

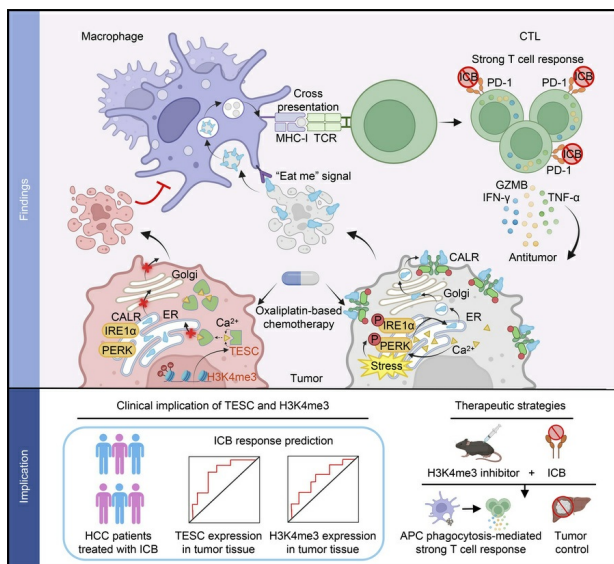
# Tescalcin is a phagocytic checkpoint driving immune escape and limiting immunotherapeutic efficacy in hepatocellular carcinoma

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1     **Tescalcin is a phagocytic checkpoint driving immune escape and limiting**  
2             **immunotherapeutic efficacy in hepatocellular carcinoma**

3  
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32

### 33 **Keywords**

34 Hepatocellular carcinoma, Immunotherapy, Phagocytic checkpoint, Macrophage, TESC,  
35 CALR membrane exposure, Immune escape

36

### 37 **Conflict of interest**

38 The authors have declared that no conflict of interest exists.

39

40

41 **Abstract**

42 Immunotherapies achieve durable responses in several cancers but show limited efficacy in  
43 refractory hepatocellular carcinoma (HCC). The mechanisms by which hepatoma cells evade  
44 immune recognition and limit immune checkpoint blockade (ICB) efficacy are incompletely  
45 defined. Here, we identified tumor-intrinsic tescalcin (TESC) as a previously unrecognized  
46 phagocytic checkpoint that contributes to immune evasion and ICB resistance in HCC.  
47 Mechanistically, H3K4 methylation drove TESC expression in hepatoma cells, facilitating  
48 cytosolic Ca<sup>2+</sup> buffering and attenuating endoplasmic reticulum (ER) stress-induced calreticulin  
49 (CALR) plasma membrane exposure, an essential “eat-me” signal. Consequently, this process  
50 abrogates membrane CALR-directed phagocytosis by antigen-presenting cells (APCs),  
51 including macrophages and dendritic cells, thereby impairing antigen presentation and  
52 subsequent T-cell activation. Clinically, elevated H3K4me3-TESC signaling was a promising  
53 prognostic biomarker for poor ICB response of HCC. Importantly, in vivo disruption of this  
54 axis restored APC phagocytic function and enhanced the antitumor effects of ICB therapy. Thus,  
55 targeting TESC-driven immune escape and its underlying epigenetic regulation may restore  
56 APC function and offer a precise therapeutic strategy to enhance immunotherapy efficacy in  
57 HCC.  
58

## 59 **Introduction**

60 Immune checkpoint blockade (ICB) therapy has revolutionized cancer treatment by restoring  
61 and amplifying T-cell-mediated antitumor responses (1–4). However, the majority of patients  
62 fail to benefit from ICB, owing to the complex interactions between tumor cells and the  
63 surrounding immune microenvironment (5–7). Elucidating these interactions is critical for  
64 identifying the cellular and molecular determinants of immune evasion, revealing potential  
65 therapeutic targets, and guiding the development of rational combination strategies. While  
66 tumor-intrinsic genetic and epigenetic alterations, such as DNA methylation, histone  
67 modifications, and chromatin remodeling, impair IFN- $\gamma$  signaling, antigen presentation, and T-  
68 cell effector function (8–11), how these changes impact upstream processes such as  
69 phagocytosis by antigen-presenting cells (APCs) remains poorly defined.

70     APCs prime and activate tumor-associated antigen (TAA)-specific T cells and are critical for  
71 defining the success of checkpoint therapy (12). This process depends on whether APCs,  
72 including macrophages and dendritic cells (DCs), can efficiently capture antigens from dead  
73 tumor cells via phagocytosis, present sufficient antigens to T cells, and activate T cells (13, 14).  
74 However, cancer cells exploit a suite of immune-evasive strategies by upregulating “don’t eat  
75 me” signals, including CD47, CD24, and MHC-I, to subvert phagocytic clearance (15). In  
76 parallel, tumor-associated macrophages (TAMs) often adopt immunosuppressive phenotypes  
77 that blunt antigen uptake and presentation. Clinically, the blockade of phagocytic checkpoints,  
78 including the CD47–SIRP $\alpha$  and CD24–Siglec-10 axes, has shown promise in restoring APC-  
79 mediated phagocytosis and enhancing the ICB response (16–18). In addition to suppressing  
80 inhibitory signals, tumors may also regulate prophagocytic “eat me” cues, such as calreticulin  
81 (CALR), a key signal required for efficient APC-mediated uptake by dying tumor cells (19).  
82 Despite increasing recognition of the role of CALR in immunogenic clearance, the upstream  
83 regulatory mechanisms governing its surface expression in human cancer cells remain elusive.

84 Elucidating these regulatory networks may reveal previously unrecognized axes of immune  
85 evasion and inform therapeutic approaches aimed at increasing sensitivity to ICB.

86 Hepatocellular carcinoma (HCC) usually occurs in inflamed fibrotic and/or cirrhotic livers  
87 with extensive leukocyte infiltration, where the inflammatory status in the tumor milieu  
88 profoundly shapes tumor cell behavior and modulates therapeutic responses (20–24). In this  
89 study, we identified tescalcin (TESC) as a previously unrecognized phagocytic checkpoint that  
90 contributes to immune evasion and ICB resistance. Mechanistically, the EF-hand calcium-  
91 binding protein TESC, which was intrinsically expressed by hepatoma cells driven by H3K4  
92 methylation, buffered cytosolic Ca<sup>2+</sup> and suppressed endoplasmic reticulum (ER) stress, thereby  
93 inhibiting CALR translocation to the plasma membrane. The resulting reduction in surface  
94 CALR expression impaired APC-mediated phagocytosis, suppressed antigen presentation, and  
95 attenuated T-cell activation, ultimately resulting in immune evasion and ICB resistance. In  
96 addition, impeding H3K4 methylation-driven TESC expression in vivo restored phagocytic  
97 function and enhanced the therapeutic efficacy of ICB, revealing a therapeutically actionable  
98 mechanism of immune resistance in HCC.

99

## 100 **Results**

### 101 **Tumor-intrinsic TESC contributes to resistance to antitumor immunity and** 102 **immunotherapy**

103 In our prospective clinical trial (NCT03869034) for locally advanced HCC, oxaliplatin-based  
104 chemotherapy combined with anti-PD-1 therapy elicited heterogeneous responses: 48.3% of  
105 patients achieved substantial tumor regression according to RECIST 1.1 criteria, whereas others  
106 derived limited benefit or exhibited disease progression (**Figure 1A**). To investigate  
107 mechanisms of resistance and uncover potential therapeutic targets, we performed  
108 transcriptomic profiling of tumors from 7 responders and 7 non-responders. Integrated with The  
109 Cancer Genome Atlas (TCGA) data on CD8<sup>+</sup> T-cell infiltration identified six genes consistently  
110 upregulated in nonresponding tumors (**Figure 1B**). Among these, TESC emerged as the top  
111 candidate, given its high expression in malignant cells and strong inverse correlation with  
112 patient survival (**Figure 1, C and D**). The remaining candidates were either expressed at low  
113 levels or lack association with clinical outcomes (**Figure 1C and Supplemental Figure 1A**).  
114 Multiplex immunofluorescence and single-cell RNA sequencing (GSE166635) demonstrated  
115 that TESC was predominantly expressed in tumor cells rather than immune cells (**Figure 1E**  
116 **and Supplemental Figure 1B**). Importantly, ROC analysis revealed that TESC expression  
117 exhibited robust predictive performance for response to oxaliplatin-based chemotherapy plus  
118 sintilimab (AUC = 0.88,  $P < 0.05$ ) (**Supplemental Figure 1C**). Consistent with this observation,  
119 elevated TESC expression in this same cohort was significantly associated with shorter overall  
120 survival by Kaplan-Meier and Cox regression analyses (**Figure 1F**) and with reduced response  
121 rates to ICB therapy (**Supplemental Figure 1D**). These associations were independently  
122 validated using single-cell RNA sequencing data (Mendeley Data: skrx2fz79n), which  
123 confirmed a correlation between high TESC expression and diminished efficacy of  $\alpha$ PD-1  
124 therapy (**Figure 1G**). Notably, in contrast to its consistent association with immunotherapeutic

125 outcomes, TESC expression showed no significant relationship with treatment response in an  
126 independent cohort of HCC patients receiving oxaliplatin-based chemotherapy alone, despite a  
127 non-significant trend toward higher TESC levels in non-responders (**Supplemental Figure 1E**).  
128 Collectively, these findings suggest that TESC may play a deleterious role in antitumor  
129 immunity and immunotherapeutic response rather than chemotherapy sensitivity.

