subset (C0) in GBM tumor-bearing

346   mice treated with GSK-J4 (Fig. 6E). C1, characterized by the expression of markers of both

347   activated CNS associated macrophages and disease associated microglia (*Ms4a7, Ccl8, Cd74,*

348   *Tmem119, Hexb, Cx3cr1, Trem2, C1qa, C1qb, C1qc, H2-Ab1*) increased in abundance post

349   treatment with GSK-J4 (Fig. 6D,E). We also identified a *Gpnmb+* phagocytic cluster (C4) which

350   increased in abundance in GSK-J4 treated mice (Fig. 6D,E). Importantly, we also noted an

351   increase in the *Cd8+Gzmb+Ifng+* cytotoxic T cells (C9) and *Cd4+Icos+Ifng+Cxcr6+* T cell cluster

352   (C10) in response to GSK-J4 treatment (Fig. 6D,E).

353   To corroborate the findings from the GL261 model, we used the CT-2A GBM model and treated

354   them with GSK-J4. MRI studies revealed lower tumor burden in GSK-J4 treated CT-2A tumor

355   bearing mice as compared with control (Extended Data Fig.13A-C). Additionally, interrogation of

356   the TME of CT-2A GBM tumors using CyTOF (Extended Data Fig.13D,E) also showed

357   significantly lower abundance of immune-suppressive myeloid clusters (C3,9) and higher

358   abundance of NK cells (C12) and effector/memory CD8 T cells (C6) in GSK-J4 treated mice

359   (Extended Data Fig.13F). Thus cumulatively, scRNA seq and CyTOF data from two distinct GBM

360   tumor bearing murine models revealed a significant reduction in intratumoral immune-suppressive

361   monocytic macrophage populations with a concomitant increase in effector CD8 T cell subset in

362   response to GSK-J4 treatment.

363   Myeloid heavy tumor types such as GBM tumors often demonstrate primary resistance to immune

364   checkpoint therapy. To test if KDM6B inhibition mediated pro-inflammatory skewing of the tumor

365   immune microenvironment could increase the efficacy of ICT in a murine model of GBM, we

366   treated GL261 tumor-bearing mice with vehicle, anti-PD1, GSK-J4 and the combination of anti-

367   PD1 plus GSK-J4. We found that the combination therapy of anti-PD1 plus GSK-J4 led to a

368   significant reduction in tumor weight (Fig. 6F) as well as in improvement in overall survival (Fig.

369   6G). In order to understand the changes in the TME, we performed CyTOF analysis on these

370   tumors (Fig. 6H, Extended Data Fig.14). We noted that CD8+CD86+CD44+ effector memory T

371   cell cluster (C17) was significantly higher in mice receiving a combination of anti-PD1 plus GSK-

372   J4 as compared with the control, GSK-J4 and anti-PD1 monotherapy groups (Fig. 6I, Extended

373   Data Fig.14). Also, there was a significant decrease in a CD11b+F4/80+ monocytic macrophage

374   cluster expressing CD115/CSF1R (C4) and a Ly6c+Ly6g-CD11b+F4/80+ monocytic-myeloid

375   derived suppressor cell (M-MDSCs) cluster expressing TGFβ (C28) following treatment with the

376   combination of anti-PD1 plus GSK-J4 (Fig. 6I, Extended Data Fig.14).

377   Together, these findings demonstrate that pharmacological inhibition of KDM6B by GSK-J4 can

378   skew the TME of GBM tumor-bearing mice to a pro-inflammatory phenotype and reduce the

379   frequency of several pro-tumorigenic myeloid cell subsets including M-MDSCs and *Cxcl2*+TAMs,

380   thus, improving overall survival and enhancing sensitivity to anti-PD1 therapy.

381

382   **Discussion**

383   This study identified a selective expression of KDM6B in the intra-tumoral myeloid cell subsets in

384   the GBM tumors resected from patients utilizing single-cell transcriptomic and spatial analysis.

385   Reverse translational studies using pre-clinical models of GBM demonstrated that targeting

386   KDM6B-mediated epigenetic pathways in the myeloid cells via genetic deletion and

387   pharmacological inhibition resulted in upregulation of pro-inflammatory pathways, cumulatively

388   improving survival and enhancing sensitivity to anti-PD1 therapy (Fig. 6J).

389

390   Over the years, multiple myeloid cell-specific pathways have been explored individually as

391   potential targets for the treatment of cancer [15-17]. However, these pathways are highly interrelated

392   and often redundant, therefore targeting a single pathway often fails to elicit optimum clinical

393   benefit. We found that KDM6B is upstream of multiple pathways and inhibition of KDM6B

394   enhanced interferon response pathways, antigen presentation, and phagocytosis in macrophages

395   as well as in DCs. Additionally, our mechanistic studies identified certain critical regulators of the

396   above mentioned pathways including *Mafb, Socs3* and *Sirpa* as direct targets of KDM6B mediated

397   H3K27me3 demethylation. MAF BZIP Transcription Factor B (MAFB) which encodes a basic

398   leucine zipper myeloid cell-specific transcription factor acts as a rheostat to inhibit type I IFN

399   induction by physically blocking IRF3 from binding to its target genes [35,48,49], which might provide

400   a possible explanation for the enhanced interferon response observed upon KDM6B deletion.

401   Further, suppressor of cytokine signaling 3 (Socs3) inhibits cytokine-induced JAK-STAT signaling

402   pathways[50] and cytokines including IFN pathways have been shown to regulate both antigen

403   presentation and phagocytosis by myeloid cells [51-55]. Additionally, KDM6B regulates *Sirpa* which

404   acts as a negative regulator of phagocytosis by generating "don't-eat-me" signals [56]. Thus,

405   KDM6B regulates phagocytosis by modulating both activators and inhibitors of the phagocytosis

406   pathway.   Of note, the changes observed in the phagocytosis assays, though statistically

407   significant were quantitatively modest. This might possibly be due to the in-vitro setting in which

408   the experiments were performed, as opposed to the in-vivo intratumoral environment. Overall,

409   our findings suggest that KDM6B functions upstream of several critical functional pathways (Fig.

410   6J). Therefore, targeting KDM6B to reprogram the immune-suppressive myeloid population into

411   an immune-stimulatory phenotype could potentially be an important therapeutic strategy rather

412   than targeting individual pathways.

413   Considering the heterogeneity of the myeloid cell subsets, a single cre-flox model system can not

414   target all the myeloid cell subsets simultaneously. We used the LysM-cre model to evaluate

415   KDM6B-mediated epigenetic regulation of myeloid cells since *Lyz2* expression is generally

416   observed in monocytes and macrophages, granulocytes and in some dendritic cells [57]. Although

417   we noted an increase in the microglial-like cells expressing antigen presentation molecules

418   following deletion of KDM6B, LysM-cre model is not adequate for a comprehensive interrogation

419   of microglial cells.

420

421   Overall, based on the single-cell and spatial transcriptomic analysis of human GBM samples and

422   the series of reverse translational studies using multiple pre-clinical model systems, we identified

423   a KDM6B-mediated immunoregulatory program in myeloid cells, providing a strong rationale to

424   consider evaluating KDM6B inhibition as a therapeutic strategy to overcome myeloid-derived

425   immune suppression and enhance response to immune-based therapies. The strategy of

426   inhibiting KDM6B, proposed in this study, not only adds to the existing repertoire of myeloid cell

427   targeting strategies, it proposes a new paradigm of regulating the epigenetic machinery to target

428   intratumoral myeloid cell plasticity thus reprogramming them to a pro-inflammatory phenotype.

429

430   **Methods:**

431   **Patients.** Patient samples were collected after appropriate informed consent was obtained on MD

432   Anderson internal review board (IRB)-approved protocol PA13-0291. All patients signed informed

433   consents for participation in PA13-0291 before surgery or sample collection. The clinical

434   characteristics of individual patients are shown in Supplementary Table 1.

