

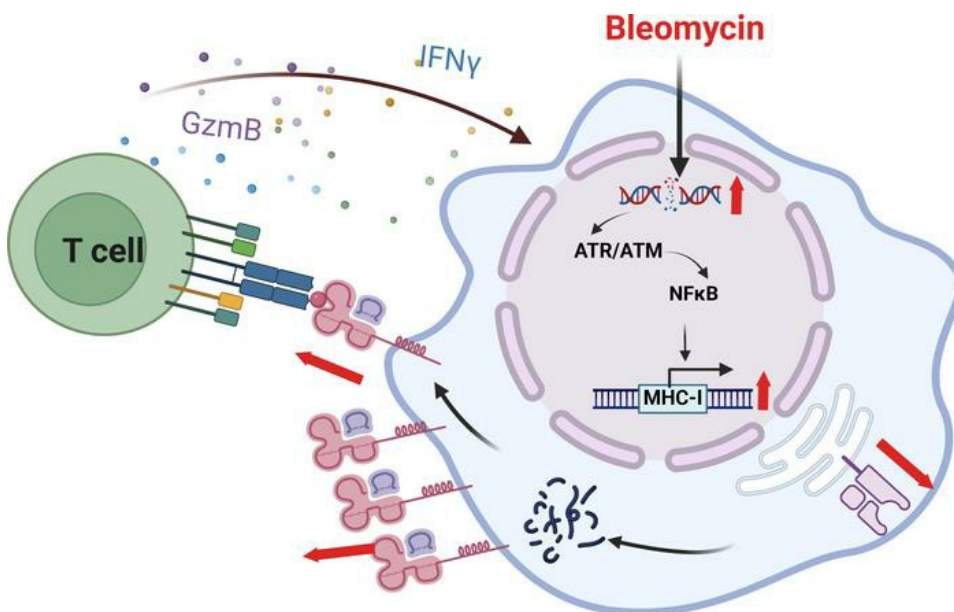
## Pharmacological induction of MHC-I expression in tumor cells revitalizes T cell anti-tumor immunity

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### Graphical abstract



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1 **Title: Pharmacological induction of MHC-I expression in tumor cells**  
2 **revitalizes T cell anti-tumor immunity**

3

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31 exists.

32

33 **Abstract**

34       Antigen presentation by Major Histocompatibility Complex Class I (MHC-I)  
35 is crucial for T-cell-mediated killing, and aberrant surface MHC-I expression is  
36 tightly associated with immune evasion. To address MHC-I downregulation,  
37 we conducted a high-throughput flow cytometry screen, identifying bleomycin  
38 (BLM) as a potent inducer of cell surface MHC-I expression. BLM-induced  
39 MHC-I augmentation renders tumor cells more susceptible to T cells in co-  
40 culture assays and enhances anti-tumor responses in an adoptive cellular  
41 transfer mouse model. Mechanistically, BLM remodels the tumor immune  
42 microenvironment, inducing MHC-I expression in an ATM/ATR-NF- $\kappa$ B-  
43 dependent manner. Furthermore, BLM improves T-cell-dependent  
44 immunotherapeutic approaches, including bispecific antibodies therapy,  
45 immune checkpoint therapy (ICT), and autologous tumor-infiltrating  
46 lymphocytes (TILs) therapy. Importantly, low-dose BLM treatment in mouse  
47 models amplified the anti-tumor effect of immunotherapy without detectable  
48 pulmonary toxicity. In summary, our findings repurpose BLM as a potential  
49 inducer of MHC-I, enhancing its expression to improve the efficacy of T-cell-  
50 based immunotherapy.

51

## 52 **Introduction**

53 Immunotherapy has revolutionized cancer treatment, demonstrating  
54 remarkable clinical efficacy across diverse tumor types (1). Despite these  
55 achievements, challenges persist, including variable response rates and the  
56 evasion of immune surveillance by malignant cells representing marked  
57 hurdles (2, 3). A pivotal mechanism in immune evasion is the downregulation  
58 of cell surface Major Histocompatibility Complex Class I (MHC-I),  
59 compromising T-cell-mediated killing (2, 4, 5).

60 Human MHC-I molecules, commonly known as HLA, play a crucial role in  
61 antigen presentation to T cells and tumor immune escape (6). Previous  
62 studies underscore a positive correlation between MHC-I expression and  
63 patient prognosis across various cancer types (7). Conversely,  
64 downregulation of MHC-I has been associated with disease progression and  
65 unfavorable prognosis in diverse cancers, such as breast carcinoma (8), colon  
66 carcinoma (9), Hodgkin Lymphoma (10), non-small cell lung cancer (11), and  
67 bladder carcinomas (12). Importantly, reduced MHC-I expression has been  
68 linked to resistance to immune checkpoint therapy (ICT) (13), where the  
69 therapeutic efficacy relies on cytotoxic T cells recognizing cytosolic antigens  
70 presented by MHC-I on the tumor cell surface (14, 15).

71 Diverse strategies employed by malignant cells to subvert immune  
72 surveillance underscore the crucial need for identifying effective small  
73 molecules capable of overcoming these evasion mechanisms. While immune

74 checkpoint blockage has proven to be a pivotal therapeutic strategy to thwart  
75 immunosurveillance, the focus has predominantly centered on antibodies.  
76 Small molecules targeting PD-1/PD-L1 function have also emerged and  
77 entered to clinical trials. However, investigations into molecules specifically  
78 regulating MHC-I expression have been comparatively limited. Notably,  
79 previous observations have demonstrated increased MHC-I expression  
80 following treatment with cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitors  
81 or the EZH2 inhibitor GSK126 for seven days (16, 17), indicating the potential  
82 of small molecules in regulating MHC-I expression. However, there is a need  
83 to explore effective strategies for improving MHC-I expression and function.

84 The expression of MHC-I is regulated by multiple regulators and pathways  
85 including IRF1, STAT1 and NRF5 (18). Among them, the transcription factor  
86 NF- $\kappa$ B plays a crucial role in the regulation of MHC-I (19). Activation of NF- $\kappa$ B  
87 was shown to promote increased expression of MHC-I and counter the  
88 immune evasion employed by cancer cells (20, 21).

89 In this context, the present study aims to address the challenge of low  
90 MHC-I expression on tumor cells. Leveraging a high-throughput flow  
91 cytometry system, we systematically screened for small molecules capable of  
92 rapidly enhancing surface MHC-I expression. Our investigations led to the  
93 identification and validation of bleomycin (BLM) as a potent inducer of MHC-I  
94 expression in tumor cells. Renowned as an antibiotic chemotherapeutic agent  
95 with established applications in various cancers, the clinical use of BLM has

96 been constrained by its side effect on lung injury. This investigation delves  
97 into the multifaceted effects of BLM. Our study unveils BLM's ability to  
98 promote CD8<sup>+</sup> T cell activation through antigen-dependent mechanisms, a  
99 phenomenon that is substantiated both ex vivo and in vivo. Moreover, BLM  
100 emerges as a regulator of the tumor immune microenvironment, inducing  
101 MHC-I expression in a manner dependent on ATM/ATR-NF-κB signaling.  
102 Importantly, our findings indicate the synergistic potential of low-dose BLM  
103 treatment when combined with immunotherapy and DNA methyltransferase  
104 inhibitors, all while avoiding detected toxicity. To validate the translational  
105 impact of our study, we extended our findings to a clinically relevant  
106 experimental setting. Here, BLM demonstrates its ability to heighten the  
107 susceptibility of patient-derived tumor cells to cytotoxicity mediated by  
108 autologous tumor-infiltrating lymphocyte (TILs). This not only underscores the  
109 potential clinical relevance of BLM but also repurposes it as a key player in  
110 enhancing the efficacy of immunotherapeutic interventions.  
111

## 112 **Results**

### 113 **BLM upregulates MHC-I expression**

114 To identify drugs with the potential to notably upregulate MHC-I expression,  
115 we conducted a screening of 2,112 FDA-approved drugs utilizing a high-  
116 throughput flow cytometry system based on tumor cell surface MHC-I  
117 expression. This screening identified several compounds, including BLM  
118 sulfate, etoposide, cabazitaxel, entinostat, and CI994, as robust enhancers of  
119 MHC-I expression (Supplemental Figure 1). Notably, the ability of paclitaxel to  
120 promote MHC-I expression in tumor cells has been previously reported (7),  
121 explaining the similar capability observed in cabazitaxel, an analog of  
122 paclitaxel. Etoposide (7) and HDAC inhibitors such as entinostat and CI994  
123 have also demonstrated their ability to modulate MHC-I expression in cancer  
124 cells (22-24). Therefore, we focused our further investigations on BLM, an  
125 antibiotic chemotherapeutic drug.

126 Flow cytometry analysis unequivocally demonstrated a dose- and time-  
127 dependent increase in MHC-I cell surface expression in SU-DHL-4 cells  
128 following BLM treatment (Figure 1, A-D). In addition, HLA-A protein levels  
129 showed a parallel increase in a time- and dose-dependent manner in both  
130 SU-DHL-4 (Figure 1, E and F) and SK-BR-3 (Supplemental Figure 2A) cells.  
131 Specifically, genes encoding MHC-I molecules (*HLA-A*, *HLA-B*, *HLA-C*, and  
132 *B2M*), peptide transport (*TAP1* and *TAP2*), transporter-MHC interactions  
133 (*TAPBP*), and peptide degradation (*PSMB8* and *PSMB9*) were upregulated in



134 BLM-treated SU-DHL-4 (Figure 1G) and SK-BR-3 (Supplemental Figure 2A)  
135 cells. Moreover, in vitro BLM treatment induced increased MHC-I expression  
136 in various human tumor cell lines, including T-47D, MDA-MB-231, and BT549  
137 (Supplemental Figure 2, B-D). Also, besides HLA-A, the expression level of  
138 HLA-B and HLA-C were also increased following BLM treatment in SK-BR-3  
139 cells, indicating that BLM acted in an allele-independent manner  
140 (Supplemental Figure 2E). Importantly, BLM exhibited a lasting effect on the  
141 induction of MHC-I expression, persisting even after drug retrieval  
142 (Supplemental Figure 2F). Analysis of The Cancer Genome Atlas (TCGA)  
143 data confirmed that the BLM-treated signature correlated with MHC-I  
144 expression in human cancers, showing a positive correlation across  
145 numerous cancer types with gene signatures of the MHC Class I pathway  
146 (Supplemental Figure 2F). Notably, in certain cancer types, such as LUSC  
147 and SARC, the BLM-treated signatures exhibited a negative correlation with  
148 the pathway. (Supplemental Figure 2F), which was possibly due to the  
149 heterogeneity between different tumor types.

150 The BLM-induced increase in cell surface MHC-I expression was also  
151 evident in murine tumor cell lines (B16F10, MC38, and MB49) (Supplemental  
152 Figure 3, A-C). Western blot analysis further confirmed a dose- and time-  
153 dependent increase in murine B2M protein levels on B16F10 cells following  
154 BLM treatment (Supplemental Figure 3D). Similarly, genes encoding murine  
155 MHC-I molecules (*H2d1*, *H2k1*, and *B2m*), those governing peptide transport

156 (*Tap1* and *Tap2*), and those involved in peptide degradation (*Psmg9*) were  
157 upregulated in BLM-treated murine cells (Supplemental Figure 3E).  
158 Collectively, these findings underscore the effective role of BLM in enhancing  
159 MHC-I expression in tumor cells.