130 We engineered Hepa1-6 cells to overexpress TESC and used an shRNA to silence TESC in  
131 H22 cells to investigate the function of TESC (**Supplemental Figure 1, F–H**). These cells were  
132 orthotopically implanted into immunocompetent C57BL/6, BALB/C, or immunodeficient nude  
133 mice (**Figure 1, H and I**). In immunocompetent mice, TESC overexpression significantly  
134 accelerated Hepa1-6 hepatoma growth and shortened overall survival, whereas TESC silencing  
135 markedly inhibited H22 hepatoma progression and prolonged survival (**Figure 1, H and I, and**  
136 **Supplemental Figure 1, I and J**). In contrast, manipulation of TESC, either by silencing or  
137 overexpression, did not affect hepatoma growth in nude mice (**Figure 1, H and I**), indicating  
138 that TESC exerts its pro-tumorigenic effects through a T cell-dependent mechanism. Consistent  
139 with this finding, anti-PD-1 therapy suppressed tumor growth in mice bearing either Hepa1-6  
140 or H22 hepatoma, and this effect was enhanced by TESC silencing but attenuated by TESC  
141 overexpression (**Figure 1, J–N**). Notably, although anti-PD-1 therapy showed no therapeutic  
142 efficacy in the *sgP53/Myc*-driven spontaneous hepatoma model, TESC knockout sensitized  
143 tumors to anti-PD-1 therapy, thereby substantially suppressing tumor development (**Figure 1,**  
144 **O and P**). Additionally, silencing or overexpressing TESC had little effect on the growth of  
145 H22 or Hepa1-6 cells in vitro (**Supplemental Figure 1, K and L**), and the addition of  
146 oxaliplatin did not confer further synergistic benefit when combined with TESC silencing and  
147 anti-PD-1 treatment (**Figure 1, J–P**). Thus, tumor-derived TESC may function as an intrinsic  
148 resistance mechanism of spontaneous and immunotherapy-induced tumor immunity.

149

150 **Tumor TESC impairs CD8<sup>+</sup> T-cell-mediated antitumor immunity**

151 To elucidate the immunological basis for TESC-mediated immune evasion, we assessed  
152 immune cell infiltration and function in TESC-manipulated tumors. While the modulation of  
153 TESC expression did not alter tumor growth in nude mice, it profoundly influenced tumor  
154 progression in immunocompetent hosts (**Figure 1, H and I**), suggesting a T-cell-dependent  
155 mechanism. In support of these findings, TESC silencing in hepatoma led to increased  
156 infiltration of CD8<sup>+</sup> T cells, whereas other immune cell populations remained largely unchanged  
157 (**Figure 2A**). The flow cytometry analysis further revealed that CD8<sup>+</sup> T cells in TESC-deficient  
158 tumors presented an increased proliferative capacity and higher expression of effector  
159 molecules, including IFN- $\gamma$ , TNF- $\alpha$ , and Granzyme B (**Figure 2, B–D**). Conversely, TESC  
160 overexpression reduced the abundance and functionality of CD8<sup>+</sup> T cells (**Figure 2, E and F**).  
161 CD8<sup>+</sup> T-cell depletion abrogated the tumor-suppressive effect of TESC knockdown (**Figure 2G**  
162 **and Supplemental Figure 2, A and B**), establishing a direct role for CD8<sup>+</sup> T cells in mediating  
163 TESC-dependent immune escape. To examine the impact of TESC on tumor antigen-specific  
164 CD8<sup>+</sup> T-cell responses, we implanted Ovalbumin (OVA)-expressing Hepa1-6 tumors harboring  
165 either control or TESC-overexpressing constructs into C57BL/6 mice to examine. As expected,  
166 tumors overexpressing TESC induced significantly fewer SIINFEKL–H-2Kb tetramer<sup>+</sup> CD8<sup>+</sup>  
167 T cells, as well as reduced proportions of IFN- $\gamma$ <sup>+</sup> and Granzyme B<sup>+</sup> cells within the tetramer<sup>+</sup>  
168 CD8<sup>+</sup> population, compared with control tumors (**Figure 2H**). Consistently, IFN- $\gamma$  ELISPOT  
169 analysis of tumor-infiltrating lymphocytes revealed markedly fewer IFN- $\gamma$  spots in TESC-  
170 overexpressing tumors (**Figure 2I**). Thus, tumor TESC impairs tumor-specific CD8<sup>+</sup> T-cell  
171 responses in vivo.

172 We further investigated how intracellular TESC expression in tumor cells affects T-cell  
173 activation. Although the exact mechanism remains unclear, previous studies have suggested  
174 that the immunogenicity of dying tumor cells can shape antigen-specific CD8<sup>+</sup> T-cell responses

175 (10). We loaded OVA into Hepa1-6 cells and TESC-overexpressing Hepa1-6 cells and then  
176 induced apoptosis by oxaliplatin exposure to test whether TESC modulates tumor  
177 immunogenicity (**Supplemental Figure 2, C and D**). These apoptotic cells were then  
178 cocultured with splenocytes isolated from OT-I transgenic mice, which express an OVA-  
179 specific TCR (**Supplemental Figure 2C**). Coculture with dead OVA-loaded control Hepa1-6  
180 cells resulted in a dose-dependent increase in IFN- $\gamma$  production, whereas coculture with dead  
181 OVA-loaded *Tesc* Hepa1-6 cells failed to elicit similar responses (**Figure 2J**). In addition to  
182 OT-I CD8<sup>+</sup> T cells, OT-I splenocytes contain antigen-presenting cells (APCs), including  
183 macrophages and myeloid dendritic cells, which are capable of processing OVA from dead  
184 tumor cells and presenting it to T cells. The markedly reduced IFN- $\gamma$  production in cultures with  
185 *Tesc* tumor cells suggests that TESC may impair the capacity of APCs to cross-present antigens  
186 and activate CD8<sup>+</sup> T cells. We further assessed whether TESC directly modulates T-cell  
187 activation by stimulating splenic T cells from C57BL/6 mice with anti-CD3 and anti-CD28  
188 antibodies in the presence of apoptotic control or TESC-overexpressing tumor cells. Under  
189 these conditions, the expression levels of IFN- $\gamma$ , TNF- $\alpha$ , and Granzyme B were comparable  
190 between the two groups (**Figure 2K**). Consistently, the single-cell RNA sequencing analysis  
191 (GSE151530) of human HCC samples showed reduced cytotoxic T-cell infiltration in tumors  
192 with high TESC expression (**Figure 2L, and Supplemental Figure 2, E and F**). Notably, T  
193 cells within TESC-high tumors exhibited a marked downregulation of TCR signaling-  
194 associated cytotoxic programs and antigen presentation-related pathways (**Figure 2M**). The  
195 reduced infiltration of IFN- $\gamma$ <sup>+</sup> and Granzyme B<sup>+</sup> CD8<sup>+</sup> T cells in TESC-high tumor tissues from  
196 HCC patients, compared with TESC-low tumor tissues, further corroborated the above findings  
197 (**Supplemental Figure 2G**). Collectively, these findings suggest that tumor-intrinsic TESC  
198 may not directly affect T-cell activation but rather potentially target APCs and reduce tumor  
199 immunity in an antigen presentation-dependent manner.

200

201 **TESC attenuates tumor immunogenicity by disrupting antigen presentation by APCs**

202 Damage-associated molecular patterns (DAMPs) dynamically shape the phenotype and  
203 function of APCs during tumor antigen uptake and processing, notably via macrophage- and  
204 dendritic cell (DC)-mediated phagocytosis (25–27). To investigate the potential role of tumor-  
205 intrinsic TESC in modulating APC function, we cocultured bone marrow-derived macrophages  
206 (BMDMs), CellTrace™ Violet (CTV)-labeled OT-I T cells, and dead OVA-expressing Hepa1-  
207 6 tumor cells either with or without TESC overexpression (**Supplemental Figure 3A**). We  
208 observed an increase in OT-I cell activation in a dose-dependent manner via CTV dilution in  
209 OT-I cells and an increase in the intracellular expression of IFN- $\gamma$  and granzyme B after  
210 coculture with dead Hepa1-6 cells (**Figure 3, A–C**). However, the magnitude of OT-I cell  
211 activation was reduced following coculture with dead *Tesc* Hepa1-6 cells, suggesting that TESC  
212 impairs the capacity of dying tumor cells to trigger effective T-cell activation. We performed an  
213 identical experiment with NC and *shTesc* H22 cells (**Figure 3, D and E**). In this setting, OT-I  
214 cell activation was higher in the presence of dead *shTesc* H22 cells than with dead H22 cells  
215 (**Figure 3, D and E**). We further extended these studies from macrophages to myeloid-derived  
216 DCs. Under comparable experimental conditions, DCs were substituted for macrophages  
217 (**Supplemental Figure 3, B and C**). Consistently, OT-I cell activation was significantly higher  
218 in the presence of dead TESC-deficient H22 cells than with dead wild-type H22 cells (**Figure**  
219 **3, F and G**). Importantly, OT-I cells failed to exhibit activation in the absence of either  
220 macrophages or DCs (**Figure 3, A–C, F and G**). Thus, tumor-intrinsic TESC results in reduced  
221 T-cell activation by targeting APCs.

222 We next examined the in vivo impact of tumor-derived TESC on the immune responses  
223 mediated by APCs, with a particular focus on tissue-resident macrophages. We orthotopically  
224 implanted control or *shTesc* H22 cells into the livers of immunocompetent mice with or without

225 anti-CSF1R treatment to deplete macrophages (**Supplemental Figure 3, D and E**). Silencing  
226 TESC significantly suppressed tumor growth, but this effect was partially reversed upon  
227 macrophage depletion (**Figure 3H**). Consistent with these findings, CD8<sup>+</sup> T cells from  
228 macrophage-depleted mice presented reduced proliferative and cytotoxic activities, as indicated  
229 by decreased Ki-67, IFN- $\gamma$  and Granzyme B expression (**Figure 3, I and J**). Collectively, these  
230 findings suggest that tumor-intrinsic TESC compromises antitumor immunity by functionally  
231 targeting both DCs and macrophages.