435   **H&E and IHC staining.** Hematoxylin and Eosin (H&E) and IHC staining were performed on

436   formalin-fixed, paraffin-embedded tissue sections. Tissues were fixed in 10% formalin, embedded

437 in paraffin, and sectioned at four-micron–thickness. For IHC, sections were antigen retrieved with

438 ER solution 1 (Leica Microsystems, catalog no. AR9961), protein block was applied for 30 mins

439 (Leica Microsystems, catalog no. RE7102) and stained with KDM6B (ThermoFisher, catalog no.

440 PA5-32192) at 1:200 dilution followed by rabbit anti-human secondary. 3′-3-diaminobenzidine

441 (DAB) substrate (Leica Microsystems) was used as a chromogen followed by hematoxylin

442 counterstain. Slides were scanned and digitalized using the scanscope system from Scanscope

443 XT, Aperio/Leica Technologies. IHC staining was interpreted in conjunction with H&E stained

444 sections.

445 **Immunofluroscence.** Using the Opal multiplex immunofluorescence staining protocol [54] on a RX-

446 BOND (Leica) autostainer, GBM tissue sections were stained for CD3 (Dako, A0452, 1:200

447 dilution), CD8 (LS-Bio, C8/144b, 1:100 dilution), CD68 (Dako, PGM-1, 1:25 dilution) and CD163

448 (Leica Microsystems, 10D6, 1:20 dilution). Subsequent visualization was performed using Akoya

449 Opal fluorophores (620, 520, 480, 570 respectively), DAPI (1:2000 dilution) and cover-slipped

450 using Vectashield Hardset895 mounting medium. Slides were scanned using a Vectra/Polaris

451 slide scanner (PerkinElmer) and images acquired at 20X magnification were spectrally unmixed

452 using Inform software (Akoya).

453 **Spatial Transcriptomics Assay (10X Genomics, Visium).** GBM tumors were paraffin

454 embedded and serially sectioned (thickness 5 μm). Formalin-fixed paraffin-embedded (FFPE)

455 tissue from 3 GBM samples were used for spatial transcriptomics analysis. FFPE samples were

456 tested for RNA quality with an DV200 > 30% (Agilent). The samples were then processed

457 according to the standard Visium Spatial Gene Expression protocol (10x Genomics) using the

458 Visium Spatial Gene Expression Slide & Reagent Kit (10x Genomics).

459 Libraries were cleaned up using SPRI select reagent and quantified using the High Sensitivity

460 DNA Kit run on Agilent 2100 Bioanalyzer and also KAPA Illumina library quantification kit (Roche,

461 7960140001) run on LightCycler 480. Library pool was quantified on Bioanalyzer and with

462 quantitative PCR and sequenced using Illumina NextSeq 500.

463 **Visium spatial transcriptomics data analysis using Spaceranger and CellTrek.** The raw

464 spatial sequencing data was processed in the Spaceranger workflow (10X Genomics). The

465 spaceranger (version 2.0.0) mkfastq pipeline was used to convert Illumina sequencer's binary

466 base call (BCL) files into FASTQ format. Samples were then run through the spaceranger count

467 pipeline, which performs alignment, tissue detection, fiducial detection, and barcode/unique

468 molecular identifier counting. Human GBM scRNA seq data analyses were performed using

469 standard Seurat data analysis pipeline including log normalization, scaling, variable genes

470 selection (n=3,000) using vst, dimension reduction using principal component analysis (PCA) and

471 UMAP. Spatial transcriptomics (ST) data (10x Genomics Visium) was analyzed similar to the

472 scRNA seq data with Seurat data analysis package (log normalization, scaling, variable genes

473 identifcation and dimensionality reduction). To analyze ST data using CellTrek[38], we first ran traint

474 to coembed the data into a shared feature space with default parameters. Then we ran Celltrek

475 on the coembedded traint data with following parameters: intp_pnt =5,000 spots, nPCs =30, ntree

476 =1,000, top_spot =5, spot_n =5 and repel_r =3 with ten iterations. To visualize the ST data, we

477 used celltrek_vis tool which allows mapping any continuous or categorical cell features to the

478 spatial map with different colors.

479 **Mice.** 5–7-weeks old C57BL/6 mice were purchased from the National Cancer Institute (NCI). 5–

480 7-weeks old KDM6b^fl/fl(B6.Cg-Kdm6b^tm1.1Rbo/J, Stock no. 029615) mice and LysM^cre (B6.129P2-

481 Lyz2^tm1(cre)Ifo/J, Stock no. 004781) mice were purchased from the Jackson Laboratory. Female

482 mice were used for the experiments. All mice were kept in specific pathogen-free conditions at

483 the Animal Resource Center, University of Texas MD Anderson Cancer Center. Animal protocols

484 were approved by the Institutional Animal Care and Use Committee of the University of Texas

485 MD Anderson Cancer Center.

486   LysM[cre]Kdm6b^{fl/fl} mice were generated by breeding KDM6b^{fl/fl} and LysM[cre] mice [39,40]. PCR based

487   genotyping study was done to confirm *Kdm6b* deficiency using primers with the following

488   sequences

    Forward- 5'-CAG CGA TCC TGA CTT GTT CA-3'

    Reverse- 5'-GTG CCA AGG CTG GAG GA-3'

489   **Mass cytometry based immunophenotyping assay.** Spleen, bone marrow and lymph node

490   were collected from control and LysM[cre]Kdm6b^{fl/fl} mice. Single-cell suspensions were generated

491   by physical dissociation and passage through 70μm filters. Cells were washed in RPMI media by

492   centrifugation at 2,000 rpm, 4 °C for 5 minutes. Upto 3×10^6 cells were washed with FACS buffer

493   containing 5% FBS (Fetal Bovine Serum) in PBS (Phosphate Buffered Saline) and then incubated

494   with blocking buffer containing a mixture of 2% of bovine, murine, rat, hamster, rabbit serum and

495   25 μg/mL 2.4G2 antibody (Fc block) in PBS at 4 °C for 10 min. Next, surface staining was done

496   with antibody mixture (Supplementary Table 2) at 4 °C for 30 min. Following incubation,194Pt

497   monoisotopic cisplatin (Fluidigm) in PBS at a final concentration of 5 μM, was incubated with the

498   samples for 3 min. Next, samples were washed twice with FACS buffer followed by fixation and

499   permeabilization for 1 hour at 4°C. After a wash with permeabilization buffer (Invitrogen)

500   intracellular staining was done for 30 mins at 4°C (Supplementary Table 2). Following staining,

501   samples were washed twice with Maxpar barcode perm buffer (Fluidigm) and labeled using

502   palladium barcoding as per the manufacturer's protocol for 30 min at room temperature. Following

503   2 washes with FACS buffer, samples were fixed using 1.6% paraformaldehyde in PBS

504   supplemented with 100 nM iridium nucleic acid intercalator (Fluidigm) and left overnight. Next

505   day, cells were washed twice with PBS, filtered, and resuspended in nuclease free water.

506   Barcoded samples were then acquired in a Helios mass cytometer (Fluidigm).

507   **Cell lines and tumor models.** The murine GBM cancer cell line GL261 was obtained from NCI

508   and the CT-2A cell line was obtained from Millipore Sigma. GL261 and CT-2A cells were cultured

509 in complete (supplemented with 10% FBS) DMEM media at 37 °C and 5% CO_2. Cells in the

510 logarithmic phase of growth were harvested by trypsinization and washed twice with PBS before

511 intracranial inoculation. $5X10^4$ GL261 cells were resuspended in 3µl of 70% PBS and 30%

512 Matrigel while $5X10^4$ CT-2A cells were resuspended in 3µl of DMEM media (without FBS) for

513 injection with a stereotactic apparatus (Stoelting) in the cranial window- 2mm posterior and 2mm

514 lateral to the bregma and 3mm deep into the mouse cerebrum.