160

161 **BLM-mediated increase in peptide-MHC-I complexes primes CD8<sup>+</sup> T cell**  
162 **activation by antigen-dependent mechanism**

163 Given the observed upregulation of antigen presentation gene expression  
164 by BLM, we hypothesized that it might enhance the function of MHC-I in  
165 antigen presentation. To investigate this hypothesis, we employed a peptide  
166 pulsing assay to evaluate cell surface expression of peptide-MHC-I  
167 complexes. Following peptide pulsing, B16F10 cells exhibited inadequate  
168 presentation of MHC-I-bound SIINFEKL complexes, an 8-amino acid peptide  
169 derived from OVA. However, pre-treatment with BLM dramatically enhanced  
170 the expression of these complexes (Supplemental Figure 4, A-D). Similarly,  
171 the murine cell line B16OVA, expressing ovalbumin (OVA), exhibited an  
172 increase in MHC-I-bound SIINFEKL complexes upon BLM treatment,  
173 indicating that BLM enhances antigen presentation. (Supplemental Figure 4,  
174 E and F).

175 Solid tumors often evade anti-tumor immunity by downregulating MHC-I  
176 surface expression, resulting in reduced recognition and responses by CD8<sup>+</sup> T  
177 cells (7). To investigate whether the increase in peptide-MHC-I complexes

178 induced by BLM enhanced CD8<sup>+</sup> T cell activation and tumor-killing capability,  
179 we conducted a co-culture assay of B16OVA tumor cells and OT-I T cells.  
180 Importantly, BLM pre-treated B16OVA tumor cells exhibited increased  
181 susceptibility to the cytotoxicity of MHC-I-restricted OVA-specific CD8<sup>+</sup> T cells  
182 (pre-activated OT-I T cells) compared to the control group. This was  
183 evidenced by a lower number of viable tumor cells, a higher apoptosis rate,  
184 and increased Interferon-gamma (IFN- $\gamma$ ) production (Figure 1, H-J). In  
185 contrast, B16F10 cells, owing to the absence of the cognate antigen OVA,  
186 remained resistant to OT-I T cell killing and failed to induce T cell cytokine  
187 production, even when exposed to relatively low concentrations of BLM  
188 (Figure 1, H-J). In line with this, TCGA analysis revealed a positive correlation  
189 between the BLM-treated signature and CD8<sup>+</sup> T cell activation in several  
190 human cancers (Supplemental Figure 4G). These results clearly demonstrate  
191 that BLM treatment sensitizes tumor cells to CD8<sup>+</sup> T cell-mediated killing.

192 Additionally, we utilized human CD8<sup>+</sup> T cells that were engineered with a  
193 recombinant T cell receptor (TCR) targeting the NY-ESO-1 antigen  
194 (specifically the NY-ESO-1:157–165 epitope) in an HLA-A\*02-restricted  
195 fashion (referred to as ESO T cells) (25). Then we performed the co-culture  
196 assay of NY-ESO-1<sup>+</sup> SK-BR-3 cells and ESO T cells to examine the effect of  
197 BLM in the scenario of human cancer (Supplemental Figure 5, A and B).  
198 Consistent with the results from the co-culture assay of B16OVA cells and  
199 OT-I T cells, NY-ESO-1<sup>+</sup> SK-BR-3 cells pre-treated with BLM showed

200 significantly increased apoptosis rates compared to the control group  
201 (Supplemental Figure 5, C and D). This indicates that BLM treatment  
202 sensitizes human cancer cells to CD8<sup>+</sup> T cell-mediated killing.

203 To examine whether BLM treatment affects antigen-independent activation  
204 of T cells, an ex vivo splenocyte culture assay was employed (26).  
205 Splenocytes treated with BLM or Concanavalin A (Con A, an antigen-  
206 independent mitogen) for 24 h were analyzed using flow cytometry to assess  
207 the frequency of CD44<sup>+</sup> and CD69<sup>+</sup> markers, which are indicative of T cell  
208 activation among CD8<sup>+</sup> or CD4<sup>+</sup> T cells (Supplemental Figure 6A). As  
209 expected, Con A induced the expression of CD44<sup>+</sup> and CD69<sup>+</sup> markers in  
210 CD8<sup>+</sup> cells or CD4<sup>+</sup> T cells, while BLM treatment had no such effect  
211 (Supplemental Figure 6, B-E). This firmly rules out the possibility of antigen-  
212 independent activation of T cells following BLM treatment.

213 In summary, these results confirm that BLM treatment promotes CD8<sup>+</sup> T  
214 cell activation through antigen-dependent mechanisms.

215

### 216 **BLM potentiates the anti-tumor responses of T cells in vivo**

217 To investigate the anti-tumor effect of BLM in vivo, we used combination of  
218 BLM and adoptive T cell transfer. To determine an effective anti-tumor  
219 concentration, we noticed in a previous study that a low dose (5 mg/kg) of  
220 BLM moderately reduced tumor volume without significant changes in mouse  
221 weight or evident lung toxicity (27). Therefore, we selected 3 mg/kg BLM for

222 subsequent experiments.

223 We investigated whether the combination of BLM and the infusion of OT-I  
224 cytotoxic T lymphocytes could enhance the killing efficacy of OT-I cells in an  
225 adoptive cellular transfer mouse model (Figure 2A). All treated groups  
226 exhibited body weights similar to those of the vehicle group (Figure 2B). To  
227 assess potential lung toxicity, a primary adverse effect of clinical BLM uses in  
228 cancer treatment (27), we conducted H&E staining, revealing no discernible  
229 lung damage in either the BLM treatment group or the combination treatment  
230 group (Supplemental Figure 7).

231 Mice treated with a relatively lower dose of BLM alone did not exhibit a  
232 remarkable anti-tumor response. Infusion of OT-I cells alone led to a  
233 slowdown in tumor growth, whereas the combination treatment resulted in  
234 further reductions in tumor weight (Figure 2C) and tumor volume (Figure 2D)  
235 compared to mono-OT-I cell therapy. Additionally, western blot analysis  
236 demonstrated that BLM elevated B2M expression in tumor samples (Figure  
237 2E). Immunofluorescence staining of tumor tissues revealed slightly increased  
238 infiltration of granzyme B<sup>+</sup> cells in the BLM-treated group compared to that in  
239 the vehicle group. Importantly, the combination treatment group exhibited a  
240 higher percentage of granzyme B<sup>+</sup> cells than the mono-OT-I cell therapy  
241 group (Figure 2F). Further analysis indicated that combination treatment  
242 significantly upregulated the gene expression of granzyme B, IFN- $\gamma$ , and  
243 perforin, secreted by cytotoxic CD8<sup>+</sup> T cells (Figure 2G). BLM also sensitized

244 B16OVA melanomas to OT-I cell transfer therapy, resulting in a substantial  
245 survival benefit (Figure 2H).

246 To assess whether BLM influenced T cell homing to tumors, we employed  
247 BrdU analysis to track T cells division in tumors after intravenous transfer of  
248 pre-activated CD45.1<sup>+</sup> OT-I cells into B16-OVA tumor-bearing mice for three  
249 days. Notably, the combination treatment group exhibited a higher density of  
250 OT-I cells in tumors than the monotherapy group with OT-I cell infusion  
251 (Figure 2, I and J). However, there was no difference in the percentage of  
252 proliferating of OT-I cells among tumors in tumor-bearing mice following  
253 treatment with OT-I cells alone or in combination with BLM and OT-I cells, as  
254 measured by BrdU incorporation (Figure 2K), indicating that the increased T  
255 cell homing, instead of T cell proliferation, was responsible for the effect of  
256 BLM.

257 In summary, these findings suggest that BLM treatment enhances T cell  
258 homing to tumors, thereby amplifying the anti-tumor responses of OT-I cells in  
259 adoptive T cell therapy in a mouse model.

260

261 **MHC-I is indispensable for the effect of BLM on promoting T cell anti-**  
262 **tumor immunity**

263 To ascertain the role of antigen recognition by cancer cells in BLM's anti-  
264 tumor effects, we conducted knockdown (KD) experiments targeting *B2m*, a  
265 pivotal component of MHC-I molecules, in B16OVA cells (Supplemental

266 Figure 8A). When co-cultured with OT-I T cells, BLM enhanced the  
267 susceptibility of B16OVA cells to CD8<sup>+</sup> T cell killing, whereas *B2m* KD  
268 B16OVA cells remained resistant to OT-I T cells (Figure 3A). T cell activation,  
269 as indicated by IFN- $\gamma$  release, returned to the control levels when *B2m* was  
270 disrupted in B16OVA cells (Figure 3B). Moreover, we observed that the levels  
271 of STAT1 phosphorylation and PD-L1 expression after IFN- $\gamma$  treatment were  
272 unaffected by MHC-I inhibition (Supplemental Figure 8, B-D).

273 Subsequently, we implanted *B2m* KD B16OVA clones into mice and  
274 assessed the anti-tumor effect of BLM in combination with OT-I cytotoxic T  
275 lymphocyte infusion in adoptive T cell transfer therapy (Figure 3C). While BLM  
276 treatment alone led to a modest decrease in tumor growth in the negative  
277 control clone, this effect was significantly amplified when combined with OT-I  
278 T cells (Figure 3, D and E). In contrast, the disruption of *B2m* in B16OVA cells  
279 attenuated the anti-tumor effect mediated by OT-I T cells, rendering the  
280 combination treatment with BLM ineffective in slowing tumor growth (Figure 3,  
281 D and E).

282 To further confirm that the increased T cell killing effect of BLM was  
283 dependent on the upregulation of MHC-I expression, we overexpressed the  
284 *H2k1* gene in B16OVA cells to increase the surface expression of H-2K<sup>b</sup>  
285 (Figure 3F). In B16OVA cells overexpressing the *H2k1* gene pretreated with  
286 BLM, no significant increase in T cell-mediated cytotoxicity was observed  
287 when co-cultured with OT-I T cells compared to the control group (Figure 3, G

288 and H). This suggests that the effect of BLM is dependent on the upregulation  
289 of MHC-I.

290 In summary, these findings underscore the substantial impact of MHC-I on  
291 anti-tumor effect of BLM.

292

### 293 **BLM treatment remodels the tumor microenvironment**

294 To investigate the potential molecular mechanism of anti-tumor responses  
295 of BLM treatment in B16-F10 melanoma, we performed unsorted Single-cell  
296 RNA sequencing, yielding 26,954 high-quality transcriptomes after quality  
297 control and filtering (Supplemental Figure 9A). To determine which cellular  
298 compartments that account for the highest BLM efficacy, we analyzed single-  
299 cell transcriptomes for the expression of melanoma, immune, fibroblast, and  
300 stromal marker genes. Consistent with above results, BLM treatment group  
301 exhibited an overrepresentation of immune cell transcriptomes compared to  
302 the control group (Figure 4A). Next, we subset and re-clustered immune cells  
303 into macrophages, monocytes, dendritic cells (DC), T cells, and neutrophils  
304 (Supplemental Figure 9B). We employed the Cellchat package to compute the  
305 total number of interactions and interaction strength of the inferred cell-cell  
306 communication networks, which were both significantly increased after BLM  
307 treatment (Figure 4B). We also observed significantly increased cell-cell  
308 interaction strength and interaction numbers among different cell types,  
309 especially signals sent from melanoma to other cell types, in the BLM group



310 compared with those in the control groups (Supplemental Figure 9, C and D).  
311 To further dissect the influence of BLM within the melanoma cell compartment,  
312 melanoma cell transcriptomes were subset and re-clustered into six  
313 subclusters named Mel0 to Mel5 by UMAP analysis (Figure 4C). The CNV  
314 scores in the BLM group among the six melanoma subclusters (Mel0 to Mel5)  
315 were significantly lower than those in the control group (Figure 4D). Using  
316 CytoTRACE, we observed that the Mel3 subcluster with the highest  
317 CytoTRACE score (Supplemental Figure 9, E and F), which was regarded as  
318 the starting point of the trajectory by monocle3. We suggested that the ends  
319 of the pseudotime trajectories of the other melanoma subclusters were the  
320 different end states of the cancer cells (Supplemental Figure 9G). Additionally,  
321 we found that the BLM group was more differentiated, which indicated a less  
322 malignant phenotype (28) (Supplemental Figure 9H).