232

### 233 **TESC restrains CALR membrane translocation to inhibit macrophage function**

234 The phagocytosis of dead tumor cells is the initial step for APCs to capture, process, and present  
235 antigens to T cells (13, 28). To dissect the mechanism by which intrinsic TESC downregulates  
236 tumor immunogenicity, we hypothesized that tumor-derived TESC affects the nature of APC  
237 phagocytosis and, in turn, impairs APC-mediated antigen presentation and T-cell activation. We  
238 tested this hypothesis by coculturing macrophages with GFP-labeled dead H22 cells or TESC-  
239 deficient H22 cells for 20 hours and assessed phagocytic uptake by quantifying intracellular  
240 GFP levels. Compared with control H22 cells, macrophages exposed to sh*Tesc* cells presented  
241 a marked increase in GFP accumulation (**Figure 4A**), a result also observed in vivo  
242 (**Supplemental Figure 4A**). This suggests that tumor-derived TESC inhibits macrophage-  
243 mediated engulfment and phagocytosis of dead tumor cells.

244 We next evaluated the effects of tumor-derived TESC on macrophage-mediated engulfment  
245 and phagocytosis over time (29). Similar to the above experiment (**Figure 4A**), we incubated  
246 macrophages with dead H22 or sh*Tesc* H22 cells for 20 hours, pulsed them with pHrodo Red-  
247 labeled particles for 20 minutes and monitored them for 40 minutes. pHrodo Red dye is pH  
248 sensitive and its fluorescence increases in acidic phagosomes (30). We monitored red  
249 fluorescence in the macrophages and observed more beads in the phagosomes of the

250 macrophages cultured with dead TESC-deficient cells than in those cultured with dead H22  
251 cells (**Figure 4B**). We performed similar experiments with dead Hepa1-6 cells and *Tesc* Hepa1-  
252 6 cells (**Supplemental Figure 4B**). These data suggest that tumor-derived TESC potentially  
253 alters persistent macrophage-mediated phagocytosis. We stained macrophages with  
254 LysoTracker Deep Red, a fluorescent dye that labels acidic lysosomal compartments, to further  
255 validate this result. Confocal microscopy revealed the beads within the macrophages (**Figure**  
256 **4C**), and the LysoTracker fluorescence intensity at the bead-containing phagosomes served as  
257 a readout for maturation. Compared with macrophages cultured with dead wild-type H22 cells,  
258 we detected an increase in LysoTracker fluorescence intensity at the bead areas in phagosomes  
259 in macrophages incubated with dead TESC-deficient H22 cells, suggesting induced phagosome  
260 maturation (**Figure 4C**). We further questioned whether the negative effect of tumor-derived  
261 TESC on macrophage phagocytosis is involved in impaired antigen presentation. We tested this  
262 hypothesis by coculturing macrophages with dead OVA-expressing H22 cells and *shTesc*-OVA  
263 H22 cells. Compared with those cocultured with wild-type H22 cells, macrophages exposed to  
264 *shTesc*-OVA H22 cells presented elevated levels of OVA–MHC-I complexes (**Figure 4D**). In  
265 contrast, macrophages incubated with TESC-overexpressing Hepa1-6 cells presented reduced  
266 OVA–MHC-I presentation (**Supplemental Figure 4C**), which is consistent with the findings  
267 described above, demonstrating the negative role of tumor-derived TESC in antigen-specific T-  
268 cell activation (**Figure 3, A–C**). Consistent with the in vitro findings, TESC silencing in H22  
269 tumors markedly increased the proportion of MHC-I<sup>+</sup> and MHC-II<sup>+</sup> F4/80<sup>+</sup> macrophages  
270 (**Supplemental Figure 4D**), whereas TESC overexpression in Hepa1-6 tumors decreased this  
271 population (**Supplemental Figure 4E**), indicating the upregulation of TESC impaired  
272 macrophage antigen-presenting capacity. In support, the single-cell RNA sequencing analysis  
273 (GSE151530) of human HCC tumors revealed that high TESC expression in malignant cells  
274 was associated with reduced phagocytosis and antigen presentation scores in tumor-associated

275 macrophages (**Supplemental Figure 4, F and G**). Taken together, these results suggest that  
276 tumor-intrinsic TESC targets macrophage phagocytosis, resulting in a reduced antigen  
277 presentation and T-cell activation.

278 We next investigated how tumor-intrinsic TESC affected macrophage phagocytosis. Given  
279 that TESC had no direct effect on APC-mediated T-cell activation, we hypothesized that tumor-  
280 intrinsic TESC may indirectly regulate macrophage phagocytosis through an interaction partner.  
281 To identify the potential partners of TESC at the protein level, we performed data-independent  
282 acquisition (DIA) proteomic profiling of *shTesc* and control H22 cells. The Gene Ontology  
283 analysis revealed the significant upregulation of MHC class I peptide-loaded components,  
284 particularly TAP1, in *shTesc* cells (**Figure 4E and Supplemental Figure 4, H and I**). However,  
285 TAP1 silencing failed to alter macrophage-mediated phagocytosis or OVA–MHC-I presentation  
286 in the *shTesc* context (**Figure 4F and Supplemental Figure 4, J and K**), suggesting that TAP1  
287 is not essential in this process. Notably, proteomic analysis revealed calreticulin (CALR) as the  
288 most prominently altered phagocytosis-related signal upon TESC knockdown (**Supplemental**  
289 **Figure 4L**), whereas other canonical “eat-me” or “don’t-eat-me” molecules showed no  
290 significant changes, suggesting a selective effect of TESC on the CALR axis. Consistently,  
291 surface CALR exposure was markedly increased in TESC-deficient H22 cells but reduced in  
292 TESC-overexpressing Hepa1-6 cells (**Figure 4, E, G, and H, and Supplemental Figure 4, H,**  
293 **I and M**), whereas CD47 expression remained comparable between *shTesc* H22 and TESC-  
294 overexpressing Hepa1-6 cells and their respective controls (**Supplemental Figure 4, H, I, and**  
295 **N**). Given that membrane CALR functions as an “eat-me” signal and facilitates APC  
296 phagocytosis (19, 31, 32), we hypothesized that TESC may regulate phagocytosis through the  
297 modulation of CALR membrane translocation. As expected, CALR silencing in TESC-deficient  
298 H22 cells markedly diminished phagocytic bead uptake and antigen presentation, which was  
299 comparable to the reduction observed in *shCalr* H22 cells (**Figure 4I and Supplemental**

300 **Figure 4, O and P**). To extend these findings to human HCC, we generated TESC-silenced  
301 HepG2 cells and TESC-overexpressing Huh7 cells (**Supplemental Figure 4Q and 4R**), and  
302 found that manipulation of TESC expression in both models resulted in concordant changes in  
303 CALR surface exposure: TESC silencing in HepG2 significantly increased CALR membrane  
304 localization, whereas TESC overexpression in Huh7 markedly reduced CALR surface exposure  
305 (**Figure 4J and Supplemental Figure 4S**). Consistently, macrophages and DCs differentiated  
306 from human peripheral blood monocytes exhibited increased phagocytosis of GFP-labeled  
307 TESC-silenced HepG2 cells, accompanied by elevated HLA-ABC and HLA-DR expression,  
308 whereas the opposite effect were observed in the co-culture system with TESC-overexpressing  
309 Huh7 cells (**Figure 4, K–M, and Supplemental Figure 4, T–V**).

310 To determine the functional consequence of CALR in CD8<sup>+</sup> T-cell immunity, OT-I cells were  
311 cultured with macrophages in the presence of OVA-loaded dead tumor cells. TESC-deficient  
312 H22 cells induced robust OT-I proliferation and activation, as evidenced by increased Ki-67,  
313 IFN- $\gamma$ , and granzyme B expression, whereas CALR silencing abolished these effects (**Figure 4,**  
314 **N and O**). Similarly, in vivo, CD8<sup>+</sup> T-cell-mediated cytotoxicity toward TESC-deficient  
315 hepatomas was abrogated by concomitant CALR silencing, leading to tumor growth rates  
316 comparable to those of controls (**Figure 4, P–R**). Moreover, RNA sequencing of macrophages  
317 that had engulfed TESC-overexpressing versus control tumor cells (GSA: CRA037701)  
318 revealed that broad suppression of gene programs related to actin cytoskeletal dynamics, ion  
319 channel activity, APC costimulation and maturation (CD40, CD86, MHC class I and II), and  
320 cytokine production, including IL6 (**Supplemental Figure 4W**). Collectively, these results  
321 demonstrate that tumor-intrinsic TESC restrains CALR membrane translocation, thereby  
322 suppressing macrophage phagocytosis and antigen presentation and dampening CD8<sup>+</sup> T-cell-  
323 mediated antitumor immunity

324

325 **TESC regulates CALR surface exposure by modulating calcium homeostasis and**  
326 **attenuating ER stress**