515 **MRI image quantification.** The MRI images were quantified using ImageJ (NIH) v.1.52a. First,

516 images were imported, and their brightness/contrast was adjusted. Image slices were then

517 scanned to identify tumor sections. A gate was drawn around the tumor in each section and the

518 area was measured. Image geometry indicated the slice thickness to be 0.75 mm and the distance

519 between two sections to be 1 mm. The tumor area in each section was multiplied by 0.75 and the

520 average between the tumor area in two sections was taken and multiplied by 0.25 (1 − 0.75; this

521 gave the value for depth). The volume for each tumor was obtained by multiplying the tumor area

522 and depth from the section-containing tumor. All values were added to determine tumor volume

523 in mm^3.

524 **Tumor harvesting and processing.** GL261 tumors were harvested on day 17 post tumor

525 inoculation and CT-2A tumors were dissected on day 22 post tumor inoculation. Following

526 dissection, tumor samples were enzymatically digested with 0.66mg/ml Liberase TL (Roche) and

527 20mg/ml DNase I (Roche) in RPMI cell culture media for 30 minutes at 37 °C. Single-cell

528 suspensions were generated by passing digested tumors through 70-µ filters and washed in

529 complete RPMI media by centrifugation at 2,000 rpm 4°C for 5 minutes. Percoll gradient

530 centrifugation at 512g for 20 minutes at 18°C was used to deplete the myelin layer and the single

531 cell suspension obtained was counted in an automated cell counter for downstream analysis.

532 **Single cell RNA sequencing.** Single cell suspension of human and murine GL261 GBM tumors

533 were made using the protocol described above. Single cells were incubated with a surface

534   staining cocktail of fluorescently conjugated antibodies, which included CD45 Pacific Blue (clone

535   30-F11, Biolegend, 103126), and live/dead discrimination viability dye Pacific Orange (Invitrogen,

536   L34968). CD45+ cells were sorted directly into 5% FBS using a FACS AriaFusion cell sorter (BD).

537   Cells from each sample were counted before 16,000 cells per sample were loaded on the 10x

538   chromium chip (Chromium platform,10x Genomics), with a target of 10,000 cells per sample for

539   the downstream analysis. Single-cell mRNA libraries were built using the Chromium Next GEM

540   Single Cell 3' Library Construction V3 Kit, libraries sequenced on NovaSeq 6000 using 100cycle

541   kit, flow cell type – S2-100, run format- 28/91 and 8 i7 index.

542   **Single cell RNA sequencing data analysis.** Cellranger v3.0.2 software (10x Genomics) was

543   used to process the sequencing reads. The "cellranger count" pipeline was used to align the reads

544   to the mouse mm10 genome and compute the count matrix. The Seurat R package was used to

545   perform the analysis including filtering out low-quality cells, normalizing the data, and clustering

546   the cells. Genes presented in less than 3 cells and cells with less than 200 genes or more than

547   6000 genes, or with more than 10% mitochondrial gene counts were excluded from downstream

548   analysis. Potential doublets were removed with the DoubleFinder R package [58]. Then, the

549   "SCTransform" function was used to normalize and log transform the raw gene counts. Anchors

550   identified by the "FindIntegrationAnchors" function were used to integrate the datasets. Principal

551   component analysis (PCA) was applied to the top 3000 highly variable genes and the first 30

552   components were used for constructing a KNN graph, clustering and UMAP projection.

553   **Single cell ATAC sequencing.** GL261 tumors were dissociated from control, LysM^{cre}Kdm6b$^{fl/fl}$

554   mice.and single cell suspension of cells were made using the protocol described above. Single

555   cells were incubated with a surface staining cocktail of fluorescently conjugated antibodies, which

556   included CD45 Pacific Blue (clone 30-F11, Biolegend, 103126), CD3ε FITC (clone 17A2,

557   eBioscience, 11-0032-82), CD11b APC (clone M1/70, Biolegend, 101212) and live/dead

558   discrimination viability dye Pacific Orange (Invitrogen, L34968). CD45+ cells were sorted directly

559 into 5% FBS using a FACS AriaFusion cell sorter (BD). Cell nuclei were isolated from the sorted

560 CD45+ cells using Nuclei Isolation for Single Cell ATAC Sequencing Protocol (CG000169 Rev

561 D). 16,000 nuclei per sample were loaded on the 10x chromium chip (Chromium platform,10x

562 Genomics), with a target of 10,000 nuclei per sample for the downstream analysis. Single cell

563 ATAC libraries were built according to the manufacturer's protocol (Chromium Next GEM Single

564 Cell ATAC Reagent Kits (v1) User Guide - CG000168 Rev D). The libraries were pooled and

565 sequenced using NovaSeq6000 instrument with Read1 (50 cycles), Read2 (49 cycles), Index1 (8

566 cycles), and Index2 (16 cycles). The sequencing reads were demultiplexed based on sample

567 index barcodes.

568 **Single cell ATAC sequencing data analysis.** Cellranger-atac v1.2 software (10x Genomics)

569 was used to process the sequencing reads. The "cellranger-atac count" pipeline was used to align

570 the reads to the mouse mm10 genome and to perform peak calling with the default parameters.

571 In case the detected cell number from the auto cell detecting algorism was unexpected, the "force-

572 cells" parameter for the "cellranger-atac count" was manually set according to the Barcode Rank

573 Plot in the web summary result. Peak barcode count matrices from all the samples were

574 aggregated using the "cellranger-atac aggr" pipeline function and normalized to sequencing

575 depth. The single-cell ATAC data analysis mainly was done using the Signac (version 1.1.0)

576 (https://github.com/timoast/signac) and Seurat [59](version 3.2.0) R packages.

577 Cluster-wise peak calling was performed with MACS2 as previously described[60]. Briefly, the

578 mouse genome was tiled into 2.5 kb size windows and a cell-by-window sparse matrix was

579 computed by the Signac "FeatureMatrix" function. The matrix was binarized and the top 20000

580 most accessible sites across all cells were used to cluster the cells. Peak calling for each cluster

581 was performed by the Signac "CallPeaks" function and a union peak set of 180413 was created.

582 Then, a cell-by-peak sparse count matrix was computed by the Signac "FeatureMatrix" function

583 and used for downstream analysis.

584   Peaks presented in less than 10 cells and cells with less than 200 peaks were removed from the

585   downstream analysis. Quality control matrixes including percentage reads in peaks, blacklist ratio,

586   nucleosome signal and TSS enrichment score were calculated following the Signac vignettes.

587   Cells with peak region fragment count between 3000 ~ 50000, percentage reads in peaks > 50,

588   blacklist ratio < 0.025, nucleosome signal < 4, and TSS enrichment score > 2 were considered

589   for further analysis (total 17380 cells).

590   The peak barcode count matrix was binarized and was normalized by term frequency-inverse

591   document frequency (TF-IDF) using the Signac "RunTFIDF" function. Dimensional reduction was

592   performed with a singular value decomposition (SVD) on the TD-IDF normalized matrix using the

593   Signac "RunSVD" function including all the peaks. Since the first LSI component usually captures

594   the sequencing depth variation, it was removed for the downstream analysis. Graph-based

595   clustering, and non-linear dimension reduction for visualization were performed using the Seurat

596   "RunUMAP", "FindNeighbors", and "FindClusters" functions with the 2 to 50 LSI components and

597   resolution of 1.4. To find differentially accessible perks between two groups of cells, the Seurat

598   "FindMarkers" function was used to with the parameter "test.use = "LR", latent.vars =

599   "peak_region_fragments". The identified peaks was annotated on the basis of its nearest gene

600   using the Signac "ClosestFeature". Peak visualization was performed with the Signac

601   "CoveragePlot" function.