323 Subsequently, we performed GO enrichment analysis to investigate the  
324 various biological processes of melanoma sub-clusters (Supplemental Figure  
325 10, A-C). The Mel1 subcluster enriched DNA proliferative pathways such as  
326 “DNA replication,” “nuclear division,” “chromosome segregation,” suggesting  
327 that it was at a relatively high proliferative status (Supplemental Figure 10, A-  
328 C). The Mel3 subcluster enriched MHC-I related pathways such as “TAP2  
329 binding,” “TAP1 binding,” “TAP binding,” “MHC class I peptide loading  
330 complex” (Figure 4E). Therefore, we regarded the Mel3 sub-cluster as the  
331 MHC-I-active sub-cluster. Additionally, compared with the control group, the

332 BLM group showed an increase in Mel3 subcluster transcriptomes and a  
333 decrease in Mel1 subcluster transcriptomes (Figure 4C). Subsequently, we  
334 used high-dimensional weighted gene co-expression network analysis  
335 (hdWGCNA) to determine the main molecular characteristics of Mel3. We  
336 identified thirty gene modules, and the functions of the M25 module were  
337 associated with the MHC class I protein complex pathway (Figure 4F).  
338 Moreover, the hub genes (*H2-D1*, *H2-K1*, *B2m*, *H2-T22*, and *H2-T23*) of M25  
339 were also closely related to the MHC-I pathway (Figure 4G).

340 We quantified oncogenic signal strengths using pathway target gene  
341 signature expression and discovered that the BLM group exhibited elevated  
342 activity in many signaling pathways, including JAK-STAT, NF- $\kappa$ B, TNF $\alpha$ , p53,  
343 VEGF, EGFR, TGF $\beta$ , and WNT, as well as in hypoxia-induced pathways. In  
344 contrast, the control group exhibited low activity for these pathways  
345 (Supplemental Figure 10D). The activities of TGF $\beta$ , VEGF, EGFR, WNT, p53,  
346 NF- $\kappa$ B, TNF $\alpha$  signaling, and hypoxia-induced pathways increased to varying  
347 degrees after BLM treatment in the Mel3 subcluster, suggesting that these  
348 pathways might be involved in the regulation of MHC-I expression (Figure 4H).

349 Consistent with our previous experimental results, single-cell RNA  
350 analysis further verifies that BLM treatment enhances immune cell infiltration,  
351 reduces the degree of malignancy and differentiation potential of melanoma  
352 cells, and promotes MHC-I-active subcluster expression, which reveals the  
353 anti-tumor efficacy of BLM.

354

355 **MHC-I upregulation caused by BLM depends on ATM/ATR-NF-κB**  
356 **activation**

357 We used LISA (29) to identify key transcription factors that drove changes  
358 in gene expression caused by BLM treatment, highlighting specific  
359 transcription factors linked to BLM. This analysis predicted that RELA most  
360 likely influenced the upregulated differentially expressed genes (Figure 5A).  
361 Additionally, MHC-I expression is regulated by various transcription factors  
362 that bind to the MHC-I promoter (30). Among these, IRF-1 (31), NF-κB (7),  
363 and NLRC5 (32) are crucial for the transcriptional upregulation of MHC-I  
364 genes following cytokine stimulation. Intriguingly, NF-κB was significantly  
365 induced following BLM treatment (Supplemental Figure 11A). Moreover, the  
366 levels of phosphorylated p65 significantly increased after BLM treatment  
367 (Figure 5B). To investigate whether NF-κB plays a role in BLM-induced MHC-I  
368 upregulation, we pre-treated SK-BR-3 cells with or without the NF-κB inhibitor  
369 BAY11-7082. BAY11-7082 reversed the BLM-induced upregulation of HLA-A  
370 and phosphorylated p65 caused by BLM treatment (Figure 5C). Notably, HLA-  
371 A expression showed no significant change upon BLM treatment in the *P65*  
372 knockdown groups (Figure 5D). qRT-PCR analysis confirmed that the  
373 increased mRNA expression of *HLA-A*, *HLA-B*, and *HLA-C* after BLM  
374 treatment was also blocked by the knockdown of *P65* (Figure 5E).  
375 Furthermore, BLM treatment led to dose- and time-dependent reduction in IκB

376 protein levels (Supplemental Figure 11, B and C). Collectively, these data  
377 indicate that NF- $\kappa$ B activation plays a critical role in BLM-induced MHC-I  
378 upregulation.

379 BLM possesses radiomimetic properties, induces DNA double-strand  
380 breaks, and is widely used in clinical chemotherapy for various cancers (33).  
381 Consistent with previous studies, we observed dose- and time-dependent  
382 increases in the expression of DNA damage response markers, including  
383  $\gamma$ H2AX (S139), phosphoATM (S1981), and p53 (S15), following BLM  
384 treatment (Supplemental Figure 11, D and E). Furthermore,  
385 immunofluorescence revealed an elevated number of  $\gamma$ H2AX foci in SK-BR-3  
386 cells after BLM treatment (Figure 5F). To assess whether MHC-I upregulation  
387 due to BLM was influenced by the DNA damage response pathway, we pre-  
388 treated cells with an ATM inhibitor (Ku60019), ATR inhibitor (AZD6738), or  
389 DNA-PKcs inhibitor (NU7441) before subjecting them to BLM treatment for 48  
390 h. BLM-induced MHC-I upregulation was diminished in ATM or ATR inhibitor-  
391 pre-treated cells (Figure 5G), but not in DNA-PKcs inhibitor-pre-treated cells  
392 (Supplemental Figure 11F). In summary, these findings suggest that MHC-I  
393 upregulation following BLM treatment is contingent on ATM/ATR-NF- $\kappa$ B  
394 activation.

395 The activated cyclic GMP-AMP synthase (cGAS)/stimulator of interferon  
396 genes (STING) pathway also promotes MHC-I mRNA expression by  
397 increasing the expression of type I interferon (IFN- $\alpha/\beta$ ) (34, 35). Therefore, we

398 explored whether the cGAS-STING pathway is activated by BLM treatment.  
399 Our results, following *STING* knockdown, indicated that the cytosolic DNA-  
400 sensing pathway was not necessary for MHC-I induction after BLM treatment  
401 in SK-BR-3 cells (Supplemental Figure 11G). Similarly, MHC-I expression  
402 remained unchanged after BLM treatment in the suppression of *TRAF6*  
403 (Supplemental Figure 11H). Additionally, the tumor suppressor p53, a crucial  
404 effector of the DNA damage response, is phosphorylated and activated by  
405 various DNA damage-inducible kinases including ATM(36). Previous studies  
406 have shown that p53 expression increases following BLM treatment (37). We  
407 speculated that p53 played a role in BLM-induced MHC-I upregulation.  
408 However, MHC-I expression showed no significant difference after BLM  
409 treatment in *TP53* knockdown cells (Supplemental Figure 11I). Consequently,  
410 p53 was deemed unnecessary for MHC-I induction following BLM treatment.

411 IFN- $\gamma$  secreted by activated T cells, plays a pivotal role in the activation of  
412 cellular immunity and, consequently, the stimulation of anti-tumor immune  
413 responses (38). We investigated whether BLM treatment enhances the IFN- $\gamma$ -  
414 induced transcriptional response in tumor cells. Gene Set Enrichment  
415 Analysis (GSEA) showed that IFN- $\alpha/\gamma$  and inflammatory pathways were  
416 transcriptionally activated in BLM-treated tumor cells (Figure 5H). Furthermore,  
417 we observed that BLM induced additional MHC-I upregulation in the presence  
418 of a relatively low dose of IFN- $\gamma$ , suggesting that BLM enhances HLA  
419 presentation through an IFN-independent mechanism (Figure 5I and

420 Supplemental Figure 11, J and K).

421 In summary, our data demonstrate that BLM-induced MHC-I upregulation  
422 in tumor cells relies on ATM/ATR-NF- $\kappa$ B activation.

423

424 **DNA methyltransferase inhibition synergizes with BLM to induce anti-**  
425 **tumor immune responses**

426 Cytidine methylation reduces the efficiency and alters the pattern of BLM-  
427 mediated cleavage of double-stranded DNA (39). Thus, we posited that DNA  
428 methyltransferase inhibitors (DNMTi) would promote the anti-tumor immune  
429 effect of BLM. Sequential treatment with DNMTi and BLM demonstrated a  
430 synergistic and robust potentiation of BLM by DNMTi in SK-BR-3 cells (Figure  
431 6A and Supplemental Figure 12A). Among the DNMTi tested, decitabine  
432 (DAC) in combination with BLM exhibited the most potent antiproliferative  
433 effect compared to azacitidine (AZA), and was therefore chosen for further  
434 investigation. MHC-I expression was increased with the combined treatment  
435 of BLM and DNMTi (Supplemental Figure 12B). Consistent with previous data,  
436 both BLM and DAC (39, 40) induced a DNA damage response, as evidenced  
437 by  $\gamma$ H2AX foci accumulation in SK-BR-3 cells (Supplemental Figure 12, C and  
438 D). Furthermore, DNMTi potentiated BLM-mediated DNA damage, as  
439 indicated by higher expression of  $\gamma$ H2AX S139 and increased  $\gamma$ H2AX foci  
440 accumulation (Supplemental Figure 12, C and D).

441 We also observed a potent augmentation of the BLM antiproliferative

442 effect in B16F10 tumor cells when used in combination with DAC  
443 (Supplemental Figure 12E). Additionally, we explored the potential of DAC  
444 treatment to enhance BLM-induced T cell activation. Neither BLM nor DAC  
445 treatment alone resulted in fewer viable cancer cells than the control group  
446 after co-culture with OT-I T cells. However, there was a significant potentiation  
447 of the BLM-induced T cell activation when used in combination with DAC  
448 (Figure 6, B and C). We further examined the impact of DAC on tumor  
449 response to BLM treatment in vivo by treating established B16F10 melanoma  
450 tumors with DAC and/or BLM. There were no significant differences in mouse  
451 body weight among the different treatment groups (Figure 6D). Consistent  
452 with the in vitro co-culture results, DAC treatment alone moderately slowed  
453 tumor growth in vivo, and the combination treatment of DAC and BLM further  
454 reduced tumor progression (Figure 6, E and F).

455 In summary, DNMTi enhances the upregulation of MHC-I in tumor cells  
456 induced by BLM and facilitates cancer cell killing by T cells, thereby  
457 complementing the therapeutic effects of BLM in B16F10 xenografts.

458

#### 459 **BLM-mediated potentiation of anti-tumor responses for T-cell-based** 460 **immunotherapy**

461 We further investigated the potential of combination therapies with BLM in  
462 immunotherapy approaches, such as bispecific antibody, immune checkpoint  
463 therapy and TILs therapy in solid tumors, which efficacy were all relied on T

464 cells and were restricted by MHC-I expression levels.