327 Having established the importance of TESC in modulating CALR surface exposure and thereby  
328 governing macrophage-mediated phagocytosis and antigen presentation, we next sought to  
329 elucidate the underlying molecular mechanism. CALR is a key endoplasmic reticulum (ER)-  
330 resident chaperone that is essential for maintaining protein-folding homeostasis (33). Upon ER  
331 stress activation, CALR can be aberrantly translocated to the cell surface via the unfolded  
332 protein response (UPR), where it acts as a canonical “eat-me” signal to facilitate immune  
333 recognition and phagocytosis (33, 34). To determine whether TESC influences CALR  
334 membrane exposure by regulating ER stress, we first examined UPR pathway activation in cells  
335 with different levels of TESC expression. Immunoblotting analyses revealed that TESC  
336 silencing robustly activated UPR signaling, whereas TESC overexpression markedly attenuated  
337 ER stress (**Figure 5A**). Treatment of sh*Tesc* and NC H22 cells with the ER stress inhibitor  
338 TUDCA abolished the increase in CALR surface exposure induced by TESC depletion (**Figure**  
339 **5B**), indicating that TESC limits CALR membrane translocation through the suppression of ER  
340 stress. TESC contains a canonical EF-hand domain capable of binding cytosolic Ca<sup>2+</sup> (35).  
341 Given the central role of calcium signaling in UPR activation and ER homeostasis (36, 37), we  
342 hypothesized that TESC buffers excess cytosolic Ca<sup>2+</sup>, thereby preventing ER Ca<sup>2+</sup> overload  
343 and subsequent stress responses. We tested this hypothesis using the ER-specific calcium probe  
344 Mag-Fluo-4-AM to quantify ER luminal Ca<sup>2+</sup> levels. Notably, TESC deficiency increased ER  
345 Ca<sup>2+</sup> accumulation, whereas TESC overexpression reduced ER Ca<sup>2+</sup> levels (**Figure 5C and**  
346 **Supplemental Figure 5, A and B**), suggesting that TESC restricts Ca<sup>2+</sup> influx into the ER to  
347 maintain homeostasis. We next asked whether cytosolic calcium is required for the ability of  
348 TESC to regulate macrophage phagocytosis and antigen presentation. The chelation of cytosolic  
349 Ca<sup>2+</sup> with BAPTA significantly alleviated ER stress and CALR membrane exposure in TESC-

350 deficient cells (**Figure 5, D and E**). Importantly, the direct alleviation of ER stress did not  
351 significantly alter ER Ca<sup>2+</sup> levels (**Figure 5F and Supplemental Figure 5C**), indicating that  
352 Ca<sup>2+</sup> overload precedes UPR activation, not vice versa. These findings support a model in which  
353 TESC regulates CALR membrane translocation by maintaining cytosol-to-ER calcium  
354 homeostasis.

355 Calcium overload may also perturb mitochondrial homeostasis, leading to elevated  
356 mitochondrial Ca<sup>2+</sup> and ROS production, which secondarily activate the UPR (37-39). We  
357 further assessed mitochondrial Ca<sup>2+</sup> levels using Rhod-2-AM and intracellular ROS levels in  
358 cells with different TESC expression levels to exclude this alternative pathway. Neither  
359 parameter changed significantly (**Figure 5G and Supplemental Figure 5, A and B**),  
360 suggesting that TESC regulates UPR predominantly through the cytosol-ER calcium axis rather  
361 than via mitochondrial dysfunction. To further determine whether the calcium-binding ability  
362 of TESC is essential for its function, we generated a point mutation (D128A) in the EF-hand  
363 domain known to disrupt calcium coordination (35). This mutant failed to suppress ER Ca<sup>2+</sup>  
364 accumulation (**Figure 5H**), reinstated UPR activation (**Figure 5I**), and restored CALR  
365 membrane translocation (**Figure 5J**), despite its overexpression. Consistent with our previous  
366 results (**Figure 3, A-C, and Supplemental Figure 4C**), macrophages exposed to TESC-  
367 overexpressing cells presented significantly suppressed antigen presentation and T-cell  
368 activation than Hepa1-6 cells did, while the D128A mutation abolished these effects (**Figure 5,**  
369 **K-M**). Thus, TESC restricts ER stress-induced CALR exposure by buffering cytosolic Ca<sup>2+</sup>,  
370 thereby suppressing macrophage phagocytosis and antitumor immunity in HCC.

371

372 **Targeting H3K4me3-induced TESC overcomes immune suppression and potentiates the**  
373 **efficacy of PD-1 combination therapy in HCC**

374 Epigenetic modifications, including DNA methylation, histone modifications, and chromatin

375 remodeling, are critical regulators of gene regulation and cellular plasticity (40). To explore  
376 whether TESC expression is epigenetically regulated, we analyzed HCC epigenetic alterations  
377 from TCGA dataset and observed a strong positive correlation between TESC expression and  
378 the expression of multiple histone methyltransferases (**Figure 6A**), suggesting a role for histone  
379 methylation in the control of TESC transcription. Consistently, an analysis of the  
380 MethMarkerDB database revealed a positive association between TESC expression and  
381 promoter DNA methylation (**Figure 6B**). Chromatin immunoprecipitation (ChIP) sequencing  
382 data from normal human liver tissues (ENCSR000FYQ) showed the marked enrichment of  
383 H3K4me1 and H3K4me3 at the TESC promoter region (**Supplemental Figure 6A**). Targeted  
384 inhibition of H3K4me3 using the selective antagonist OICR-9429 significantly suppressed  
385 TESC expression in both H22 and HepG2 cells in a dose-dependent manner (**Figure 6C and**  
386 **Supplemental Figure 6B**), whereas pharmacologic blockade of H3K4me1 had no effect  
387 (**Figure 6D and Supplemental Figure 6C**). Consistently, ChIP-qPCR analysis confirmed  
388 robust H3K4me3 occupancy at the TESC promoter, supporting a direct role for H3K4me3 in  
389 driving TESC transcription (**Figure 6E**). Notably, TESC knockdown and overexpression did  
390 not alter global H3K4me3 levels (**Supplemental Figure 6D**), suggesting that H3K4me3 acts  
391 upstream of TESC and not vice versa. Similarly, in mice bearing Hepa1-6 hepatoma, injecting  
392 OICR-9429 effectively reduced tumor TESC expression (**Supplemental Figure 6, E and F**).  
393 Importantly, OICR-9429 treatment restored antigen presentation by tumor-associated  
394 macrophages and rescued CD8<sup>+</sup> T-cell effector responses previously suppressed by TESC  
395 (**Figure 6, F–H**), indicating that pharmacological targeting of TESC can reinvigorate antitumor  
396 immunity. Notably, in HCC tissues derived from patients receiving anti-PD-1 therapy, low  
397 TESC expression was associated with reduced H3K4me3 levels, increased ER stress, increased  
398 CALR surface exposure, enhanced macrophage antigen presentation, and increased CD8<sup>+</sup> T-  
399 cell cytotoxic activity (**Figure 6, I and J**). Accordingly, intratumoral H3K4me3 expression

400 showed an acceptable ability to predict response to immunotherapy (AUC = 0.75,  $P < 0.05$ ),  
401 and reduced intratumoral H3K4me3 levels in HCC patients significantly correlated with  
402 improved overall survival after immunotherapy (**Figure 6K and Supplemental Figure 6G**),  
403 suggesting that this epigenetic axis is a key determinant of immune responsiveness.

404 Since H3K4me3 drives transcription and impairs immune activation, we next tested whether  
405 targeting this epigenetic axis could increase the efficacy of PD-1 blockade in tumors. In an  
406 orthotopic *Tesc* H22 hepatoma model, treatment with the selective H3K4me3 inhibitor OICR-  
407 9429, the anti-PD-1 antibody, or their combination led to the suppression of TESC expression  
408 and tumor growth (**Figure 6L and Supplemental Figure 6H**), with the greatest therapeutic  
409 benefit observed in the combination group (**Figure 6L**), and the combination of OICR-9429  
410 and anti-PD-1 antibody was found to be safe (**Supplemental Figure 6I**). This response was  
411 associated with enhanced macrophage-mediated antigen presentation and improved CD8<sup>+</sup> T-  
412 cell effector function, as indicated by increased expression of H-2Kb, Ki-67, IFN- $\gamma$ , and  
413 granzyme B (**Figure 6, M–O**). Notably, these immune enhancements and antitumor effects  
414 were abolished in TESC-overexpressing tumors, confirming that the therapeutic efficacy of  
415 OICR-9429 depends on effective TESC inhibition (**Figure 6, M–O**). In a *sgP53/Myc*-driven  
416 spontaneous hepatoma model characterized by intrinsic resistance to ICB, enforced TESC  
417 overexpression further exacerbated resistance to both anti-PD-1 monotherapy and combined  
418 OICR-9429 and anti-PD-1 treatment, accompanied by reduced macrophage antigen-presenting  
419 capacity and impaired CTL function (**Supplemental Figure 6J–O**). Moreover, in an acquired  
420 anti-PD-1-resistant orthotopic hepatoma model established through iterative cycles of anti-PD-  
421 1 treatment followed by tumor re-isolation and re-implantation (**Supplemental Figure 6P**),  
422 TESC expression was markedly elevated in the resistant tumor (**Supplemental Figure 6Q**),  
423 and pharmacological inhibition of TESC using OICR-9429 significantly restored sensitivity to  
424 anti-PD-1 therapy (**Supplemental Figure 6R**). Thus, H3K4me3-driven TESC functions as an

425 epigenetic barrier to antitumor immunity in HCC and represents a target for combination

426 therapy.

427

## 428 **Discussion**

429 Immune checkpoint blockade (ICB) has revolutionized cancer therapy, yet its clinical efficacy  
430 in hepatocellular carcinoma (HCC) remains limited due to the intrinsic resistance mechanisms  
431 embedded within the immunosuppressive tumor microenvironment (TME) (4, 41-43). Here, we  
432 identified tescalcin (TESC), a calcium-binding EF-hand protein, as a previously unrecognized  
433 tumor-intrinsic modulator and phagocytic checkpoint that promotes immune evasion and  
434 compromises the efficacy of ICB in HCC. We used multiple complementary strategies to map  
435 the regulation, pathogenic influences, and clinical relevance of this molecule to cancer  
436 progression and ICB therapy. These findings highlight a pivotal role for tumor-intrinsic  
437 molecules in shaping the immune characteristics of HCC and provide insights into the  
438 mechanism underlying immunotherapy resistance.