602   Gene activity score was calculated using the Cicero (version 1.3.4) and Monocle3 (version 0.2.2)

603   R package as described previously[61]. The binary filtered peak counts matrix from the Seurat

604   object was used to build a Monocle3 Cell Data Set (cds) object. A Cicero cds object was created

605   using the Cicero "make_cicero_cds" function with the parameter reduced_coordinates equal to

606   the UMAP coordination in the Seurat object. Co-accessibility scores between peaks were

607   calculated using the Cicero "run_cicero" function with the mouse mm10 genome. The gene

608   activity score was calculated using the Cicero "build_gene_activity_matrix" function and

609 normalized with the Cicero "normalize_gene_activities" function. The gene activity score was

610 transformed with log(score*1000000 + 1). The unnormalized and normalized gene activity matrix

611 were used to create a "RNA" assay in the Seurat object for downstream analysis. To find

612 differentially gene activity between two groups of cells, the Seurat "FindMarkers" function was

613 used.

614 **BMDM generation**. Dissected femurs from 6-8 weeks old control and LysM^{cre}KDM6B $^{fl/fl}$ mice

615 were collected in ice cold complete RPMI 1640 media. Both epiphyses were removed before the

616 bones were placed in sterile microfuge tubes and centrifuged at 500g for 5 minutes at 4 °C to

617 extrude the bone marrow. The collected bone marrow was homogenized by pipetting followed by

618 RBC lysis. The single cell suspension of bone marrow cells obtained, was counted in an

619 automated Vicell cell counter before $2X10^6$ cells/well were cultured in Iscove's Modified

620 Dulbecco's Medium (IMDM) supplemented with 10% FBS and 10ng/ml Macrophage colony-

621 stimulating factor (M-CSF) (Biolegend), (growth media) in a 12 well plate. On Day 3, cells were

622 resuspended in fresh growth media and on Day 7, cells were passaged in fresh complete IMDM

623 containing 100ng/ml LPS (InvivoGen). On Day 8, the generated BMDMs were subjected to

624 antigen presentation and phagocytosis assay as outlined below.

625 **ChIP sequencing.** Both LPS stimulated and unstimulated BMDMs generated as described

626 above, were subjected to ChIP using the MAGnify Chromatin Immunoprecipitation System

627 (Applied Biosystems) according to the Manufacturer's protocol. Briefly, following crosslinking with

628 formaldehyde for 10 minutes, cells were resuspended in lysis buffer and subjected to sonication

629 to shear the DNA to 150-300 kb fragments. For each immunoprecipitation reaction 10μg of anti-

630 KDM6B (Active Motif) and anti H3K27me3 antibodies (Active Motif) were used. Following antibody

631 incubation, samples were washed and the DNA was purified and eluted. DNA concentration was

632 measured in Qubit using the dsDNA HS Assay kit (Invitrogen) and 10μg of DNA was sent for

633   sequencing to the MD Anderson Cancer Center Advanced Technology Genomics Core where

634   sequencing was done using the NextSeq500 instrument.

635   **ChIP sequencing data analysis.** The quality of CHIPseq FASTQ sequences generated as

636   described above, were assessed using FastQC, followed by mapping by bowtie2[62] with mouse

637   reference genome mm10. The bam files obtained from mapping were further processed using

638   SAMBLASTER[63] and SAMTOOLS[64], for duplicate removal, sorting and indexing. SAMBAMBA[65]

639   was used to Normalize bam files per read counts by performing random sampling. The ChIP-seq

640   signal enrichment over "Input" background was identified using Model based analysis of ChIP-

641   seq (MACS) version 3[66].The identified peaks were annotated using CHIPseeker[67],

642   clusterProfiler[68] and AnnotationDbi. Quantitative comparisions of different datasets were

643   performed with MAnorm[69]. The profile plots for specific genes were plotted using computeMatrix

644   and plotProfile programs of deepTools[70] and the gene tracks were plotted using EAseq version

645   1.111[71].

646   **RNA isolation and real-time PCR.** Total RNA was isolated from LPS stimulated BMDMs

647   generated from control and LysM[cre]KDM6B^{fl/fl} mice using the TriZol (Invitrogen) method according

648   to the Manufacturer's protocol. 1µg of the RNA was reverse transcribed into complementary DNA

649   (Superscript III cDNA kit from Invitrogen, USA) and the cDNA was used to measure the

650   expression of genes of interest via Real Time PCR (Applied Biosystems 7500 Fast, USA). Primers

651   used for real time PCR are as follows-

    MuSocs3 Forward- 5'-CGCCCAGGTCCTTTGCCTGA-3'

    MuSocs3 Reverse- 5'-CCGCATCCCGGGGAGCTAGT-3'

    MuMafb Forward- 5'-GGCAGGGAGTCTCTGTCGGC-3'

    MuMafb Reverse- 5'-CAGGCCCTCCGACCCCATCT-3'

652   **Phagocytosis assay with beads.** Uncoated carboxylate modified polystyrene fluorescent

653   orange beads (Sigma Aldrich) were added to 1×10^5 BMDMs generated using the protocol outlined

654   above, at a ratio of 500:1. The cells were incubated at 37 °C and 5% CO_2 for 4 hours before being

655   washed in PBS, fixed in 1% PFA and acquired in a flow cytometer to monitor uptake of beads by

656   the cells.

657   **Phagocytosis assay with GL261 cells.** CellTrace Far Red (Invitrogen) labelled GL261 cells

658   were mixed with CTV labelled BMDMs (generated as described above) at at ratio of 1:1 and

659   incubated at 37°C and 5% CO_2 for 2 hours before the mixture was washed with PBS, fixed in 1%

660   PFA and acquired in a flow cytometer to measure the uptake of GL261 cells by the BMDMs.

661   **Antigen presentation assay.** BMDMs generated above were pulsed with GP100 (1μg/ml) or

662   LCMV (1μg/ml, negative control) peptides (AnaSpec). Additionally, naïve CD8 T cells were

663   isolated from the spleen of pmel mice (having CD8 T cells bearing T cell receptors specific for the

664   gp100 antigen) by magnet-assisted cell sorting (Naïve CD8+ T cell isolation kit, Miltenyi Biotec)

665   and stained with Cell Trace Violet (CTV, Invitrogen). Subsequently, the antigen pulsed

666   macrophages and stained naïve CD8 T cells were co-cultured at a ratio of 1:2 for 3 days, washed,

667   fixed and subjected to flow cytometry (BD LSR II) to measure CTV dilution as a measure of T cell

668   proliferation in response to antigen presentation by the different BMDMs**.** FlowJo software v10

669   was used for analysis.

670   **GSK-J4 and anti-PD-1 treatment regimen:** 5–7-weeks old C57BL/6 mice bearing GL261 or

671   CT-2A tumors were treated with KDM6B inhibitor (GSK-J4; Sigma-Aldrich); 1mg per mouse, in

672   200ul volume (3% DMSO+ 97% sterile water - vehicle) via oral gavage daily starting from day 3

673   post tumor inoculation to the end of the experiment. GL261 tumor bearing mice were also injected

674   intraperitoneally with 200μg, 100μg and 100μg of α-PD1 (RMP1-14; Bio X Cell) on day 7, 10 and

675   13 respectively and sacrificed on day 17 for downstream analysis.

676 **Mass cytometry to study TME.** GL261 and CT-2A tumor tissues were dissected, processed,

677 stained with antibodies and acquired in a mass cytometer as previously described. Surface and

678 intracellular antibodies used for this mass cytometry are mentioned in Supplementary Table 2.

679 **Mass cytometry analysis.** Files were manually gated in FlowJo v10 by using iridium for cells,

680 event length for singlets, cisplatin for live/dead discrimination and using CD45 lineage marker for

681 immune cells. Clustering analysis was performed using the FlowSOM and ConsensusClusterPlus

682 packages as previously described [72]

683 **Statistical analyses.** R v4.0.2 and GraphPad Prism software v9 was used for the statistical

684 analyses. The individual tests performed have been indicated in the figure legends. All in-vivo

685 experiments had two to three independent replicates.