465 A recent study introduced a bispecific antibody (H2-scDb) designs to  
466 specifically target the most common p53 mutation (R175H) along with a  
467 common HLA-A allele (HLA-A\*02:01) on the cell surface. The efficacy of H2-  
468 scDb is highly correlated with the levels of HLA-A allele (HLA-A\*02:01)  
469 expressed in tumor cells (41). We demonstrated dose- and time-dependent  
470 increase in surface HLA-A2 levels in SU-DHL-4 (p53<sup>WT</sup> and HLA-A\*02:01) and  
471 SK-BR-3 (p53<sup>R175H</sup> mutation and HLA-A\*02:01) cells after BLM treatment  
472 (Supplemental Figure 13, A and B). We then examined whether BLM  
473 treatment could enhance the ability of H2-scDb to activate T cells. Consistent  
474 with previous studies, H2-scDb had no impact on SK-BR-3 cells, which  
475 harbored the p53<sup>R175H</sup> mutation and exhibited relatively low expression of  
476 HLA-A\*02:01. However, pre-treatment with BLM enhanced T cell activation  
477 mediated by H2-scDb, resulting in a higher number of tumor cells killing and  
478 higher production of IFN- $\gamma$  (Figure 7, A-C).

479 Many solid tumors resistant to checkpoint blockade are characterized by a  
480 lack of cytotoxic T cells recognition and infiltration (42). Therefore, we  
481 hypothesized that BLM might enhance the efficacy of checkpoint blockade by  
482 increasing MHC-I expression and promoting T-cell infiltration. To investigate  
483 whether the anti-tumor efficacy of PD-L1 blockade could be improved by  
484 combination with BLM treatment, we treated B16F10 melanoma-bearing mice  
485 with BLM, anti-mouse PD-L1 antibody, or a combination of both. The body



486 weight remained stable across the different treatment groups (Figure 7D).  
487 Furthermore, mice treated with BLM or PD-L1 antibody alone showed partial  
488 tumor growth inhibition, while BLM sensitized B16F10 melanomas to  
489 checkpoint blockade with a PD-L1 antibody, resulting in substantially reduced  
490 tumor growth and tumor weight (Figure 7, E and F). Additionally, the effect of  
491 BLM was examined in MC38 mouse model and similar results were obtained  
492 (Supplemental Figure 14, A-D).

493 We next expanded our results to a clinically relevant experimental setting.  
494 Tumors with high levels of somatic mutations, such as melanoma and bladder  
495 cancer, respond well to immunotherapy with checkpoint blockade therapy or  
496 the adoptive transfer of anti-tumor lymphocytes (43, 44). Therefore, we  
497 investigated whether BLM treatment improves the activation of autologous  
498 TILs in primary patient-derived bladder cancer cells. Firstly, we successfully  
499 established bladder cancer cells from patient tumor tissues and urine samples  
500 using a conditional reprogramming technique (45-47). In this study, eight  
501 patients diagnosed with bladder cancer were enrolled (Supplemental Table 5).  
502 Among these, four primary patient-derived cancer cell lines were established  
503 from the patient urine samples (BCC3, BCC16, BCC38, and BCC49),  
504 whereas the others were established from the tumor samples (BCC1, BCC15,  
505 BCC101, and BCC102). MHC-I expression could be induced by BLM in most  
506 of the primary patient-derived cancer cell lines with very low toxic effects  
507 (Supplemental Figure 15, A and B).

508        Among the four tumor samples, we selected BCC101 (high response) and  
509        BCC102 (low response) based on their response to BLM induced MHC-I  
510        expression. Subsequently, TILs from different fragments of the two tumor  
511        samples (labelled F1, F2, F3...) were expanded ex vivo and the phenotypes  
512        of the expanded TILs were assessed by flow cytometry (Supplemental Figure  
513        16, A-C). As expected, pre-treatment with BLM rendered BCC101 cells more  
514        susceptible to autologous TIL-mediated cytotoxicity than untreated cells,  
515        whereas BCC102 showed reduced effect (Figure 7G and Supplemental  
516        Figure 16D). We confirmed that BLM had no toxic effects to primary cancer  
517        cells within the range of experimental concentrations (Supplemental Figure  
518        16E). Additionally, BLM pre-treated BCC101 cancer cells exhibited increased  
519        susceptibility to the cytotoxicity of autologous reactive TIL fractions, which  
520        was evidenced by a lower number of viable tumor cells, a higher apoptosis  
521        rate (Figure 7, H and I). Collectively, these results suggest that the potential of  
522        combination therapy with BLM rely on T cells as key effector cells, such as  
523        bispecific antibody, immune checkpoint therapy and TILs therapy for solid-  
524        tumor indications.

525

526 **Discussion**

527       Reduced surface expression of MHC-I stands as a formidable barrier to  
528 the success of immunotherapy. In this study, we present BLM as a promising  
529 agent, repurposed for its ability to rapidly induce surface MHC-I expression in  
530 tumor cells. This highlights MHC-I as an ideal pharmacological target for  
531 enhancing tumor immunity.

532       Utilizing a high-throughput flow cytometry system, we not only identified  
533 BLM but also demonstrated the versatility of our screening strategy to uncover  
534 potential immunotherapy-enhancing drugs. Moreover, the induction of  
535 downregulated MHC-I expression through BLM treatment presents a  
536 therapeutically valuable avenue to improve T cell anti-tumor immunity,  
537 overcoming obstacles posed by MHC-I expression limitations and opening  
538 possibilities for combination therapies.

539       BLM, traditionally an antibiotic chemotherapeutic agent with established  
540 use in various cancers, possesses anti-tumor activity attributed to its induction  
541 of specific double-strand DNA breaks (27, 39). Antigen processing and  
542 presentation in the MHC-I context is a complex, multi-step process subject to  
543 regulation at multiple levels (48). Previous studies have highlighted the  
544 involvement of the NF- $\kappa$ B and cGAS–STING pathways in cancer-related  
545 MHC-I expression (7, 49). Our results reveal that BLM treatment induces  
546 substantial DNA damage and promotes CD8<sup>+</sup> T cell activation through the  
547 specific upregulation of MHC-I expression in an ATM/ATR-NF- $\kappa$ B-dependent

548 manner. The dominant role of NF- $\kappa$ B in BLM-induced MHC-I upregulation  
549 reinforces the significance of the ATM/ATR-NF- $\kappa$ B pathway in regulating  
550 MHC-I expression in tumors. However, the specific molecular target of BLM  
551 remained unclear, and its identifying is crucial for a comprehensive  
552 understanding of its efficacy in MHC-I upregulation.

553 Building on BLM's induction of double-strand DNA breaks, the exploration  
554 of its synergy with DNA methyltransferase inhibitors in anti-tumor immunity  
555 emerges as a pivotal avenue. The experimental data presented in this study  
556 conclusively demonstrate that the combination therapy involving BLM and  
557 DNA methyltransferase inhibitors broadens the application potentials of both  
558 agents.

559 The importance of BLM in combination therapy becomes apparent as it  
560 showcases the potential to enhance various immunotherapy approaches that  
561 rely on T cells as primary effectors. This encompasses checkpoint blockade  
562 therapy, bispecific antibody therapy, and TILs therapy for solid tumor  
563 indications. The correlation established between high MHC-I expression and  
564 improved antigen presentation serves to validate the heightened efficacy of  
565 immunotherapies. Our study further establishes that BLM plays a crucial role  
566 in augmenting anti-tumor responses facilitated by these immunotherapy  
567 modalities.

568 Additionally, alternative pathways beyond MHC-I expression may  
569 contribute to the immune sensitivity of BLM-treated tumors, such as activated

570 IFN- $\alpha/\gamma$  and inflammatory pathways, which may facilitate T cell infiltration.  
571 Further research should delve into these alternative pathways to discern their  
572 impact on the immunological responsiveness of BLM-treated tumors.

573 Addressing pulmonary toxicity associated with BLM is imperative for its  
574 clinical application. While high doses (15 to 20 mg/kg) result in lung  
575 inflammation and pulmonary fibrosis (27, 50), our study indicates that a lower  
576 dosage of BLM (3 mg/kg) increases lymphocyte infiltration in tumor samples  
577 and remodels the tumor immune microenvironment without causing notable  
578 lung injury. This suggests the potential of repurposing BLM at lower doses,  
579 making it a more viable and economical option for cancer treatment.

580 TIL therapy represents an intricately personalized approach to cancer  
581 treatment, influenced by a multitude of variables and often yields  
582 unpredictable outcomes. Consequently, the inherent limitations of TIL therapy  
583 have spurred researchers to explore novel avenues for enhancing its efficacy.  
584 In this study, we reveal that BLM renders patient-derived bladder cancer cells  
585 more susceptible to cytotoxicity mediated by autologous TILs. This finding  
586 suggests a potential combination therapy to enhance the efficacy of TIL  
587 therapy.

588 This suppression of MHC-I is a critical viral strategy to avoid immune  
589 surveillance (51). The downregulation of MHC-I expression induced by  
590 Influenza A and B virus, which hinder viral clearance by CD8<sup>+</sup> T cells (52).  
591 SARS-CoV-2 can inhibit the induction of the MHC class I pathway by targeting

592 the STAT1-IRF1-NLRC5 axis (53). Restoring antigen presentation by  
593 Interferon gamma can enhance the immune system's ability to combat viral  
594 escape (54). BLM' s ability to induce MHC-I expression could, therefore,  
595 provide a dual therapeutic strategy, augmenting the immune response against  
596 both cancer cells and virally infected cells, making it a promising candidate for  
597 further antiviral research. Interestingly, BLM has been reported to inhibit  
598 multiple virus including HIV, picornavirus, herpesvirus, and poxvirus (55, 56).  
599 This highlights the potential of BLM as an antiviral agent by combating  
600 immune evasion. In summary, our data underscores the ability of BLM to  
601 augment cytotoxic T cell recognition and responses by inducing surface MHC-  
602 I expression in tumor cells. Furthermore, the potential of combination therapy  
603 with BLM extends beyond adoptive T cell transfer therapy, encompassing  
604 other immunotherapy modalities dependent on T cells as primary effectors.  
605 The demonstrated correlation between MHC-I expression and improved  
606 immunotherapy efficacy repurposes BLM as a key player in advancing the  
607 field of cancer immunotherapy.  
608

## 609 **Methods**

610 Additional details for methods are provided in the Supplemental Methods.

611

### 612 **Sex as a biological variable**

613 The mechanisms and pathways investigated are not sex-specific in our  
614 study. Female mice (C57BL/6) were exclusively used in the animal  
615 experiment due to their more docile nature, which leads to more consistent  
616 experimental conditions.

617

### 618 **High-throughput flow cytometry screening system**

619 To facilitate high-throughput screening, SU-DHL-4 cells were seeded in  
620 round-bottom 96-well plates and FDA-approved drugs were introduced into  
621 the cell plates. Additionally, each plate received 500 U/mL Interferon- $\gamma$  (IFN- $\gamma$ )  
622 (#315-05-20ug, PeproTech) and DMSO for the manual addition of positive  
623 and negative controls. After 48 h, the cells were labeled with the anti-HLA-  
624 A/B/C antibody W6/32-APC (#311410, BioLegend) at 4°C for 30 min.  
625 Subsequently, the cell samples were analyzed using the IntelliCyt iQue  
626 Screener PLUS (Sartorius).

627

### 628 **Peptide Pulsing Assay**

629 For the peptide pulsing assay, B16F10 cells were pre-treated with BLM at

630 the indicated concentrations or with PBS as a control for specified durations.  
631 Following this treatment, the cells were pulsed with 1 ng/mL of SIINFEKL  
632 (Ovalbumin peptide, OVA peptide) at 37°C for 2 h. The cell surface  
633 expression of H-2K<sup>b</sup>-SIINFEKL was assessed using flow cytometry. In the co-  
634 culture experiments, the culture media were removed, and the cells were  
635 washed with PBS to eliminate residual BLM and OVA peptide after pulsing.  
636 Subsequently, OT-I CD8<sup>+</sup> T cells were added at a 2:1 effector to target (E/T)  
637 ratio. After approximately 20 h of co-culture, all cells were harvested and  
638 analyzed using flow cytometry. Tumor cells were identified as the CD45-  
639 negative population, and the cell apoptosis rate was determined using the  
640 Annexin V/633 Apoptosis Detection Kit (AD11, DOJINDO, Japan) and  
641 analyzed with FlowJo™ v10.7 Software (BD Life Sciences, USA).