439 Studies have revealed that TESC, a calcium-binding EF-hand protein, may be involved in  
440 cellular stress, metabolism, proliferation, and tumorigenesis (44). Several studies have detected  
441 elevated levels of TESC in cancers, such as thyroid cancer, colorectal cancer, and  
442 cholangiocarcinoma (45-47). Unexpectedly, we found that TESC is an immunoregulatory  
443 molecule in the context of tumor immunity. We functionally validated the relationship between  
444 TESC and tumor immunity in multiple murine hepatoma models. Hence, our work revealed  
445 that tumor-derived TESC functions as a previously unappreciated intrinsic resistance  
446 mechanism in tumor immunity and immunotherapy.

447 Although TESC exerted potent immunosuppressive effects on various hepatoma-bearing  
448 models in vivo and its high expression was associated with a poor clinical response to ICB, the  
449 precise mechanisms by which TESC disrupts antitumor immunity remain unclear. We observed  
450 that TESC was markedly overexpressed in HCC cells, and its expression correlated inversely  
451 with tumor sensitivity to immune checkpoint blockade. Given that TESC is a nonsecretory  
452 protein, we hypothesized that TESC, similar to MYC or NLRC5 (48, 49), might interfere with

453 MHC-I antigen presentation and directly facilitate tumor immune escape by evading T-cell  
454 recognition. Unfortunately, TESC expression in tumor cells did not directly inhibit T-cell  
455 activation under either antigen-specific or polyclonal TCR stimulation. Intriguingly, when T  
456 cells were cocultured with APCs in the presence of either TESC-overexpressing or TESC-  
457 deficient apoptotic tumor cells, we found that T-cell activation was significantly impaired in the  
458 former group but not in the latter group. These results prompted us to speculate that tumor-  
459 derived TESC may modulate APC function and, in turn, regulate APC-mediated T-cell priming.

460 We initially examined the potential role of tumor cell-intrinsic TESC in APC-mediated  
461 phagocytosis to explore this possibility. Phagocytosis is an early step during APC-mediated  
462 antigen capture, processing, and presentation (13). Indeed, we have shown that tumor cell-  
463 derived TESC negatively affects APC-mediated phagocytosis, accompanied by reduced antigen  
464 presentation. Since no prior studies have reported the regulatory role of TESC in APC function  
465 and given its nature as a  $\text{Ca}^{2+}$  sensor (35), we speculated that TESC may modulate phagocytic  
466 activity by altering intracellular calcium-dependent signaling. Consistent with this possibility,  
467 our proteomic profile revealed close interactions between TESC and key components of the  
468 MHC class I peptide loading complex, notably TAP1 and CALR. Strikingly, silencing CALR,  
469 but not TAP1, abolished the enhanced phagocytosis and antigen presentation observed in  
470 macrophages exposed to TESC-deficient tumor cells. Mechanistically, the loss of TESC  
471 increased cytosolic  $\text{Ca}^{2+}$  levels, induced endoplasmic reticulum (ER)  $\text{Ca}^{2+}$  overload and stress,  
472 and promoted CALR exposure on the tumor cell surface—an established prophagocytic “eat-  
473 me” signal (19). These findings suggest that TESC attenuates ER stress by modulating cytosolic  
474 calcium levels, thereby reducing CALR membrane translocation and suppressing CALR-  
475 mediated APC phagocytic activity. Consistent with prior studies showing that surface CALR is  
476 essential for immunogenic cell clearance (19, 50), particularly when counteracting “don’t eat-  
477 me” signals such as CD47 or CD24, we propose that TESC functions as an intracellular

478 checkpoint that suppresses CALR-mediated phagocytosis. Indeed, in HCC patients treated with  
479 immune checkpoint blockers (ICBs), low TESC expression correlated with elevated ER stress,  
480 increased membrane CALR expression, and increased APC-driven CD8<sup>+</sup> T-cell activation. As  
481 CALR exposure is a prerequisite for immunogenic tumor cell death induced by  
482 chemotherapeutic drugs such as oxaliplatin and mitoxantrone (19), our findings extend this  
483 paradigm by positioning TESC as a key negative regulator of CALR trafficking. In addition to  
484 recent advances in identifying CD47–SIRP $\alpha$ , CD24–Siglec-10, and Siglec-15 as phagocytosis  
485 checkpoints (16, 51, 52), our study introduces TESC as an intracellular modulator of the  
486 phagocytic synapse. Together, these findings suggest that TESC represents a previously  
487 unrecognized phagocytosis checkpoint that governs the immunogenicity of tumor cells by  
488 restricting CALR surface exposure and macrophage-mediated clearance. Therefore, a better  
489 understanding of the regulatory network of TESC in cancer cells in the tumor environment  
490 would be helpful for developing rational macrophage-based designs for anticancer therapies  
491 that can amplify the antitumorigenic function of ICBs.

492 Epigenetic dysregulation is a hallmark of cancer (53). While previous studies have shown  
493 that genetic and epigenetic alterations can impair the IFN- $\gamma$ –MHC-I axis and lead to T-cell  
494 dysfunction and immune escape (11), whether these tumor-intrinsic changes also affect APC-  
495 mediated phagocytosis, thereby contributing to ICB resistance, remains unclear. In the present  
496 study, through integrated multi-omics analyses, we identify a strong positive association  
497 between TESC expression and multiple histone methyltransferases in HCC, with H3K4me3  
498 emerging as a key epigenetic driver of TESC upregulation. Selective inhibition of H3K4me3  
499 using OICR-9429 consistently suppressed TESC expression in both transplanted and  
500 spontaneous murine hepatoma, restored CALR surface exposure, reactivated the phagocytic  
501 function of macrophages, and increased the efficacy of ICB. Notably, in an acquired anti-PD-  
502 1–resistant orthotopic hepatoma model through iterative anti-PD-1 treatment and tumor re-

503 implantation, pharmacological inhibition of TESC similarly reinstated sensitivity to anti-PD-1  
504 therapy. These results suggest that TESC represents a therapeutically actionable epigenetic–  
505 immune node that mechanistically links tumor-intrinsic chromatin remodeling to impaired  
506 phagocytic surveillance and immune evasion.

507 In addition to its biological importance, our work may inform the clinical management of  
508 HCC patients receiving anti-PD-1 (L1) therapy, suggesting that combination with epigenetic  
509 inhibitors of TESC, such as OICR-9429, could enhance therapeutic efficacy. While our work  
510 establishes a tumor-intrinsic role for TESC in restraining CALR-dependent phagocytosis and  
511 immune surveillance, the generalizability of these findings across diverse HCC etiologies,  
512 distinct tumor microenvironments, or other cancer types remains to be determined. Additionally,  
513 our models primarily use murine transplanted and spontaneous hepatomas, which may not fully  
514 recapitulate human tumor heterogeneity. Future studies should evaluate the clinical translation  
515 of pharmacological TESC inhibition (e.g., OICR-9429), including therapeutic feasibility, safety,  
516 and optimal dosing in humans. Collectively, these directions highlight the potential of targeting  
517 the H3K4me3–TESC axis to improve immune checkpoint blockade outcomes and guide  
518 rational combination strategies for HCC.

519

## 520 **Methods**

### 521 *Sex as a biological variable*

522 Our study examined male and female animals, and similar findings are reported for both sexes.

### 523 *Patients and specimens*

524 Tumor samples were collected from 64 patients with pathologically confirmed HCC at the Sun  
525 Yat-sen University Cancer Center. Samples from patients with concurrent autoimmune disease,  
526 HIV, or syphilis were excluded. Tumor biopsy samples from 14 HCC patients collected prior to  
527 oxaliplatin-based chemotherapy plus ICB (anti-PD-1 antibody) treatment between May 2019  
528 and June 2020 were used for RNA sequencing (cohort 1). Patients in cohort 1 were enrolled  
529 from a clinical trial (NCT03869034). Among the 29 evaluable patients, 14 (48.3%) responded  
530 to treatment, while 15 (51.7%) did not respond. Tumor samples from 20 HCC patients who  
531 exclusively underwent surgical resection between July 2017 and September 2017 were  
532 subjected to immunofluorescence. Tumor samples from 30 patients with comprehensive follow-  
533 up data who underwent surgical resection after receiving oxaliplatin-based chemotherapy plus  
534 ICB treatment between April 2019 and July 2022 were used for both immunofluorescence and  
535 immunohistochemistry (cohort 3). Tumor biopsy specimens from 20 patients with  
536 comprehensive follow-up data who received oxaliplatin-based chemotherapy between July  
537 2020 and June 2022 were used for immunohistochemistry (cohort 4). Detailed patient  
538 information is provided in Supplemental Table 1.

### 539 *Animal studies*

540 Wild-type C57BL/6 mice, BALB/C mice, Nude mice and OT-I transgenic mice, all aged 5–8  
541 weeks, were purchased from Beijing Vital River Laboratory Animals.

542 For the orthotopic hepatoma models, Hepa1-6 cells and H22 cells were inoculated into  
543 C57BL/6, BALB/C, and nude mice, respectively. A total of  $1 \times 10^6$  hepatoma cells were  
544 suspended in 20  $\mu$ L of a mixture of PBS and Matrigel at a 1:1 volume ratio and then injected

545 into the subcapsular region of the liver. Tumor size (tumor length × width × height/2) was  
546 measured for a comparative analysis. For the spontaneous hepatoma models, a plasmid mixture  
547 containing 10 µg *sgP53*, 12 µg *Myc*, 20 µg NC/*sgTesc* or NC/*Tesc*, and SB100 transposase  
548 plasmid at 1/4 of the total plasmid mass was diluted in 0.9% saline to a final volume  
549 corresponding to 10% of the mouse body weight hydrodynamically injected into mice via the  
550 tail vein within 5–7 seconds. The maximal diameter of macroscopic tumor nodules was  
551 analyzed. For the αPD-1-resistant mouse model, mice bearing Hepa1-6 hepatomas were treated  
552 with αPD-1 or IgG in each cycle, and tumors with the highest burden were harvested for ex  
553 vivo tumor cell isolation and expansion, followed by reimplantation into mice. After four  
554 sequential cycles, αPD-1-resistant and αPD-1-sensitive mouse models were established. The  
555 rest detailed procedures of the animal experiments are shown in Figure 1, H–J, M, and O,  
556 Supplemental Figure 2A, Supplemental Figure 3D, and Supplemental Figure 6, E, J and P.