686

687 **Data availability.** Raw reads for the single cell RNA sequencing, single cell ATAC sequencing

688 and ChIP-sequencing will be deposited in European Genome-phenome Archive (EGA) which will

689 be available upon acceptance of the manuscript.

690 **Code availability.** The scRNA seq, scATAC seq and CHIP seq analyses presented in the

691 manuscript has been performed with open source algorithms as described in the method section.

692 Further details will be made available by authors upon request.

693

694 **Figure legend:**

695 **Fig. 1:** *Single-cell and spatial transcriptomic analyses of human GBM tumors demonstrated*

696 *selective expression of KDM6B in immune-suppressive myeloid cells.* **(A)** UMAP plot of scRNA

697 seq data depicting the different immune cell subsets (*CD45*+ cells) in the TME of patients with

698 GBM (n=5). **(B)** UMAP plot of myeloid cells obtained by reclustering the *CD68*+ clusters from Fig.

699 1A. **(C)** Dotplot showing the average expression of indicated genes as well as the percentage of

700 cells expressing the gene in the indicated myeloid clusters shown in Fig. 1C. **(D)** Feature plots of

701 scRNA seq data demonstrating the expression of indicated genes encoding epigenetic enzymes,

702 in intratumoral myeloid cell clusters. **(E)** Multiplex immunofluorescence staining shows distribution

703 of CD3 (red), CD8 (cyan), CD68 (yellow) and CD163 (magenta) in a GBM case. Inset shows

704 CD3+, CD8+ and CD68+CD163+ myeloid cells. **(F)** UMAP plot of scRNA seq data from patient 6

705 (Supplementary Table 1), showing *CD45*+ immune cell subsets in the GBM TME used to embed

706 single cells to their spatial coordinates in tissue sections by applying CellTrek. **(G)** Gene

707 expression data from all the different immune cell clusters from the matched patient plotted on

708 spatial coordinates. **(H)** Gene expression data from the *KDM6B* expressing myeloid cell clusters

709 (black) and CD8 T cell clusters (yellow), plotted on spatial coordinates**.**

710 **Fig. 2:** *Myeloid cell-specific deletion of Kdm6b improves survival in preclinical models of GBM*

711 *and results in a pro-inflammatory milieu in murine GBM tumors.* **(A)** Representative axial MRI

712 images taken on day 14 post tumor inoculation, of GL261 (top panel) and CT-2A (bottom panel)

713 tumors in control (left panel) and LysM^{cre}KDM6B$^{fl/fl}$ mice (right panel). Box and whisker plot

714 demonstrating the difference in tumor volume as calculated from MRI images of GL261 (top

715 panel) (n=5/group) and CT-2A (bottom panel) (n=10/group) GBM tumors from control and

716 LysM^{cre}KDM6B$^{fl/fl}$ mice. Two-tailed Student's t-test was performed (* p<0.05, ** p<0.01). **(B)**

717 Kaplan Meier plot depicting the difference in survival of GL261 (top panel) and CT-2A (bottom

718 panel) tumor-bearing control and LysM^{cre}KDM6B$^{fl/fl}$ mice (n=10/group). Log-rank test was

719   performed (***p<0.001, **** p<0.0001). Data is representative of 3 independent experiments. **(C)**

720   UMAP plot of scRNA seq data showing the different immune cell subsets *(Cd45+* cells) in the

721   GBM (GL261) TME of control and LysM^creKDM6B^fl/fl mice (pooled 4-5 samples/group). The data

722   is representative of 2 independent scRNA seq experiments. **(D)** Volcano plot representing

723   differentially expressed genes between control and LysM^creKDM6B^fl/fl mice in intratumoral

724   monocytes, macrophages and microglial-like cells (C0,1,10,12,14,15,23 in Fig. 2C). **(E)** Volcano

725   plot depicting differentially expressed genes between control and LysM^creKDM6B^fl/fl mice in

726   intratumoral DCs (C3,4,7,9,16,17,19 in Fig. 2C). Volcano Plots shows the fold change (log2 Ratio)

727   plotted against the Absolute Confidence (-log10 adjusted p value) **(F)** Plots representing GSEA

728   pathways in intratumoral monocytes, macrophages and DCs enriched in LysM^creKDM6B^fl/fl mice

729   as compared to control.

730   **Fig. 3:** *Kdm6b deletion alters the abundance and transcriptomic landscape of intratumoral*

731   *monocytes and macrophages*. **(A)** The left panel shows UMAP plot of scRNA seq data depicting

732   the different immune cell subsets (*Cd45+* cells) in the GBM (GL261) TME of control and

733   LysM^creKDM6B^fl/fl mice (pooled 4-5 samples/group, representative of two independent

734   experiments). The right panel shows UMAP plot obtained by reclustering of highlighted *Itgam*+

735   clusters from the left panel. **(B)** Dotplot showing the average expression of genes of interest as

736   well as the percentage of cells in the cluster expressing the gene in the indicated myeloid clusters.

737   **(C)** Bar plots representing the relative frequencies of indicated myeloid clusters in control and

738   LysM^creKDM6B^fl/fl mice. Volcano plots depicting differentially expressed genes in the indicated

739   intratumoral myeloid cell clusters from control and LysM^creKDM6B^fl/fl mice. The Volcano Plot

740   shows the fold change (log2 Ratio) plotted against the Absolute Confidence (-log10 adjusted p

741   value).

742   **Fig. 4:** *Myeloid cell-specific deletion of Kdm6b alters the chromatin landscape of key genes*

743   *regulating the functional phenotype of intratumoral myeloid cells.* **(A)** UMAP demonstrating the

744   different *Cd45*+ immune cell subsets in the GBM (GL261) TME of control and LysM^cre^KDM6B^fl/fl^

745   mice as determined from scATAC seq (4-5 pooled samples/group). **(B)** UMAP depicting the gene

746   activity score of the indicated genes in different immune cell subsets in the GBM TME of control

747   and LysM^cre^KDM6B^fl/fl^ mice. **(C)** Heatmap showing Z-scores of 153,638 cis-elements neighboring

748   indicated genes in the scATAC seq clusters derived from Fig.4A. **(D)** UMAP representation of

749   scATAC seq data depicting *Itgam*+ cell subsets in the GL261 TME of control and LysM^cre^KDM6B^fl/fl^

750   mice (pooled 4-5 samples/group). **(E)** Dotplot demonstrating the average gene activity score of

751   genes of interest as well as percentage of cells in the cluster expressing the gene in the indicated

752   myeloid cell clusters. **(F)** Bar graphs representing the relative frequencies of indicated myeloid

753   clusters from control and LysM^cre^KDM6B^fl/fl^ mice.