642

#### 643 **Co-culture of cancer cells and T cells for T cell cytotoxicity assay**

644 *Co-culture of mouse tumor cells and OT-I CD8<sup>+</sup> T cells.* C57BL/6-Tg  
645 (TcraTcrb)1100Mjb/J (OT-I) mice were generously shared by Jianhua Li' s lab  
646 from the Department of Pathogen Biology at School of Basic Medical  
647 Sciences, Fudan University, Shanghai. B16F10 and B16OVA cells were pre-  
648 treatment with BLM (1.25 μM or 2.5 μM, respectively) or PBS for 24 h. CD8<sup>+</sup> T  
649 cells were isolated from the spleens and lymph nodes of OT-I mice,  
650 stimulated with SIINFEKL (OVA peptide) (#HY-P1489A, InvivoGen), and  
651 cultured in RPMI 1640 medium containing 10% FBS, 1% penicillin-



652 streptomycin, 2 mM L-glutamine (#25030081, Gibco, Thermo Fisher), 10 mM  
653 HEPES (#15630080, Gibco, Thermo Fisher), MEM NEAA (#11140050, Gibco,  
654 Thermo Fisher), 50  $\mu$ M  $\beta$ -Mercaptoethanol (#444203, Sigma), and 10 ng/mL  
655 recombinant mouse IL-2 (#78081, STEMCELL, Canada). These mouse CD8<sup>+</sup>  
656 T cells were then co-cultured with BLM- or PBS pre-treated tumor cells at a  
657 ratio of 1:2 (tumor cells: T cells) for approximately 20 h. At the conclusion of  
658 the experiment, the concentration of IFN- $\gamma$  in the co-culture supernatant was  
659 determined by ELISA, and the percentage of apoptotic tumor cells was  
660 assessed by flow cytometry.

661 *Co-culture of human tumor cells and human CD8<sup>+</sup> T cells (Bispecific antibody*  
662 *treatment)*. SK-BR-3 cells were pre-treated with BLM (1.25  $\mu$ M) or PBS for 24  
663 h. Peripheral blood mononuclear cells (PBMCs) were collected from healthy  
664 donors with informed consent and isolated via density gradient centrifugation  
665 using Ficoll Paque Plus (GE Healthcare, 17144003). CD8<sup>+</sup> T cells were  
666 subsequently purified from PBMCs using negative selection with the EasySep  
667 Human CD8<sup>+</sup> T Cell Enrichment Kit (#19053, STEMCELL, Canada) following  
668 the manufacturer's protocol. Human CD8<sup>+</sup> T cells were stimulated in 12-well  
669 culture plates coated with 1  $\mu$ g/mL anti-human CD3 antibody (#300402,  
670 BioLegend) and 2  $\mu$ g/mL soluble anti-human CD28 antibody (#302902,  
671 BioLegend) along 10 ng/mL recombinant human IL-2 (#200-02-1MG,  
672 PeproTech, Thermo Fisher). Human CD8<sup>+</sup> T cells were cultured in RPMI 1640  
673 medium containing 10% FBS, 1% penicillin-streptomycin, 2 mM L-glutamine

674 (#25030081, Gibco, Thermo Fisher), 10 mM HEPES (#15630080, Gibco,  
675 Thermo Fisher), MEM NEAA (#11140050, Gibco, Thermo Fisher), and 50  $\mu$ M  
676  $\beta$ -Mercaptoethanol (#444203, Sigma). Co-culture experiments involving  
677 human CD8<sup>+</sup> T cells and BLM or PBS pre-treated tumor cells at a ratio of 1:2  
678 (tumor cells: T cells) in the presence of the appropriate bispecific antibody  
679 (0.3 nM H2-scDb) for approximately 20 h. At the conclusion of the experiment,  
680 the concentration of IFN- $\gamma$  in the co-culture supernatant was measured using  
681 ELISA.

682 *Co-culture of NY-ESO-1<sup>+</sup> human tumor cells and human CD8<sup>+</sup> T cells*  
683 *transduced with the NY-ESO-1 TCR.* Human CD8<sup>+</sup> T cells were engineered to  
684 express a recombinant T cell receptor (TCR) targeting the NY-ESO-1 antigen  
685 (specifically the NY-ESO-1:157–165 epitope) in an HLA-A\*02-restricted  
686 fashion (referred to as ESO T cells). NY-ESO-1<sup>+</sup> SK-BR-3 cell was conducted  
687 by overexpressing NY-ESO-1 gene. NY-ESO-1<sup>+</sup> SK-BR-3 cells were pre-  
688 treated with BLM (2.5  $\mu$ M, 5  $\mu$ M, 10  $\mu$ M, and 20  $\mu$ M) or PBS for 24 h. ESO T  
689 cells were then co-cultured with BLM- or PBS pre-treated NY-ESO-1<sup>+</sup> SK-BR-  
690 3 cells at a ratio of 1:2 (tumor cells: T cells) for approximately 20 h. At the  
691 conclusion of the experiment, the percentage of apoptotic tumor cells was  
692 assessed by flow cytometry.

693

#### 694 **Bispecific antibody production**

695 Bispecific antibody production was performed as previously described

696 (41). Briefly, 1 L FreeStyle 293-F cells were transfected with 1.2 mg of  
697 plasmid DNA using polyethylenimine (PEI). After three days, the culture  
698 medium was collected and filtered through a 0.45- $\mu$ m filter. Ni-NTA Agarose  
699 (#30210, QIAGEN) was pre-equilibrated with PBS and incubated overnight  
700 with the supernatant. Following this, non-specifically bound proteins were  
701 removed from the agarose by washing with PBS, 10 mM, and 20 mM  
702 imidazole before being eluted with 50 mM and 100 mM imidazole. The protein  
703 was concentrated and desalted in PBS using Amicon Ultra-15 Centrifugal  
704 Filters (#UFC901096, Sigma-Aldrich). The protein concentration was  
705 determined using Coomassie blue staining and/or the Pierce BCA Protein  
706 Assay Kit (#23225, Thermo Scientific).

707

## 708 **Adoptive T Cell Transfer Therapy and BrdU labeling**

709 B16OVA cells ( $2 \times 10^5$ /mouse) were subcutaneously injected into the right  
710 flank of C57BL/6 mice (purchased from Shanghai JieSiJie Laboratory Animal  
711 Company Limited, 6-8 weeks old). After eight days, when the tumor volume  
712 reached  $\sim 100$  mm<sup>3</sup>, the mice were randomly assigned to different treatment  
713 groups and received either PBS or BLM (3 mg/kg, dissolved in PBS). Tumor  
714 size was monitored every two days. On the 11th day, pre-activated OT-I  
715 CD45.1<sup>+</sup> cells ( $1 \times 10^6$ /mouse) were intravenously injected into tumor-bearing  
716 mice. For in vivo BrdU labeling of transferred OT-I cells, mice were injected  
717 intraperitoneally with 1 mg (0.1 mg/mL) of BrdU (BD Bioscience) in 1 $\times$  PBS 24

718 and 48 h after OT-I transfer. Tumors were harvested and BrdU staining kit  
719 was used for the flow cytometry analysis 72 h after OT-I transfer. For tumor  
720 weight and lung toxicity evaluation, all tumor-bearing mice were humanely  
721 euthanized and their tumors and lungs were collected on the 18<sup>th</sup> day. For  
722 survival studies, endpoints included death, mouse weight loss exceeding 20%,  
723 significant tumor ulceration, and tumor volume exceeding 2000 mm<sup>3</sup>. Animal  
724 survival rates were recorded daily.

725

#### 726 **Expansion of TILs and tumor cell lines from patient-derived bladder** 727 **tumor samples**

728 Fresh tumor samples were sectioned into small fragments measuring  
729 approximately 1-3 mm<sup>3</sup>, which were then placed into a culture medium  
730 containing 60,000 IU/mL of IL-2. After four weeks of expansion, the resulting  
731 TILs were either cryopreserved or subjected to a rapid expansion protocol  
732 (REP) involving irradiated human PBMCs as feeder cells. Bladder tumor cell  
733 lines were established according to previously established protocols (47).

734 The established bladder tumor cells were pretreatment with BLM or were  
735 exposed to PBS for 24 h. Simultaneously, expanded TILs were harvested  
736 post-REP and co-cultured with BLM pre-treated or PBS-exposed tumor cells  
737 at a ratio of 1:4 (tumor cells : TILs) for approximately 20 h. Cytotoxicity was  
738 assessed using CellTiter-Glo reagent (Promega), and the concentration of  
739 IFN- $\gamma$  in the co-culture supernatant was determined by ELISA.

740 **Statistical Analysis**

741 Data are represented as mean  $\pm$  SEM. Differences between two groups  
742 and among multiple groups were evaluated using a two-tailed Student's t-test  
743 and one-way ANOVA, respectively. Each experiment was repeated three  
744 times independently. Data analyses were conducted using the SPSS software  
745 (SPSS, Chicago, IL, USA). A *P* value less than 0.05 was considered  
746 significant.

747

748 **Study approval:**

749 This study enrolled eight patients diagnosed with bladder cancer, with four  
750 having high-grade and four with low-grade forms of the disease. The detailed  
751 clinical information of the patients is listed in Supplemental Table 7. All  
752 experimental procedures were approved by the Zhongshan Hospital Ethics  
753 Committee (project numbers: B2016-148 and B2017-129R) and Medical  
754 Ethics Committee of the School of Basic Medical Sciences, Fudan University  
755 (project number: 2023-C005). Informed consent was obtained from all the  
756 patients. All animal experiments were performed based on the guidelines  
757 published by the Association for Assessment and Accreditation of Laboratory  
758 Animal Care, and the animal studies were approved by the Department of  
759 Laboratory Animal Science Fudan University.

760

761 **Data availability:**

762 The single-cell RNA-sequencing data and the bulk RNA-sequencing data  
763 in this study have been deposited in CNGB Nucleotide Sequence Archive of  
764 China National GeneBank (<https://db.cngb.org/cnsa/>). The accession  
765 numbers are CRA017507 and HRA007881, respectively. Values for all data  
766 points found in graphs are in the Supporting Data Values file.