### 557 ***Cell lines and cell culture***

558 The murine hepatoma cell lines Hepa1-6 and Hepa1c1c7, the human hepatoma cell lines HepG2,  
559 SNU449, LM3, and Hep3B, and HEK293T cell line were obtained from the American Type  
560 Culture Collection (ATCC). The murine hepatoma cell line H22 and the human hepatoma cell  
561 line Huh7 were obtained from Procell. These cell lines were cultured in DMEM or RPMI 1640  
562 supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin in cell culture  
563 dishes. All the cell lines were routinely Written informed consent was obtained from each  
564 authenticated using a short tandem repeat analysis and tested for mycoplasma contamination  
565 using single-step PCR.

### 566 ***Flow cytometry (FACS)***

567 Single-cell suspensions were prepared from fresh tumor tissue as previously described (41).  
568 Briefly, tumors were cut into 2–3 mm<sup>3</sup> pieces and dissociated into single cells using a mouse  
569 tumor dissociation kit for 25 minutes at 37°C. The resulting cell suspensions were filtered

570 through a 70  $\mu\text{m}$  strainer and resuspended in PBS containing 2% BSA. Red blood cells were  
571 lysed with red blood cell lysis buffer. For surface staining, the samples were incubated with a  
572 cocktail of antibodies in cell staining buffer for 30 minutes at 4°C. For intracellular staining,  
573 the samples were first stimulated for 6 hours at 37°C with a cell stimulation cocktail, followed  
574 by treatment with a fixation/permeabilization kit and then an incubation with antibodies. For  
575 intranuclear staining, the samples were treated with a transcription factor buffer set according  
576 to the manufacturer's instructions and incubated with antibodies. Finally, the cells were washed,  
577 resuspended in PBS at 4°C, and analyzed using a flow cytometer (CytoFLEX). The data were  
578 processed using FlowJo software.

579 For apoptosis assay, H22 cells were treated with 120  $\mu\text{M}$  oxaliplatin, while Hepa1-6, HepG2,  
580 and Huh7 cells were treated with 700  $\mu\text{M}$  oxaliplatin for 48 hours. The tumor cells were stained  
581 using an Annexin V apoptosis detection kit according to the manufacturer's protocol and  
582 analyzed using a flow cytometer (CytoFLEX). The data were processed using FlowJo software.

### 583 ***Immunohistochemistry***

584 Paraffin-embedded tumor samples were cut into 4  $\mu\text{m}$ -thick slices. After the sections were  
585 baked for 1 hour at 65°C, the paraffin was removed by dewaxing in xylene, followed by  
586 rehydration through a graded ethanol series. Antigen retrieval was performed using EDTA  
587 buffer. The sections were then incubated with endogenous peroxidase blocking buffer for 15  
588 minutes to inhibit endogenous peroxidase activity and subsequently treated with blocking  
589 buffer for 30 minutes. The samples were incubated with primary antibodies overnight at 4°C.  
590 After washes with TBST, the samples were incubated with an HRP-conjugated secondary  
591 antibody for 30 minutes at 37°C. Signals were visualized using DAB substrate, and the reaction  
592 was terminated by rinses with distilled water. The sections were dehydrated through a graded  
593 ethanol series and mounted with neutral balsam before being counterstained with hematoxylin.  
594 The slides were scanned using a scanner (KONFOONG Bioinformation).

595 ***Immunofluorescence staining***

596 Immunofluorescence staining was performed using a PANO 7-plex IHC kit. The procedures,  
597 from paraffin-embedded tissue section hydration to the secondary antibody incubation, were  
598 performed according to the protocol outlined in the '***Immunohistochemistry***' methods section.  
599 Then, the sections were incubated with fluorescent dyes, diluted in the tyramine working  
600 solution, for 10 minutes at room temperature. Microwave-based antigen retrieval was  
601 performed in EDTA buffer (pH 9.0). The sections were incubated with blocking buffer for 30  
602 minutes at room temperature. The staining cycle procedure was repeated for each primary  
603 antibody until the desired number of cycles was completed. The sections were mounted with  
604 anti-fluorescence quenching sealing solution containing DAPI. The slides were scanned using  
605 the KONFOONG scanner.

606 For immunofluorescence staining of surface CALR in hepatoma cells, cells grown on  
607 confocal dishes were fixed with 4% paraformaldehyde for 15 minutes at room temperature.  
608 After three washes with PBS, the cells were blocked with 5% BSA without permeabilization.  
609 The cells were then stained with a primary anti-CALR antibody, followed by an incubation with  
610 a secondary antibody conjugated to Alexa Fluoro 488.

611 ***Immunoblotting***

612 The processed cells were washed three times with PBS, resuspended in lysis buffer and  
613 incubated on ice for 30 minutes. Following ultrasonic disruption and centrifugation at 10,000  
614 × g for 15 minutes, the supernatants were collected, mixed with loading buffer, and heated at  
615 95°C for 10 minutes. The total protein solutions were loaded onto a sodium dodecyl sulfate–  
616 polyacrylamide gel electrophoresis (SDS–PAGE) gel, and then electrotransferred to  
617 polyvinylidene fluoride (PVDF) membranes. After blocking with 5% BSA, the membranes  
618 containing the target proteins were incubated with specific antibodies overnight at 4°C. Finally,  
619 the target protein was detected using specific antibodies and a commercial ECL kit.

620 ***Cell proliferation assay***

621 Hepa1-6 or H22 hepatoma cells were plated at a density of  $2 \times 10^3$  cells per well in a 96-well  
622 cell culture plate. The cell density was measured every 24 hours using the Cell Counting Kit-8  
623 (CCK-8) reagent according to the manufacturer's protocol, and the cells were incubated for 1  
624 hour at 37°C. The optical density (OD) value was assessed at 450 nm.

625 ***ChIP assay***

626 ChIP assays were conducted to investigate the association between H3K4me3 and the TESC  
627 promoter. The assays were performed using an EZ-Magna ChIP kit according to the  
628 manufacturer's instructions. After the crosslinks were reversed, the DNA immunoprecipitated  
629 with the H3K4me3 antibody was analyzed by qRT-PCR.

630 ***Enzyme-linked immunosorbent assay (ELISA)***

631 For the analysis of splenocyte activation, splenocytes from OT-I TCR transgenic mice were  
632 activated and incubated with dead Ovalbumin (OVA)-loaded tumor cells at a 2:1 ratio for 3  
633 days. IFN- $\gamma$  concentrations in the supernatants from the incubation of splenocytes with dead  
634 tumor cells were measured using ELISA kits according to the manufacturer's instructions.

635 ***Endoplasmic reticulum and mitochondrial calcium measurements***

636 The calcium levels in the endoplasmic reticulum and mitochondria were monitored using the  
637 specific calcium-sensitive fluorescent indicators Mag-Fluo-4-AM and Rhod2-AM, respectively.  
638 After washes with HBSS, tumor cells were incubated with 2  $\mu$ M Mag-Fluo-4-AM or 5  $\mu$ M  
639 Rhod2-AM for 40 minutes at 37°C in the dark. The fluorescence intensity was measured to  
640 assess calcium levels via fluorescence microscopy or FACS.

641 ***Measurement of reactive oxygen species (ROS) levels***

642 The intracellular ROS levels were measured using the ROS-sensitive fluorescent indicator  
643 H2DCFDA. After washes with PBS, the tumor cells were incubated with 5  $\mu$ M H2DCFDA for

644 30 minutes at 37°C in the dark. ROS levels were quantified by measuring the fluorescence  
645 intensity using FACS.

#### 646 ***Detection of cell membrane CALR levels***

647 The tumor cells were collected, washed three times with PBS, and then resuspended in cell  
648 staining buffer. The cells were incubated with a CALR antibody at 4°C for 30 minutes. Next,  
649 the cells were washed three times with PBS and incubated with a secondary antibody  
650 conjugated to Alexa Fluor 488 at 37°C for 30 minutes. Finally, the cells were washed,  
651 resuspended in PBS at 4°C, and analyzed using a flow cytometer.

#### 652 ***Mouse CD8<sup>+</sup> T-cell isolation and activation***

653 CD8<sup>+</sup> T cells or OT-I cells were isolated from the spleens of wild-type mice or OT-I TCR  
654 transgenic mice. CD8<sup>+</sup> T cells or OT-I cells were obtained using the EasySep Mouse CD8<sup>+</sup> T-  
655 Cell Isolation Kit according to the manufacturer's instructions. Next, the CD8<sup>+</sup> T cells were  
656 incubated with interleukin (IL)-2 (10 ng/mL), an anti-CD3 antibody (5 µg/mL), and an anti-  
657 CD28 antibody (2.5 µg/mL) for 72 hours. In a different setting, OT-I cells were cultured with  
658 OVA-loaded dead tumor cells and IL-2 for 72 hours.