754   **Fig. 5:** *Kdm6b regulates H3K27me3 enrichment of genes regulating phagocytosis, antigen*

755   *presentation, and interferon response in myeloid cells.* **(A, D, G)** Profile plots depicting the

756   probability scores of KDM6B binding at -/+1kb regions from transcription start site (TSS) and

757   transcription end site (TES) of the *Socs3, Mafb* and *Sirpa* gene loci in bone marrow derived

758   macrophages (BMDMs) from control (grey) and LysM^cre^KDM6B^fl/fl^ (blue) mice. **(B, E, H)** Genome

759   browser view of H3K27me3 peaks at the *Socs3, Mafb* and *Sirpa* gene loci in control and

760   LysM^cre^KDM6B^fl/fl^ BMDMs. **(C, F)** Box and whisker plots showing the relative expression of

761   indicated genes normalized to the expression of β-actin as determined by quantitative PCR. One-

762   tailed Student's t-test was performed (*p<0.05, ***p<0.001, n=3-6/condition). **(I,J)** Box and

763   whisker plots representing the difference in phagocytic capacity between control and *Kdm6b*

764   deficient BMDMs, based on percentage of cells taking up beads **(I)** or GL261 cells **(J)**. Two-tailed

765   Student's t-test was performed (**p<0.01). n=5-6/group, representative of 2-4 independent

766   experiments. **(K)** Schematic representation of the antigen presentation and T cell proliferation

767   assay performed. **(L)** Representative pseudocolor flow cytometry plots showing the percentage

768   of proliferated CD8 T-cells (gated, CTV negative), upon co-culture with gp100 pulsed control and

769   *Kdm6b* deficient BMDMs. Box and whisker plot depicting the percentage of proliferated CD8 T-

770   cells upon co-culture with control versus *Kdm6b* deficient macrophages. (n=4, **p<0.01). Data is

771   representative of two independent experiments.

772   **Fig. 6:** *Pharmacological inhibition of KDM6B improves the efficacy of immune checkpoint therapy*

773   *in GBM.* **(A)** Box and whisker plot depicting the difference in the tumor volume as calculated from

774   day 14 MRI images of GBM (GL261) tumors from control (vehicle treated) and GSK-J4 treated

775   mice (n=10/group). Two-tailed Student's t-test was performed (**** p<0.0001). **(B)** Kaplan Meier

776   plot demonstrating the difference in survival of GBM (GL261) tumor-bearing mice treated with

777   vehicle and GSK-J4 (n=10/group). Log-rank test was performed (*** p<0.001). Data is

778   representative of 3 independent experiments. **(C)** UMAP plot of scRNA seq data representing the

779   different immune cell subsets (*Cd45*+ cells) in the GBM (GL261) TME of vehicle and GSK-J4

780   treated mice (pooled 4-5 samples/group). **(D)** Dotplot showing the average expression of genes

781   of interest as well as percentage of cells in the cluster expressing the genes defining the indicated

782   cell clusters. **(E)** Bar plots representing the frequencies of indicated immune cell clusters from

783   vehicle and GSK-J4 treated mice as determined by scRNA seq. **(F)** Box and whisker plot

784   representing the GBM (GL261) tumor weights from mice receiving the indicated treatments

785   (n=5/group). Two-tailed Student's t-test was performed (**p<0.01, ***p<0.001, ****p<0.0001). **(G)**

786   Kaplan Meier plot depicting difference in survival of GBM (GL261) tumor-bearing mice treating

787   with vehicle, anti-PD1, GSK-J4 and combination of anti-PD-1 plus GSK-J4. Log-rank test was

788   performed (*p<0.05, **p<0.01, ****p<0.0001). Data is representative of 2 independent

789   experiments. **(H)** t-SNE plot of CyTOF data demonstrating different immune cell subsets (CD45+

790   cells) in the GBM (GL261) TME of vehicle, GSK-J4 and anti-PD1 treated mice (n=5/group). **(I)**

791   Box and whisker plots showing the relative frequencies of indicated immune cell clusters from

792   vehicle treated and therapeutic agent treated mice as determined by CyTOF (n=5/group). Two-

793   tailed Student's t-test was performed (*p<0.05, **p<0.01, ***p<0.001,****p<0.0001). **(J)** Graphical

794   summary of the findings presented in this study depicting the role of KDM6B in regulation of

795   myeloid cell function and its importance as a therapeutic target in GBM. IFNGR - Interferon gamma

796   receptor. IFNAR - Interferon alpha receptor. GBM - Glioblastoma.

797   **Extended Data figure legend:**

798   **Extended Data Fig. 1: (A)** Dotplot showing the average expression of indicated genes as well as

799   the percentage of cells expressing the gene in the indicated *CD45*+ immune cell clusters in the

800   TME of patients with GBM (n=5) shown in Fig. 1A. **(B)** UMAP plot of scRNA seq data depicting

801   the expression of *KDM6B* gene in the different immune cell subsets (*CD45*+ cells). **(C)** Violin

802   plots demonstrating the expression level of indicated genes in the different myeloid cell clusters

803   from Fig. 1B.

804   **Extended Data Fig. 2: (A)** UMAP plot of scRNA seq data showing *CD45*+ immune cell subsets

805   in the GBM TME derived from patients with GBM (n=4)[32]. **(B)** Violin plots demonstrating the

806   expression level of indicated genes in the different immune cell clusters. **(C)** UMAP plot of scRNA

807   seq data depicting *CD45*+ immune cell subsets in the GBM TME derived from patients with GBM

808   (n=20)[37].**(D)** Violin plots representing the expression level of indicated genes in the different

809   immune cell clusters.

810   **Extended Data Fig. 3: (A)** Hematoxylin and Eosin stained GBM tumor sections. Each section

811   represent one patient. Scale included in the images. **(B)** Representative figures showing

812   immunohistochemical staining for KDM6B in GBM tissue samples from 3 patients (patient

813   number: 6, 2 and 3, Supplemental Table 1)

814   **Extended Data Fig. 4: (A, B)** UMAP plots of matched scRNA seq data from patient 2 and patient

815   3 (Supplementary Table 1) showing *CD45*+ immune cell subsets in the GBM TME used to embed

816   single cells to their spatial coordinates in tissue sections by applying CellTrek. **(C, D)** Gene

817   expression data of all the different immune cell clusters from matched patients plotted on spatial

818   coordinates. **(E, F)** Gene expression data from the *Kdm6b* expressing myeloid cell clusters (black)

819   and T cell clusters (yellow) plotted on spatial coordinates of two matched patients**.**

820   **Extended Data Fig.5(A-C)** Dotplots showing the average gene activity score of genes of interest

821   as well as percentage of cells in the cluster expressing the gene in the indicated clusters **(A)**

822   shown in Fig. 1F, and **(B,C)** shown in Fig. S4A,B.

823   **Extended Data Fig.6: (A)** Schematic representation demonstrating generation of the

824   LysM^cre^KDM6B^fl/fl^ genetic murine model. **(B)** Representative image of an agarose gel showing

825   bands depicting PCR amplified DNA from *Kdm6b* deleted homozygous mice (single 400bp band),

826   *Kdm6b* deleted heterozygous mice (both 368 and 400bp bands), and control homozygous mice

827   (single 368bp band).**(C)** t-SNE plots and box and whisker plots depicting the identity and

828   abundance of different immune cell populations present in the indicated anatomical locations in

829   control and LysM^cre^KDM6B^fl/fl^ mice as determined from CyTOF analysis (n=3/group).

830   **Extended Data Fig. 7: (A)** Heatmap showing the expression of genes of interest in the different

831   *Cd45+* immune cell clusters (shown in Fig. 2C). **(B)** Bar graphs depicting the frequencies of the

832   different intratumoral immune cell subsets in control and LysM^cre^KDM6B^fl/fl^ mice. **(C)** Bar graph

833   representing the ratio of intratumoral CTLs and Tregs in control and LysM^cre^KDM6B^fl/fl^ mice as

834   determined from scRNA seq. **(D)** Violin plots depicting the expression level of *Lyz2* in different

835   immune cell clusters in control and LysM^cre^KDM6B^fl/fl^ mice. Data representative of two

836   independent scRNA seq experiments.

837   **Extended Data Fig. 8: (A)** t-SNE plot of CyTOF data demonstrating different immune cell subsets

838   (CD45+ cells) in the GBM (CT-2A) TME of control and LysM^cre^KDM6B^fl/fl^ mice (n=10/group). **(B)**

839   Heatmap showing the expression of protein markers of interest in the indicated immune cell

840   clusters as determined by mass cytometry. **(C)** Box and whisker plots representing the relative

841   frequencies of indicated immune cell clusters from control and LysM^cre^KDM6B^fl/fl^ mice as

842   determined by CyTOF (control n=10/group, LysM^{cre}KDM6B$^{fl/fl}$ n=8/group). Two-tailed Student's t-

843   test was performed (*p<0.05, **p<0.01, ****p<0.0001). **(D)** Bar plots depicting the ratio of

844   intratumoral CTLs and Tregs in control and LysM^{cre}KDM6B$^{fl/fl}$ mice as determined by CyTOF.