767

768 **Authors' contributions:** Y.J.D., W.J. and W.X. conceived and supervised the  
769 project; Q.Y., Y.D., X.B.W. and C.X.S. conducted the experiments; S.J., Y.D.,  
770 C.X.S., and R.K.Z. provided biopsy samples and cultured primary cells and  
771 TILs; Q.Y. analyzed the data; Q.Y. wrote the manuscript; and Y.J.D. and W.J.  
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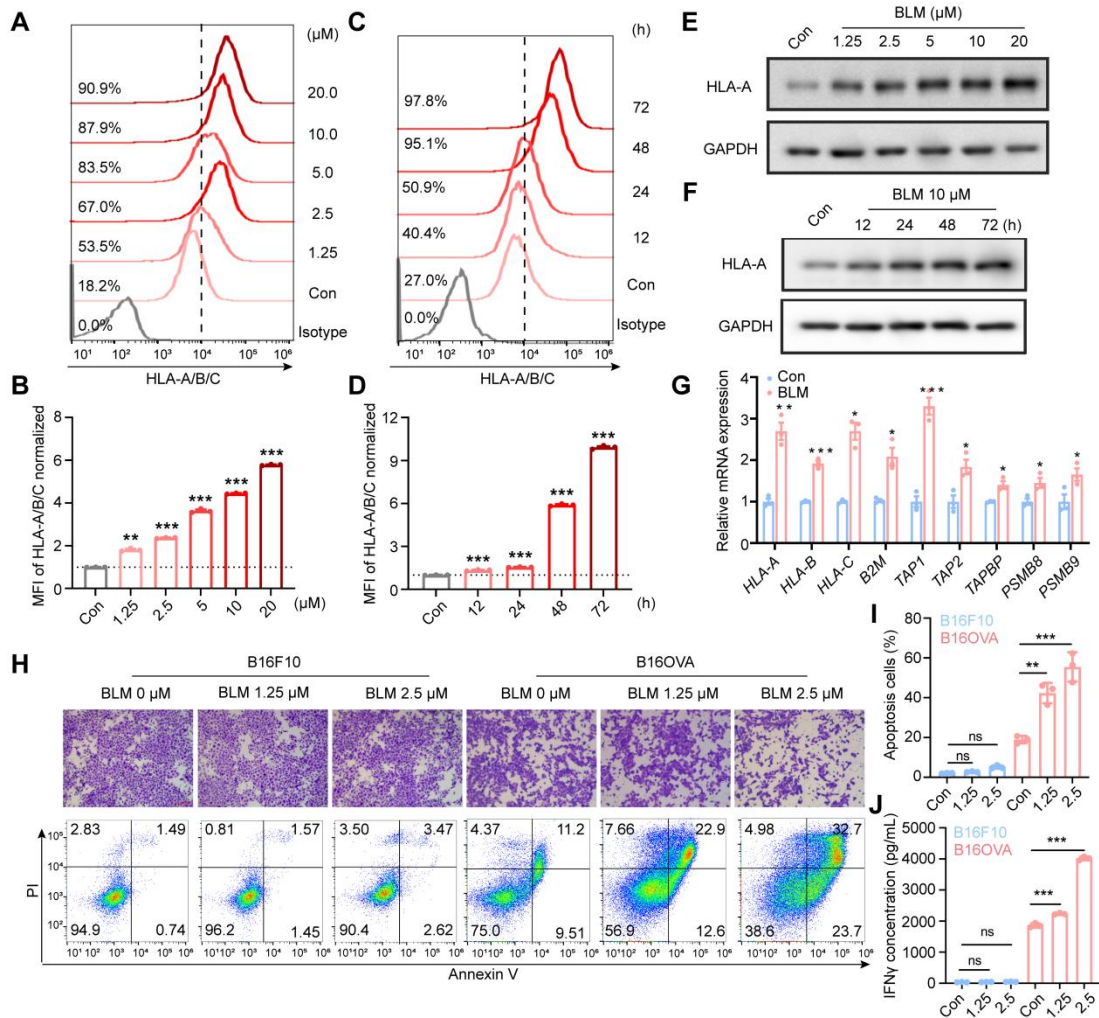


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- 952
- 953



955

956 **Figure 1. BLM-mediated increasing of MHC-I expression primes CD8<sup>+</sup> T**

957 **cell activation. (A)** Cell surface HLA-A/B/C in SU-DHL-4 cells after

958 incubation with the indicated concentrations of BLM for 48 h. **(B)**

959 Quantification of mean fluorescence intensities (MFI) of HLA-A/B/C from **(A)**,

960 n=3 per group. **(C)** Cell surface HLA-A/B/C in SU-DHL-4 cells following

961 incubation with 10 μM BLM for 12, 24, 48, and 72 h. **(D)** Quantification of MFI

962 of HLA-A/B/C from **(C)**, n=3 per group. **(E and F)** Western blot analysis of the

963 HLA-A expression in SU-DHL-4 cells after the indicated BLM concentrations

964 in **(E)** or BLM treatment times in **(F)**. **(G)** qRT-PCR analysis of the antigen

965 presentation gene expression in SU-DHL-4 cells after BLM treatment for 48 h.

966 **(H)** Co-culture of murine cancer cells and OT-I T cells for T cell cytotoxicity

967 assay. B16F10 or B16OVA cells were pre-treated with indicated

968 concentrations of BLM for 24 h prior to co-culture with OT-I T cells. The first

969 lane displays the crystal violet staining images of remaining cancer cells

970 (Scale bars, 400  $\mu$ m). The second lane presents the representative images of

971 cancer cells apoptosis after co-culture with OT-I T cells. **(I)** Quantification of

972 the percentages of early and late apoptotic cells among cancer cells from **(H)**,

973 n=3 per group. **(J)** The concentration of IFN- $\gamma$  in the co-culture supernatant as

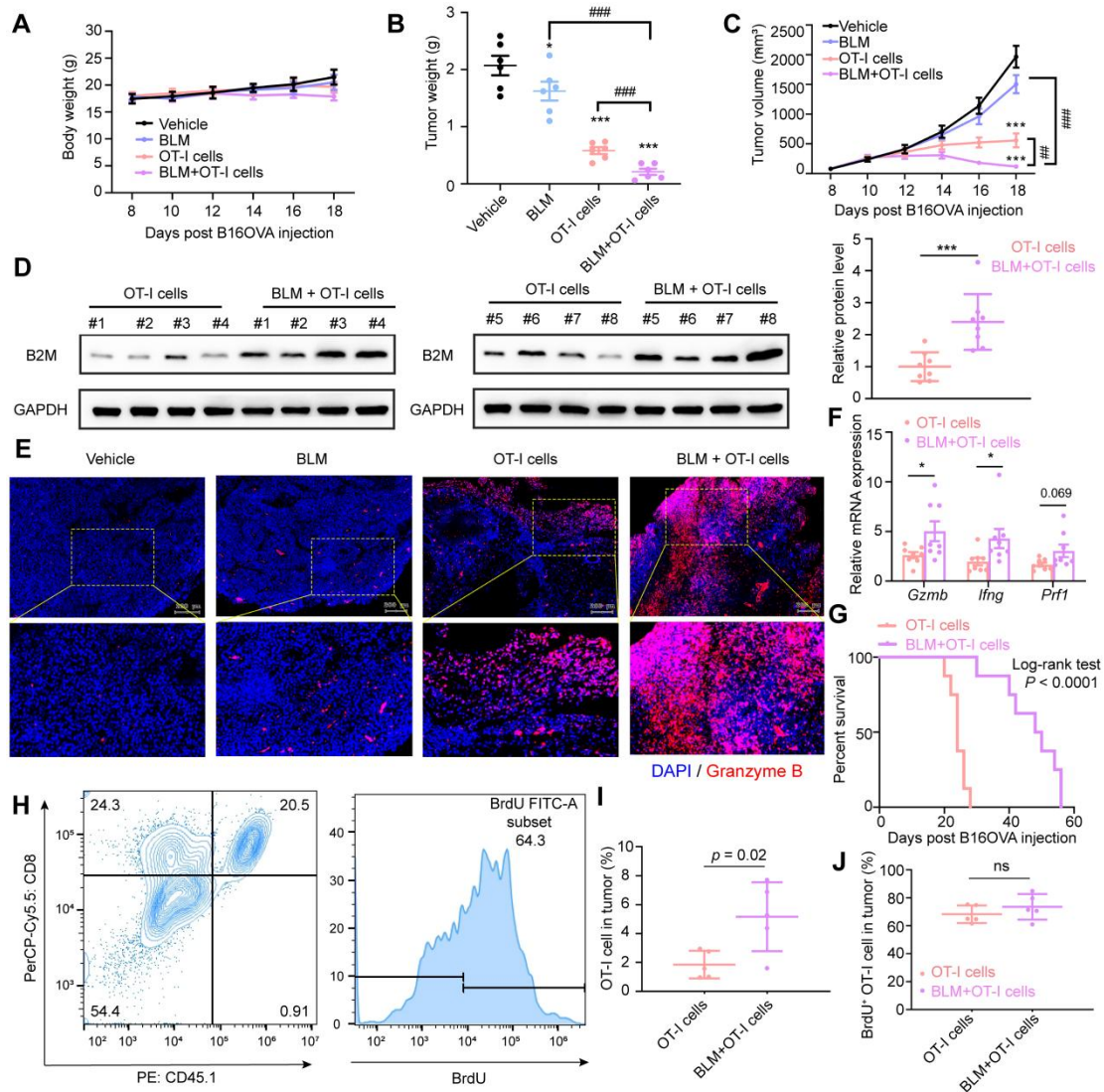
974 detected by ELISA, n=3 per group. Student's two-tailed unpaired t test, \* $P$  <

975 0.05, \*\* $P$  < 0.01 and \*\*\* $P$  < 0.001 compared to the vehicle group; ns, no

976 significance. Data indicate the mean  $\pm$  SD. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001,

977 and ns, no significance compared to the vehicle group by one-way ANOVA **(B,**

978 **D, I,** and **J)** and unpaired t test **(G)**.



979

980 **Figure 2. Potentiated anti-tumor response of T cells by BLM treatment.**

981 (A) Experimental procedure for the adoptive T cell transfer. (B-D) Mice body

982 weight (B), tumor weight (C), and tumor volume (D), n=8 per group. (E)

983 Western blot analysis of B2M level in tumor tissues as indicated. (F) Mouse

984 melanoma tissues were stained for Granzyme B (red) together with DAPI

985 (blue) (Scale bars, 200  $\mu$ m). (G) RT-PCR analysis of gene expression of anti-

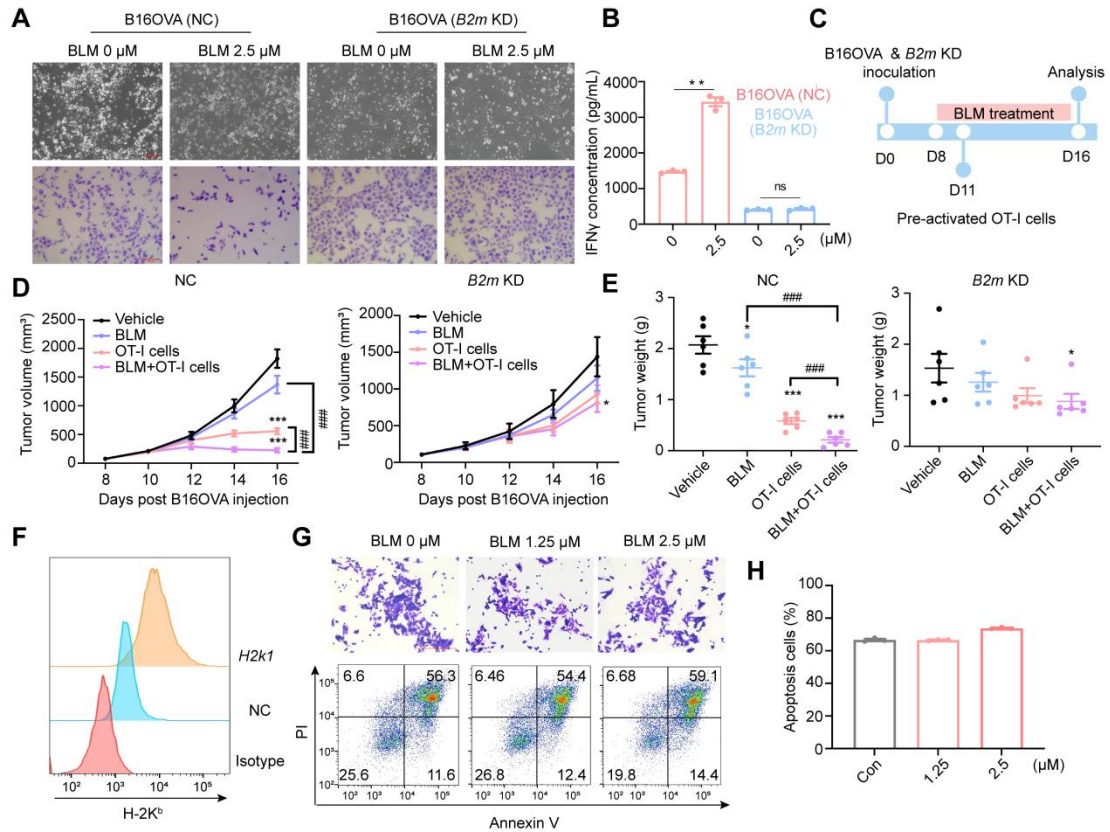
986 tumor effector molecules including granzyme B (*Gzmb*), IFN- $\gamma$  (*Ifng*), and