#### 659 ***Induction of bone marrow-derived macrophages (BMDMs) and bone marrow-derived 660 dendritic cells (BMDCs)***

661 Bone marrow cells were isolated from the hind limbs of the mice. Red blood cells in the cell  
662 suspension were lysed with red blood cell lysis buffer. BMDMs were generated from bone  
663 marrow cells by culture with M-CSF (20 ng/mL), and the culture medium was changed every  
664 3 days. The macrophages were collected between days 7 and 9 for further experimentation.  
665 BMDCs were generated from bone marrow cells by culture with GM-CSF (20 ng/mL) and IL-  
666 4 (20 ng/mL). The dendritic cells (DCs) were labeled with CD11c and sorted using a flow  
667 cytometer between days 8 and 10.

#### 668 ***Induction of peripheral blood mononuclear cell-derived dendritic cells and macrophages***

669 Peripheral blood mononuclear cells were obtained from the blood of healthy donors using  
670 Ficoll-based density gradient separation. CD14<sup>+</sup> monocytes were then purified using magnetic  
671 bead-based separation and subsequently cultured in DMEM medium supplemented with 10%  
672 fetal bovine serum. DCs were generated from monocytes by culture with GM-CSF (50 ng/mL)  
673 and IL-4 (20 ng/mL), with medium replacement every 3 days. On day 6, LPS (100 ng/mL) was  
674 added for 24 hours to induce DC maturation. Macrophages were generated from monocytes by  
675 culture with M-CSF (50 ng/mL), with medium replacement every 3 days, and were mature at  
676 day 7.

#### 677 ***Antigen presentation and T-cell activation assay***

678 Macrophages were exposed to dead OVA-loaded tumor cells for 48 hours, followed by staining  
679 with antibodies specific to the OVA-peptide-MHC-I binding epitope and CD11b to detect the  
680 surface presentation of the OVA peptide SIINFEKL on H-2Kb (MHC-I). The fluorescence  
681 intensity of the H-2Kb-OVA complex on macrophages was detected using FACS. For the T-  
682 cell proliferation and activation assays, OT-I cells were labeled with CellTrace™ Violet (CTV).  
683 A total of 4×10<sup>4</sup> macrophages or DCs pretreated with dead OVA-loaded tumor cells were  
684 cocultured with 2×10<sup>5</sup> CTV-labeled OT-I cells. Following a 3-day incubation, the cells were  
685 harvested and assessed for CTV dilution and measurements of the cytokine expression levels  
686 in OT-I cells by FACS.

#### 687 ***Phagocytosis assay***

688 The macrophages or DCs were incubated with green fluorescent protein (GFP)-labeled dead  
689 tumor cells at a 1:5 ratio. The median fluorescence intensity (MFI) of GFP in macrophages was  
690 analyzed by FACS after gating for CD11b or CD11c.

691 The macrophages were incubated with dead tumor cells at a 1:5 ratio for 24 hours, followed  
692 by a 20-minute pulse with pHrodo™-SE-labeled 3 μm latex beads. The cells were then

693 thoroughly washed with cold PBS and incubated for 40 minutes to allow for chase. The MFI of  
694 pHrodo Red fluorescence in macrophages was analyzed by FACS after gating on CD11b.

695 In a similar experimental setup, macrophages were incubated with dead tumor cells at a 1:5  
696 ratio for 24 hours and pHrodo<sup>TM</sup>-SE beads for 20 minutes, followed by a 30-minute incubation  
697 with LysoTracker dye. After extensive washes, the macrophages were fixed with 4%  
698 paraformaldehyde, and images of immunofluorescence staining were captured using a confocal  
699 microscope. The fluorescence intensity of the LysoTracker dye was quantified using ImageJ  
700 software.

#### 701 ***ELISPOT assay***

702 Tumor-infiltrating lymphocytes were isolated from tumor tissues of Hepa1-6-OVA tumor-  
703 bearing mice. A total of  $2.5 \times 10^5$  cells were cultured with OVA peptide for 48 hours, followed  
704 by incubation with detection antibody according to the manufacturer's protocol. The images  
705 and spot counts were obtained using an AID ELISPOT READER.

#### 706 ***Stable cell line generation***

707 Plasmids encoding TESC-, CALR-, or TAP1-silencing constructs, TESC or OVA  
708 overexpression constructs or the *Tesc*-D128A point mutation plasmid, along with two  
709 lentivirus-packaging plasmids (psPAX2 and pMD2.G), were cotransfected into HEK293T cells  
710 using Lipofectamine 3000. After 24 hours, the supernatant containing the lentivirus was  
711 collected. Tumor cells were then infected with lentivirus using polybrene and selected with  
712 puromycin. The expression of TESC, CALR, TAP1, and TESC (D128A) was further verified  
713 by immunoblotting.

#### 714 ***Culture system for hepatoma cells***

715 Hepa1-6 or H22 hepatoma cells were treated with BAPTA (10  $\mu$ M) for 1 hour to chelate  
716 cytosolic Ca<sup>2+</sup>. Hepa1-6 or H22 hepatoma cells were treated with TUDCA (100  $\mu$ M) for 24  
717 hours to inhibit ER stress. Hepa1-6 or H22 hepatoma cells were treated with a continuous

718 concentration gradient of OICR-9429 (0  $\mu$ M, 70  $\mu$ M, 120  $\mu$ M, 140  $\mu$ M, or 240  $\mu$ M) for 24 hours  
719 to inhibit H3K4me3 or TESC expression in vitro. Hepa1-6 or H22 hepatoma cells were treated  
720 with a continuous concentration gradient of isoginkgetin (0  $\mu$ M, 5  $\mu$ M, 10  $\mu$ M, 20  $\mu$ M, or 40  
721  $\mu$ M) for 24 hours to inhibit H3K4me1 expression in vitro.

#### 722 ***Data-independent acquisition mass spectrometry (DIA-MS) analysis***

723 Whole-cell lysates were prepared from NC or sh*Tesc* H22 cells. The DIA-MS analysis was  
724 conducted by New Creation Biotechnology (Guangzhou, China). Differentially expressed  
725 proteins were identified and subjected to a Gene Ontology (GO) biological process analysis.  
726 The criteria for selecting differentially expressed proteins were as follows:  $P < 0.05$  and  $\log_2$   
727 fold change (FC)  $> 1.2$  or  $\log_2$  (FC)  $< 0.83$ . For the analysis of “eat me” and “don’t eat me”  
728 signals, proteins shown in Supplemental Figure 4L were extracted from the proteomics dataset,  
729 and the expression levels between two groups were visualized as a heatmap.

#### 730 ***RNA sequencing (RNA-seq) and single-cell RNA sequencing (scRNA-seq) analyses***

731 For RNA sequencing analysis, total RNA was extracted from tumor biopsy specimens collected  
732 from cohort 3 patients with HCC prior to oxaliplatin-based chemotherapy plus ICB treatment  
733 and from BMDMs. RNA sequencing was conducted by Novogene (Beijing, China).

734 For the characterization of gene expression profiles of BMDMs after engulfment of NC or  
735 *Tesc* Hepa1-6 cells, genes showing distinct expression patterns across different gene sets were  
736 identified based on FPKM values and visualized using a heatmap.

737 For the analysis of the CD8<sup>+</sup> T-cell infiltration score, RNA sequencing data from TCGA  
738 dataset were utilized to assess the infiltration score with the xCell algorithm (54). The samples  
739 were divided into low- and high-infiltration groups based on these scores, and the differential  
740 gene expression analysis was performed between the two groups.

741 For the identification of the primary cell populations expressing TESC within the tumor  
742 microenvironment, the single-cell RNA sequencing data (GSE166635) from HCC patients were  
743 analyzed on the TISCH2 database.

744 For the single-cell sequencing analysis, the GEO dataset GSE151530 was utilized to  
745 investigate the correlation between tumor TESC expression and cytotoxic T lymphocyte (CTL)  
746 infiltration. Samples were included if the patients were pathologically diagnosed with HCC,  
747 had not received prior treatment and the tissues contained more than 15 tumor cells. Data  
748 normalization was performed using the NormalizeData, FindVariableFeatures and ScaleData  
749 functions in the Seurat package. Clustering was performed using FindNeighbors and  
750 FindClusters with a resolution of 0.1, followed by the identification of marker genes with  
751 FindAllMarkers. Based on these marker genes, T cells were subdivided into six clusters, which  
752 were then used to compare the proportions of CTLs. The KEGG enrichment analysis of the  
753 differentially expressed genes (DEGs) from CTLs in the high-TESC group was performed using  
754 the clusterProfiler package. Analyses of the antigen presentation and phagocytosis scores of  
755 macrophages were performed based on the antigen presentation-associated gene set and  
756 phagocytosis-associated gene set (55). The gene set is provided in Supplemental Table 2.

757 The differential gene expression analysis was performed using the DESeq2 R package. Genes  
758 with  $P < 0.01$  and a  $\log_2(\text{FC}) > 1.5$  were considered DEGs.

759 For the investigation of the correlation between TESC and the therapeutic efficacy of  
760 immunotherapy in HCC patients, Mendeleev Data (skrx2fz79n) were used to stratify responders  
761 and non-responders, and the transcriptional levels of TESC in malignant cells were  
762 subsequently analyzed.

### 763 ***Reagents and Primers***

764 The antibodies used for FACS, immunohistochemistry, immunofluorescence, immunoblotting,  
765 ChIP assay, and animal studies are detailed in the Supplemental Table 3 and 4. The recombinant

766 proteins, peptides, chemicals, and commercial assays utilized in this study are listed in  
767 Supplemental Table 5.

### 768 *Additional resources*

769 The Figure 1, H–J, M, and O, Supplemental Figure 2, A and C, Supplemental Figure 3, A, B,  
770 and D, and Supplemental Figure 6, E, J, and P were created with BioRender.com. Data graphics  
771 were generated using Prism 8.0 and R version 4.1.2.