845   **Extended Data Fig. 9: (A)** Heatmap showing the expression of genes of interest in the different

846   myeloid cell clusters (shown in Fig. 3A-right panel). **(B)** Bar plots representing the frequencies of

847   intratumoral myeloid clusters from control and LysM^{cre}KDM6B$^{fl/fl}$ mice. Data representative of two

848   independent experiments.

849   **Extended Data Fig. 10: (A)** UMAP demonstrating the CTL and Treg clusters in the GBM (GL261)

850   TME of control and LysM^{cre}KDM6B$^{fl/fl}$ mice determined by scATAC seq (as shown in Fig. 4 A-B).

851   Bar graphs depicting the frequencies and ratio of intratumoral CTLs and Tregs in control and

852   LysM^{cre}KDM6B$^{fl/fl}$ mice as determined from scATAC seq. **(B)** Coverage plots depicting the

853   chromatin accessibility of the indicated genes in the CTL and Treg clusters.

854   **Extended Data Fig. 11: (A)** Heatmap showing the expression of genes of interest in the indicated

855   myeloid cell clusters (shown in Fig. 4D). **(B, C)** Coverage plots depicting accessibility of indicated

856   chromatin regions (peaks) in genes of interest. **(D)** Representative gating strategy on FlowJo for

857   analysis of flow cytometry data showing GL261 phagocytosis by BMDMs (shown in Fig. 5J). **(E)**

858   Representative pseudocolor flow cytometry plots showing the percentage of proliferated CD8 T-

859   cells (gated, CTV negative), upon co-culture with LCMV pulsed control and *Kdm6b* deficient

860   BMDMs.

861   **Extended Data Fig.12:** Heatmap representing the expression of genes of interest in the indicated

862   *Cd45+* immune cell clusters as determined by scRNA seq (shown in Fig. 6C).

863   **Extended Data Fig. 13: (A)** Representative axial MRI images of CT-2A tumor from vehicle treated

864   mice (left panel) and GSK-J4 treated mice (right panel), taken on day 14 post tumor inoculation.

865   **(B)** Box and whisker plot showing the difference in CT-2A tumor volumes (determined from MRI)

866 between vehicle and GSK-J4 treated mice (n=10/ group). Two-tailed Student's t-test was

867 performed (*p<0.05). **(C)** Box and whisker plot depicting the difference in CT-2A tumor weight

868 (harvested on day 22 post tumor inoculation) between vehicle and GSK-J4 treated mice

869 (n=10/group). Two-tailed Student's t-test was performed (*p<0.05). **(D)** Heatmap demonstrating

870 the expression of protein markers of interest in the indicated CD45+ immune cell clusters in the

871 GBM (CT-2A) TME of vehicle & GSK-J4 treated mice as determined by mass cytometry.**(E)** t-

872 SNE plot of CyTOF data depicting different immune cell subsets (CD45+ cells) in the GBM (CT-

873 2A) TME of vehicle and GSK-J4 treated mice (n=5/group). **(F)** Box and whisker plots representing

874 the relative frequencies of indicated immune cell clusters from vehicle and GSK-J4 treated mice

875 as determined by CyTOF (n=5/group). Two-tailed Student's t-test was performed (**p<0.01).

876 **Extended Data Fig. 14:** Heatmap showing the expression of protein markers of interest in the

877 indicated CD45+ immune cell clusters as determined by mass cytometry (shown in Fig. 6H).