987 perforin (*Prf1*) in tumor tissues. (H) Kaplan–Meier curves for B16OVA tumor

988 bearing mice treated with OT-I cells or with the combination treatment of OT-I

989 cells and BLM. (I) Representative flow cytometry plot of transferred OT-I T  
990 cells (CD45.1<sup>+</sup>CD8<sup>+</sup>) and BrdU staining. (J and K) Three days after transfer,  
991 the frequencies of transferred OT-I cells (CD45.1<sup>+</sup>CD8<sup>+</sup>) (J) and BrdU<sup>+</sup> OT-I  
992 cells (K) were quantified in tumors from B16OVA mice pretreated by BLM or  
993 not, n=5 per group. Data are shown as mean ± SD. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P*  
994 < 0.001, and ns, no significance compared to the vehicle group by one-way  
995 ANOVA (C and D) and unpaired t test (E, G, J, and K); #*P* < 0.05, ##*P* < 0.01  
996 and ###*P* < 0.001 between the indicated groups by unpaired t test (C and D).  
997

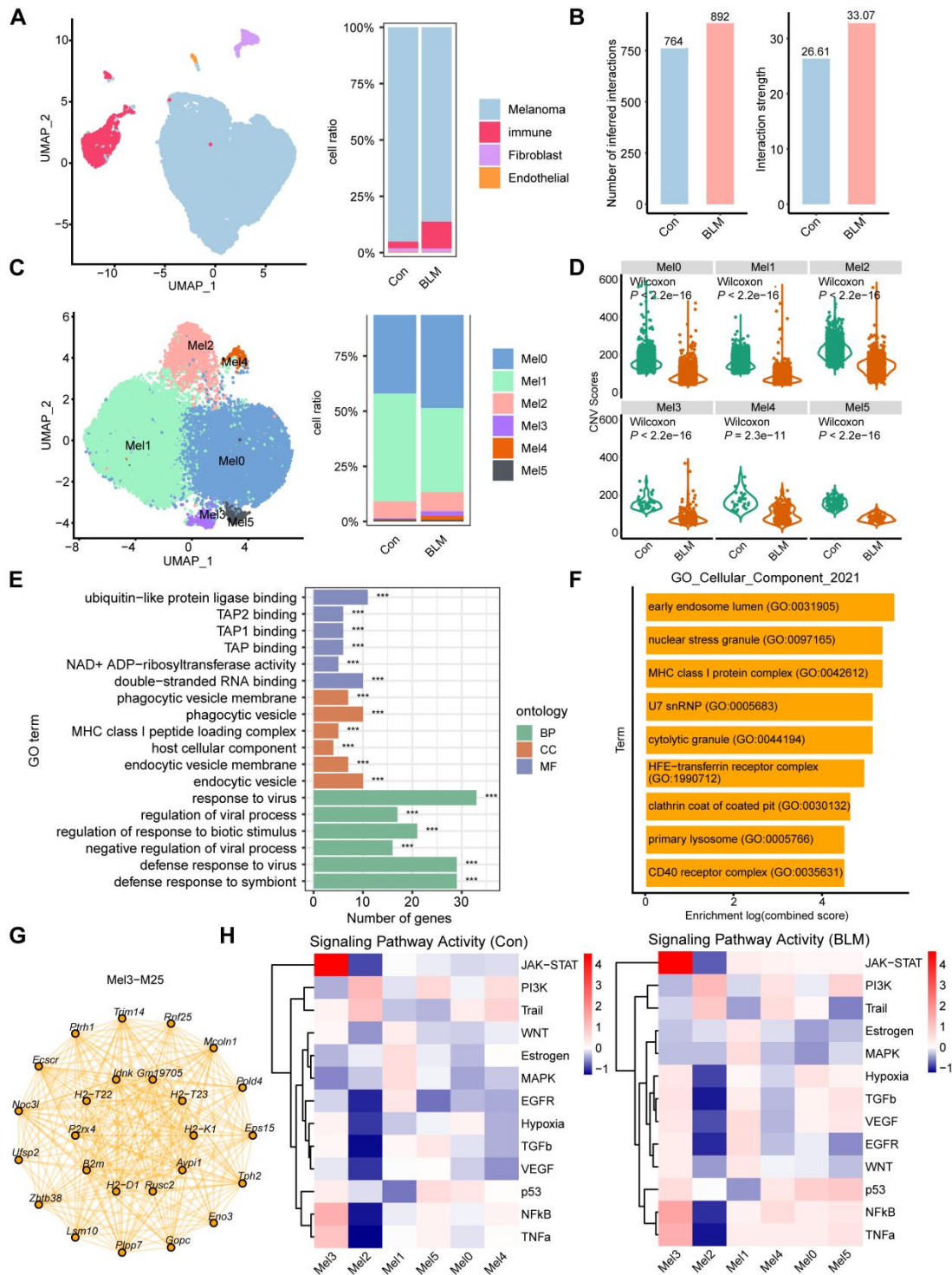




998

999 **Figure 3. MHC-I in cancer cells is indispensable for the anti-tumor effect**  
1000 **of BLM.** (A) Co-culture of non-targeting control (NC) or *B2m* knockdown  
1001 B16OVA cells and OT-I T cells for T cell cytotoxicity assay. Cells were pre-  
1002 treated with indicated concentrations of BLM for 24 h prior to co-culture with  
1003 OT-I T cells. The first lane shows the microscopy images and the second lane  
1004 displays the crystal violet staining images (Scale bars, 400  $\mu$ m). (B)  
1005 Concentration of IFN- $\gamma$  in the co-culture supernatant as detected by ELISA,  
1006 n=3 per group. (C-E) The experimental procedure (C), tumor volumes (D),  
1007 and tumor weights (E) on day 16, n=6 per group. (F) Flow cytometry detected  
1008 cell surface H-2K<sup>b</sup> expression in non-targeting control (NC) or *H2k1*  
1009 overexpressing B16OVA cells. (G) Co-culture of B16OVA cells  
1010 overexpressing *H2k1* and OT-I T cells for T cell cytotoxicity assay. B16OVA

1011 cells overexpressing *H2k1* were pre-treated with indicated concentrations of  
1012 BLM for 24 h prior to co-culture with OT-I T cells. The first lane displays the  
1013 crystal violet staining images of remaining cancer cells (Scale bars, 400  $\mu$ m).  
1014 The second lane presents the representative images of cancer cells apoptosis  
1015 after co-culture with OT-I T cells. (H) Quantification of the percentages of  
1016 early and late apoptotic cells among cancer cells from (G), n=3 per group.  
1017 Data are shown as mean  $\pm$  SD. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, and ns,  
1018 no significance compared to the vehicle group by unpaired t test (B) and one-  
1019 way ANOVA (D and E); ### $P$  < 0.001 between the indicated groups by  
1020 unpaired t test (D and E).  
1021



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1023

**Figure 4. BLM treatment remodels the tumor microenvironment. (A)**

1024

UMAP based on the top 20 principal components of all single-cell

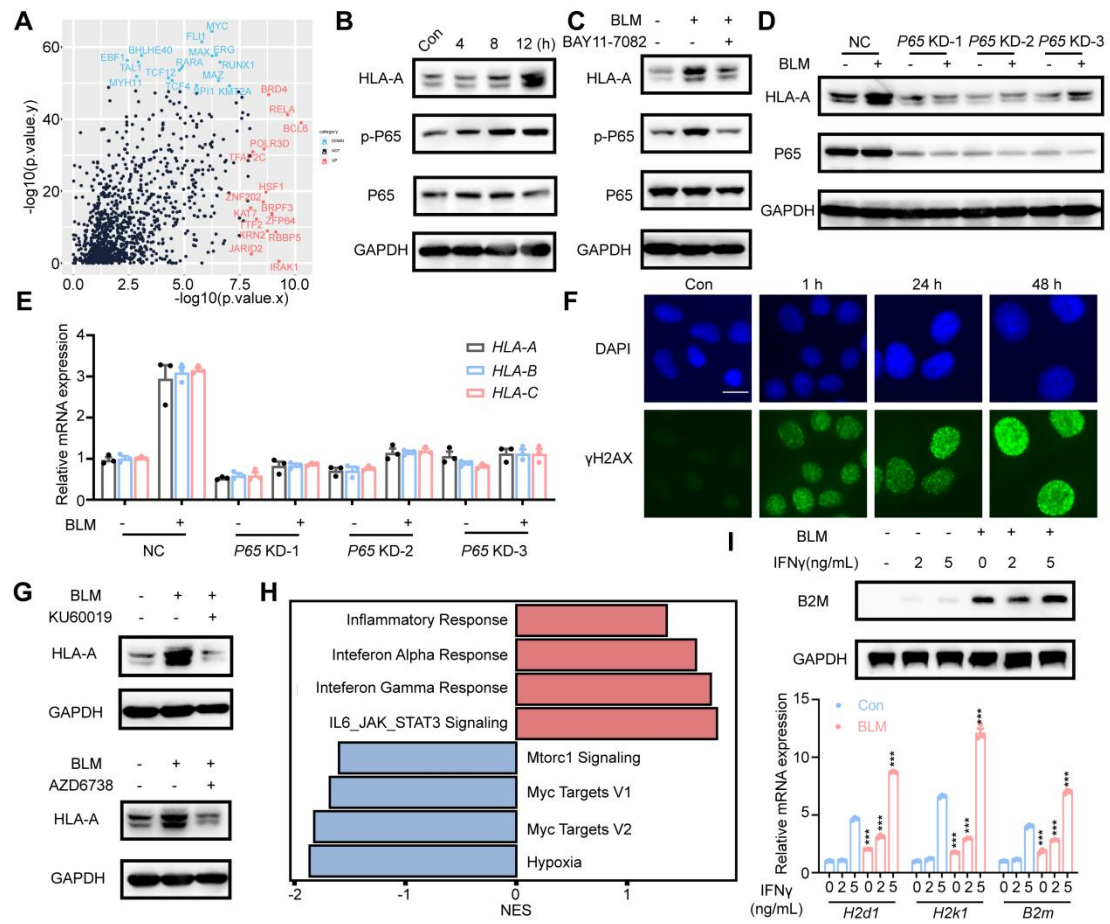
1025

transcriptomes color-coded by main cell type, and proportion of main cell type

1026

per tumor sample. (B) The number of inferred interactions and interaction

1027 strength were computed by CellChat package among control (Con) and BLM  
1028 tumor samples. (C) U-MAP based on the top 20 principal components of all  
1029 single-cell transcriptomes color-coded by melanoma subclusters, and  
1030 proportion of melanoma subclusters per tumor sample. (D) CNV scores  
1031 among melanoma subclusters (Mel0-Mel5) in different groups were computed  
1032 by infercnv package. (E) GO function enrichment analysis for melanoma  
1033 subcluster three (Mel3) was determined by clusterProfiler package. (F) The  
1034 gene set functional analyses of module25 were conducted with enrichR  
1035 package. (G) Network plot visualized the network underlying the top 25 hub  
1036 genes for module25. (H) Mean pathway activity scores of melanoma tumor  
1037 cells among different subclusters in control (Con) and BLM groups.  $***P <$   
1038  $0.001$ .  
1039



1040

1041 **Figure 5. MHC-I upregulation caused by BLM depends on ATM/ATR-NF-**

1042  **$\kappa\text{B}$  activation. (A) DEGs identified in comparisons of BLM-treated cells**

1043 **relative to control were subjected to LISA. The top 30 enriched regulators of**

1044 **up-regulated (red) and down-regulated (blue) DEGs were noted. (B) Western**

1045 **blot analysis of indicated proteins in SK-BR-3 cells treated with 10  $\mu\text{M}$  BLM for**

1046 **the indicated times. (C) Western blot analysis of the HLA-A, p-P65, and P65**

1047 **expressions. SK-BR-3 cells were pre-treated with 5  $\mu\text{M}$  BAY11-7082 for 6 h,**

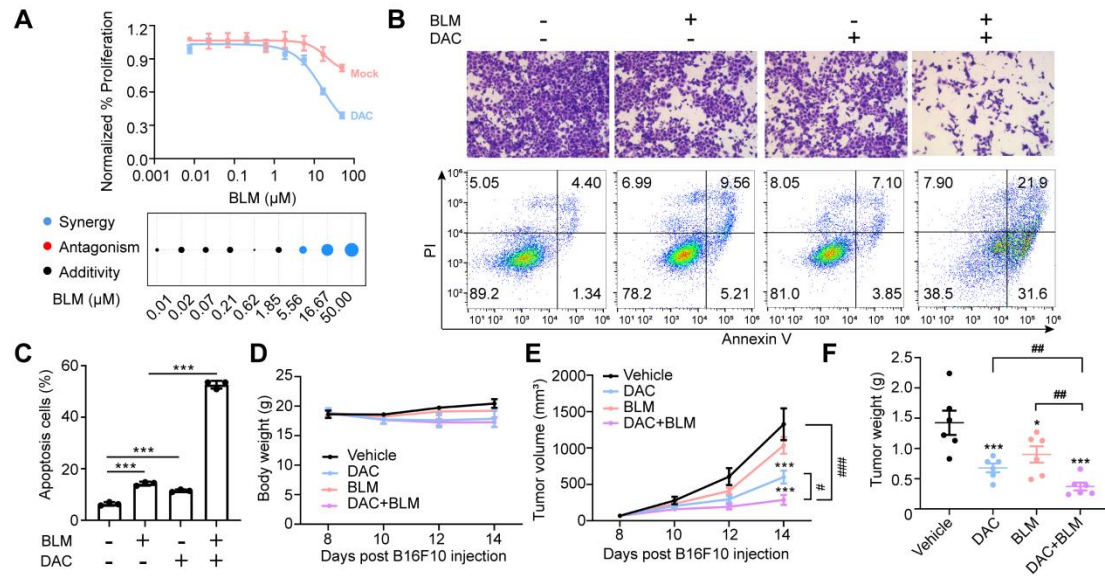
1048 **then followed by 10  $\mu\text{M}$  BLM for 12 h. (D) HLA-A protein levels examined in**

1049 **P65-depleted SK-BR-3 cells 48 h after BLM treatment. (E) qRT-PCR analysis**

1050 **of gene expressions of HLA-A, HLA-B, and HLA-C in P65-depleted SK-BR-3**

1051 **cells 48 h after BLM treatment. (F) Immunofluorescence analysis of dsDNA**

1052 damage by  $\gamma$ H2AX S139 antibody staining (Green foci; nuclei labeled with  
1053 DAPI) in SK-BR-3 cells after the indicated times of BLM treated (Scale bars,  
1054 25  $\mu$ m). (G) Western blot analysis of the HLA-A expression in SK-BR-3 cells.  
1055 Cells were pre-treated with 10  $\mu$ M KU60019 or 10  $\mu$ M AZD6738, for 6 h, then  
1056 followed with 10  $\mu$ M BLM for 12 h. (H) Gene Set Enrichment Analysis (GSEA)  
1057 analysis of significantly upregulated/downregulated pathways in BLM  
1058 treatment versus control SK-BR-3 cells. (I) Western blot and qRT-PCR  
1059 analysis of MHC-I expression levels in B16F10 cells after BLM treatment in  
1060 the presence of 2 or 5 ng/mL IFN- $\gamma$  for 48 h. Data are shown as mean  $\pm$  SD.  
1061 \*\*\* $P$  < 0.001 compared to the vehicle group by one-way ANOVA.  
1062



1063

1064

**Figure 6. DNA methyltransferase inhibition promotes BLM-induced anti-**

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**tumor immune responses. (A)** Top panel: Growth inhibition as detected by

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cell viability of SK-BR-3 cells treated with mock/100 nM DAC for five days and

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BLM for two days. Bottom panel: Combination index (CI) plots. **(B)** B16OVA

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cells pre-treated with 100 nM DAC for five days were trypsinized and plated in

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12 well plates with equal numbers of viable cells. Cells were then treated with

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BLM (0.5  $\mu\text{M}$ ) for one day prior to co-cultured with OT-I T cells. The first lane

1071

displays the crystal violet staining images (Scale bars, 400  $\mu\text{m}$ ). The second

1072

lane presents the representative images of cancer cells apoptosis after co-

1073

culture with OT-I T cells. **(C)** Quantification of the percentages of early and

1074

late apoptotic cells among cancer cells from **(B)**,  $n=3$  per group. **(D-F)**

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Treatment of B16F10 tumors with DNMT1 inhibitor (DAC) or vehicle control in

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combination with BLM or vehicle control,  $n=6$  per group. Mice weight **(D)**,

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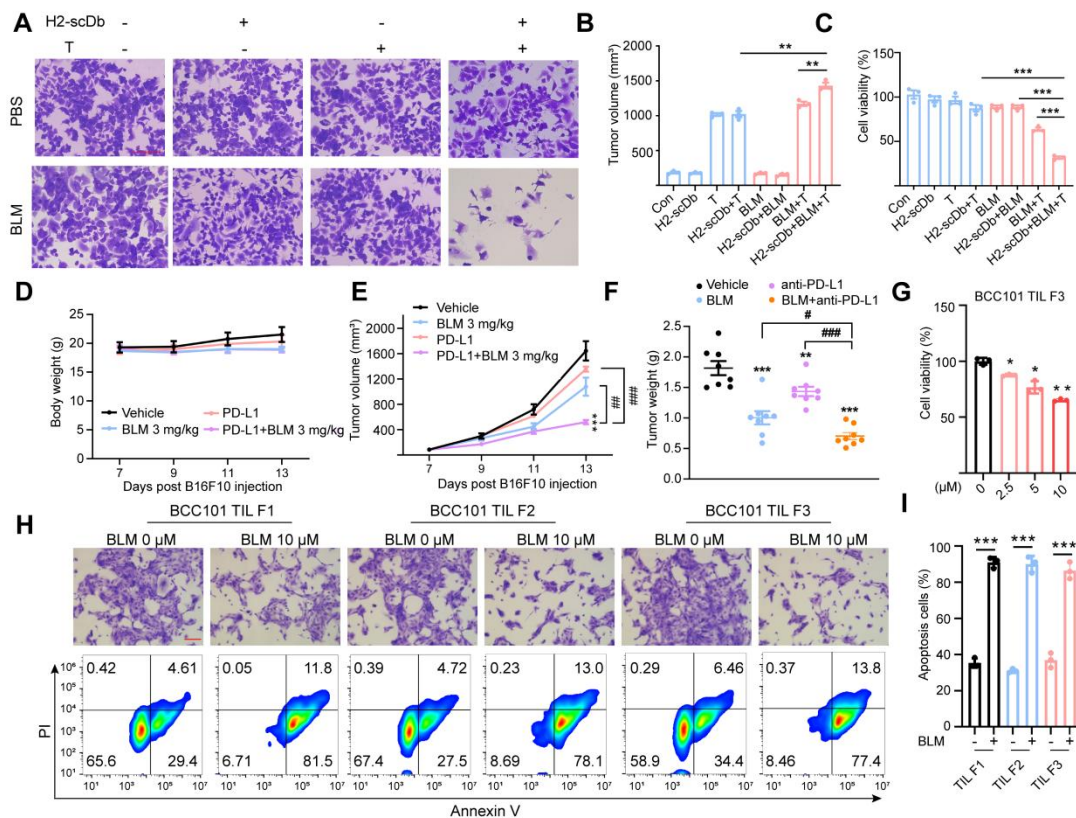
tumor volume **(E)** and tumor weight **(F)**,  $n=6$  per group. Data are shown as

1078

mean  $\pm$  SD. \* $P < 0.05$ , \*\*\* $P < 0.001$ , and ns, no significance compared to the

1079 vehicle group by one-way ANOVA (**E** and **F**) and unpaired t test (**C**); # $P <$   
1080 0.05, ## $P <$  0.01 and ### $P <$  0.001 between the indicated groups by unpaired  
1081 t test (**E** and **F**).  
1082





1083

1084 **Figure 7. BLM-mediated potentiation of anti-tumor responses for**

1085 **immunotherapy. (A) Co-culture of SK-BR-3 cells and human CD8<sup>+</sup> T cells for**

1086 **T cell cytotoxicity assay. The crystal violet staining images of remaining**

1087 **cancer cells are displayed (Scale bars, 400 μm). (B) The concentration of**

1088 **IFN-γ in the co-culture supernatant as detected by ELISA, n=3 per group. (C)**

1089 **T cell activation mediated by BLM and H2-scDb in response to SK-BR-3 cells**

1090 **at E:T ration of 2:1 as measured by the CellTiter-Glo reagent. (D-F) Treatment**

1091 **of B16F10 tumors with BLM or vehicle control in combination with PD-L1 or**

1092 **isotype control antibodies, n=8 per group. (D) Mice weight, (E) tumor volumes**

1093 **and (F) tumor weights of mice. (G) Co-culture of primary bladder cancer cells**

1094 **and TILs for T cell cytotoxicity assay. Primary bladder cancer cells were pre-**

1095 **treated with indicated concentrations of BLM for 24 h. Percent remaining live**

1096 cancer cells following 24 h incubation with autologous TILs at a 1:5 ratio. (H)  
1097 Co-culture of patient-derived tumor cells BCC101 and TILs (F1, F2 and F3  
1098 fragments) for T cell cytotoxicity assay. BCC101 tumor cells were pre-treated  
1099 with BLM (10  $\mu$ M) for 24 h prior to co-culture with TILs. The first lane displays  
1100 the crystal violet staining images (Scale bars, 400  $\mu$ m). The second lane  
1101 presents the representative images of cancer cells apoptosis after co-culture  
1102 with TILs. (I) Quantification of the percentages of early and late apoptotic cells  
1103 among cancer cells from (H), n=3 per group. Data are shown as mean  $\pm$  SD.  
1104 \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, and ns, no significance compared to the  
1105 vehicle group by unpaired t test (B, C, and I) and one-way ANOVA (E-G); # $P$   
1106 < 0.05, ## $P$  < 0.01 and ### $P$  < 0.001 between the indicated groups by  
1107 unpaired t test (E and F).  
1108