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Author information

S.G. developed the project, designed the experiments, analyzed data, wrote the manuscript and acquired funding. D.R, S.M.N, P.S performed the experiments, analyzed data and wrote the manuscript. Y.C, performed bioinformatics analyses. J.Z, M.H, S.A helped with the murine experiments. B.P.K, C.P and F.L provided human GBM tumor samples. M.M and S.J performed the H&E, IHC and IF staining of human GBM samples. S.B and Z.H helped with the human scRNA-sequencing and VISIUM analysis. P.S oversaw the study, provided scientific input, edited the manuscript and acquired funding.
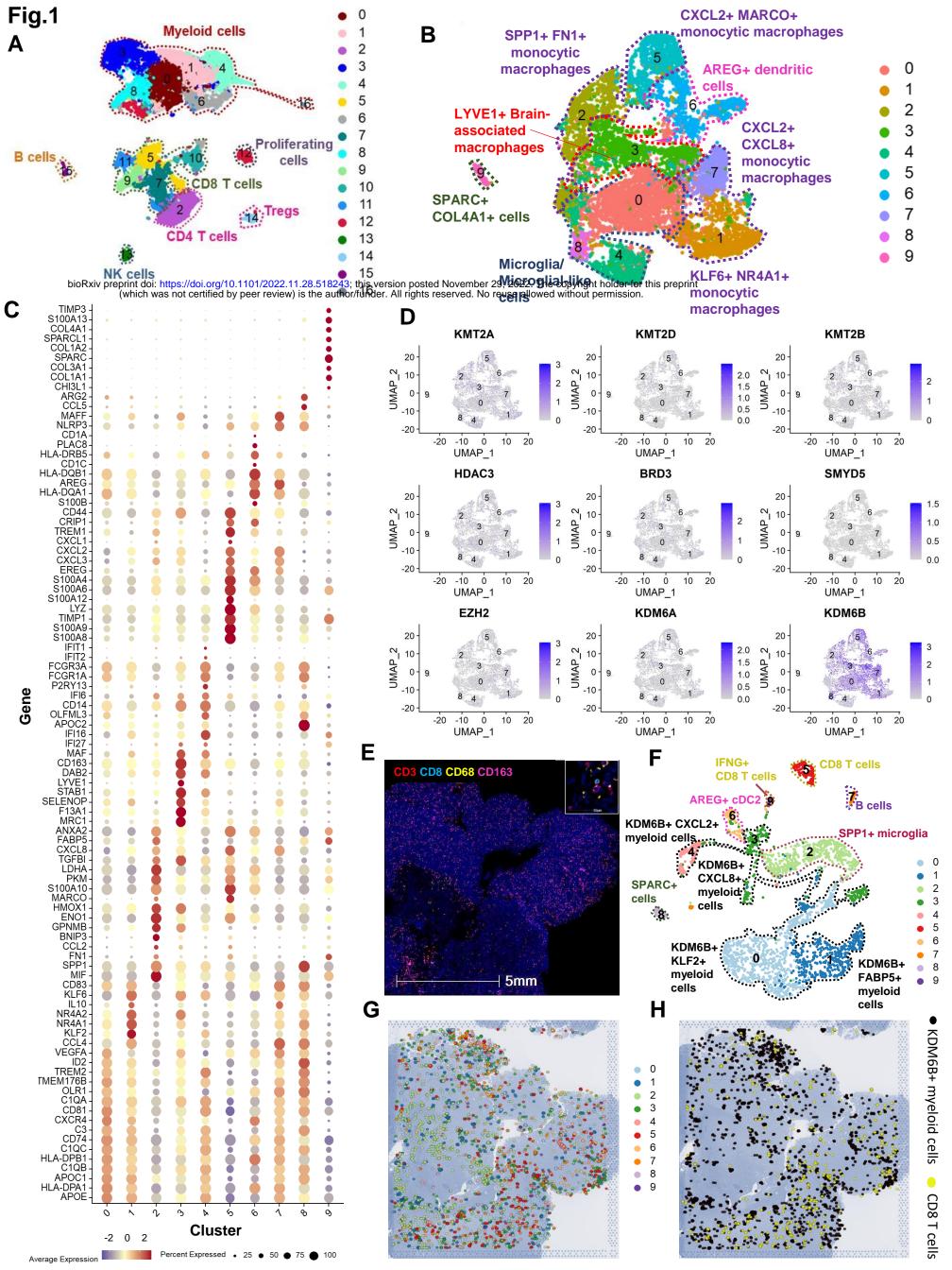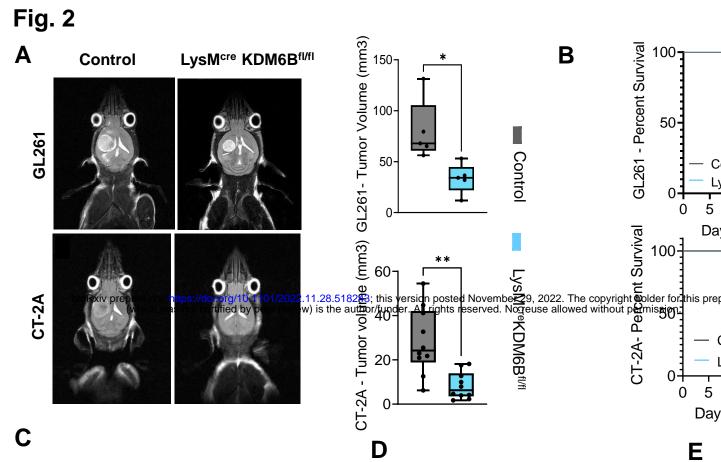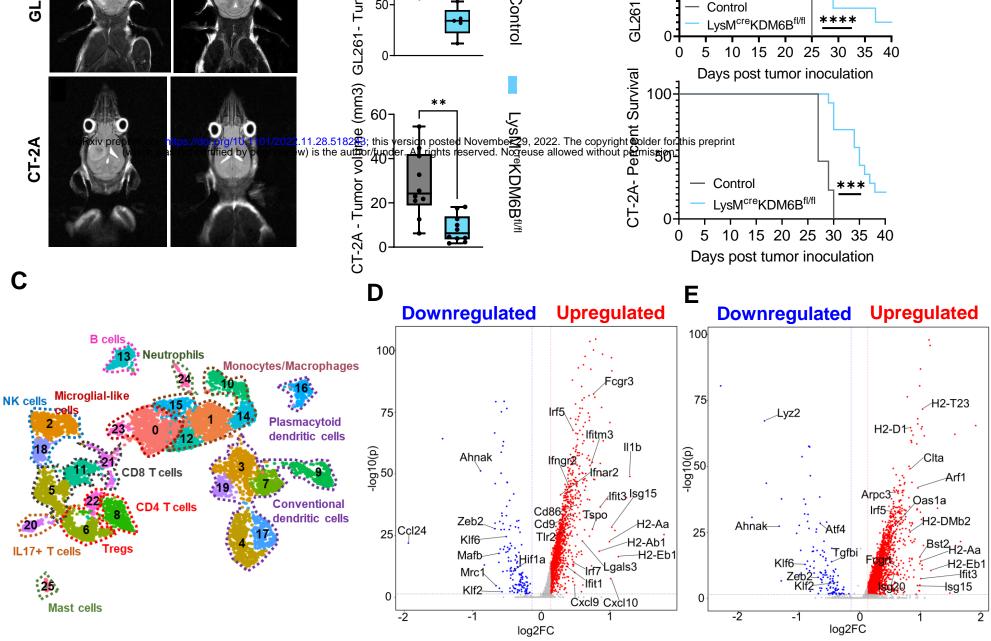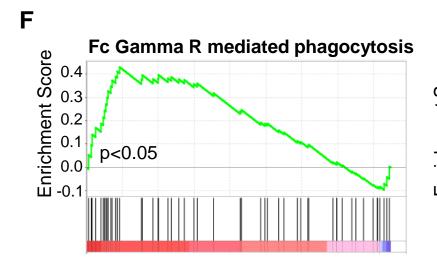
Ethics declarations

Competing interest

P.S. reports consulting, advisory roles, and/or stocks/ownership for Achelois, Apricity Health, BioAlta, Codiak BioSciences, Constellation, Dragonfly Therapeutics, Forty-Seven Inc., Hummingbird, ImaginAb, Jounce Therapeutics, Lava Therapeutics, Lytix Biopharma, Marker
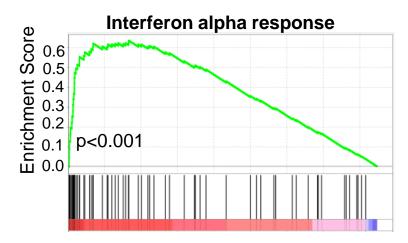
1085   Therapeutics, BioNTx, Oncolytics, Glympse, Infinity Pharma, and Polaris and owns a patent

1086   licensed to Jounce Therapeutics.

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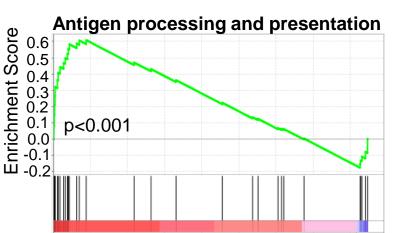
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Fig.1

A

Myeloid cells

B cells

Proliferating cells

CD8 T cells

CD4 T cells

Tregs

NK cells

B

SPP1+ FN1+ monocytic macrophages

CXCL2+ MARCO+ monocytic macrophages

AREG+ dendritic cells

LYVE1+ Brain-associated macrophages

CXCL2+ CXCL8+ monocytic macrophages

SPARC+ COL4A1+ cells

Microglia/Microglial-like cells

KLF6+ NR4A1+ monocytic macrophages

C

Gene / Cluster

D

KMT2A KMT2D KMT2B

HDAC3 BRD3 SMYD5

EZH2 KDM6A KDM6B

E

CD3 CD8 CD68 CD163

5mm

F

IFNG+ CD8 T cells

CD8 T cells

AREG+ cDC2

B cells

KDM6B+ CXCL2+ myeloid cells

SPP1+ microglia

KDM6B+ CXCL8+ myeloid cells

SPARC+ cells

KDM6B+ KLF2+ myeloid cells

KDM6B+ FABP5+ myeloid cells

G

H

KDM6B+ myeloid cells

CD8 T cells

Average Expression Percent Expressed

Fig. 2

A

Control   LysM^cre KDM6B^fl/fl

GL261

CT-2A

B

C

D Downregulated   Upregulated

E Downregulated   Upregulated

F

Fc Gamma R mediated phagocytosis

Antigen processing and presentation

Interferon alpha response

Interferon gamma response

Fig. 3

Fig.4

A

B

GA
0.0 2.5 5.0 7.5 10.0

C

Cis-regulatory elements

153,638 cluster ATAC-seq peaks

Z-score

Cluster

Macrophages DC Mast cells B cells NK cells CD4 T cells Treg CD8 T cells DC

D

0
1
2
3
4
5
6
7

E

Average Gene activity score
-1 0 1

Percent Expressed
20 40 60

Cluster

Lgals3 Trem2 Lgals3bp Cd9 Gpnmb Tlr3 Tlr12 Rsad1 Cd86 H2-Aa H2-Ab1 H2-Eb1 Cd14 Cd33

Gene

F

C0   C2   C4

% (of CD45+ cells)

Control
LysM^cre KDM6B^fl/fl

Fig. 5

A

Socs3

B

Socs3

C

***

Control

LysM^{cre}KDM6B^{fl/fl}

D

Mafb

E

Mafb

F

*

G

Sirpa

H

Sirpa

I

**

J

**

K

L

8.19%

43.8%

Control

LysM^{cre}KDM6B^{fl/fl}

CTV

**

Fig. 6