### 772 *Statistics*

773 The statistical tests are detailed in the figure legends. The data are presented as the means  $\pm$   
774 standard errors of the means (SEMs). Pearson's correlation coefficients were calculated to  
775 assess correlations between parameters. The Kaplan-Meier method was used to estimate  
776 cumulative survival time, and the log-rank test was conducted to compare the groups. ROC  
777 curve analysis was conducted to predict the response to ICB. Statistical analyses were  
778 conducted using GraphPad Prism software, with  $P < 0.05$  considered statistically significant.

### 779 *Study approval*

780 All the mice were housed under specific pathogen-free conditions in the Animal Facility of Sun  
781 Yat-sen University Cancer Center. All animal experiments were performed in accordance with  
782 the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and  
783 were approved by the Sun Yat-sen University Cancer Center Ethics Committee (ethical  
784 approval number: L025503202202009). Written informed consent was obtained from each  
785 patient, and the study protocol was approved by the Ethical Review Committee of Sun Yat-sen  
786 University Cancer Center (ethical approval number: G2025-107).

### 787 *Data availability*

788 Supporting data values are available online in the Supporting Data Values file. The raw  
789 sequencing data reported in this paper have been submitted to the Genome Sequence Archive  
790 under the accession number HRA016607 and CRA037701. The raw data of DIA-MS have been

791 submitted to the Open Archive for Miscellaneous Data under the accession number

792 OMIX014731.

793

794 **Author contributions**

795 All authors have full access to all data used in the study and take responsibility for the integrity  
796 of the data and the accuracy of the data analysis. ZGZ, DPC, and MZ conceptualized the study  
797 and supervised the project, determining order of corresponding authors. JLW, JCW, and YXP  
798 developed the methodology and performed the validation. MRH and ZKZ conducted the formal  
799 analysis. HZ, TQW, YHZ, ZLH, YZF, and WP carried out the investigation. LX, YJZ, MSC,  
800 and DDH provided the resources. ZYY and JBC curated the data. ZGZ, DPC, JLW, and JCW  
801 wrote the manuscript. MRH and JLW prepared the visualizations. ZGZ, DPC, and JCW  
802 acquired the funding. The order of the co-first authors was determined based on their relative  
803 efforts and contributions to the study.

804

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806

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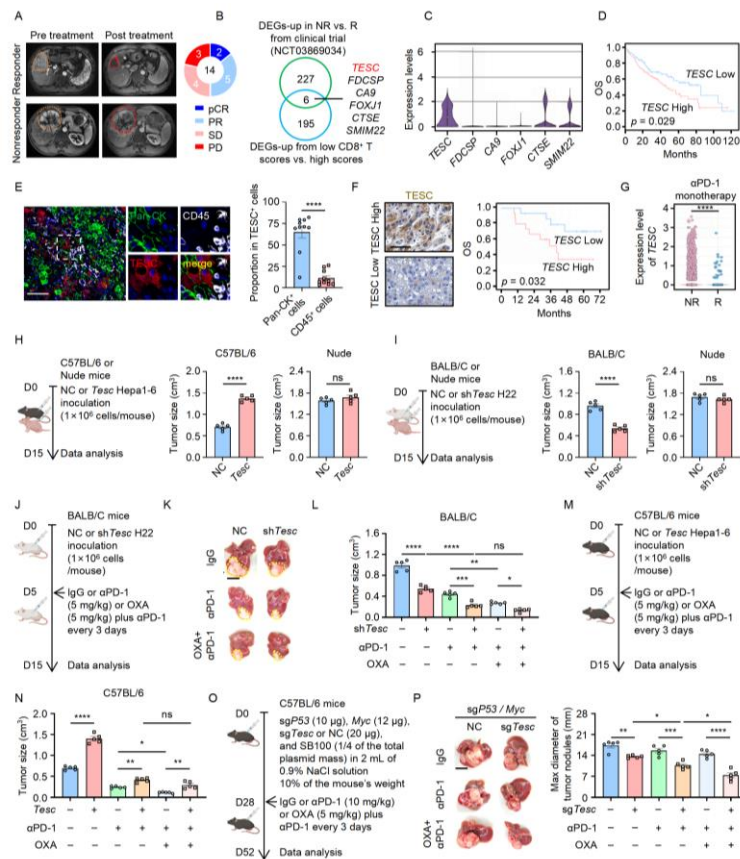
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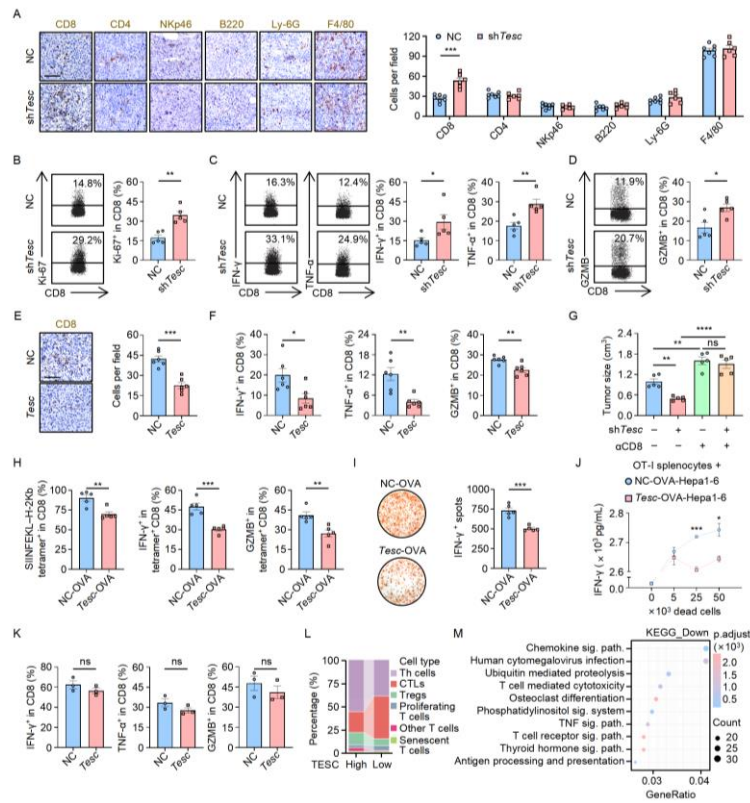
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923 **Figure legends**

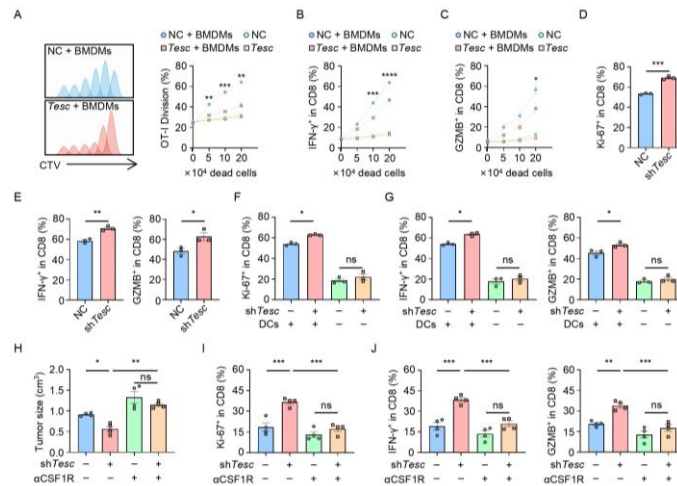


924 **Figure 1.** Tumor-intrinsic TESC contributes to resistance to antitumor immunity and  
 925 immunotherapy. (A) Representative magnetic resonance imaging enhancement scans of HCC  
 926 patients before and after oxaliplatin-based chemotherapy plus ICB (cohort 1, NCT03869034).  
 927 (B) Upregulated genes in non-responders from cohort 1 and both low CD8<sup>+</sup> T-cell infiltration  
 928 scores and upregulated genes in HCC tissue from cohort 2 (TCGA) were determined. (C)  
 929 Candidate gene expression in tumor cells from HCC patients (GEO: GSE166635). (D)  
 930 Correlations between TESC expression and OS in TCGA HCC patients grouped by median  
 931 TESC expression. Log-rank test. (E) Representative images of immunofluorescence for pan-  
 932 CK, CD45, and TESC in HCC tissues ( $n = 20$ ). Scale bar, 100  $\mu\text{m}$ . 2-tailed Student's t test. (F)  
 933 Correlation of TESC level with OS of 30 HCC patients treated with oxaliplatin-based  
 934 chemotherapy plus ICB (cohort 3). Patients were grouped by the median TESC expression.  
 935 Scale bar, 50  $\mu\text{m}$ . Log-rank test. (G) Transcript levels of TESC in HCC tumor cells from ICB  
 936 responders and non-responders [Mendeley Data (skrx2fz79n)]. 2-tailed Student's t test. (H and  
 937 I) TESC-overexpressing (*Tesc*) Hepa1-6 and TESC-silenced (*shTesc*) H22 cells were inoculated  
 938 into the livers of C57BL/6 or BALB/C and Nude mice, respectively ( $n = 5$ ). 2-tailed Student's  
 939 t test. (J–N) Sh*Tesc* H22 (J–L) or *Tesc* Hepa1-6 hepatoma-bearing mice (M and N) were treated  
 940 as described above ( $n = 5$ ). Scale bar, 1 cm. 1-way ANOVA analysis. (O and P) C57BL/6 mice  
 941 with spontaneous hepatoma were treated as described above ( $n = 5$ ). Scale bar, 1 cm. 1-way  
 942 ANOVA analysis. ns, not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ .  
 943 DEG, differentially expressed gene; HCC, hepatocellular carcinoma; ICB, immune checkpoint  
 944 blockade; NR, non-responder; OS, overall survival; OXA, oxaliplatin; pCR, pathological  
 945 complete response; PD, progressive disease; PR, partial response; R, responder; SD, stable  
 946 disease.

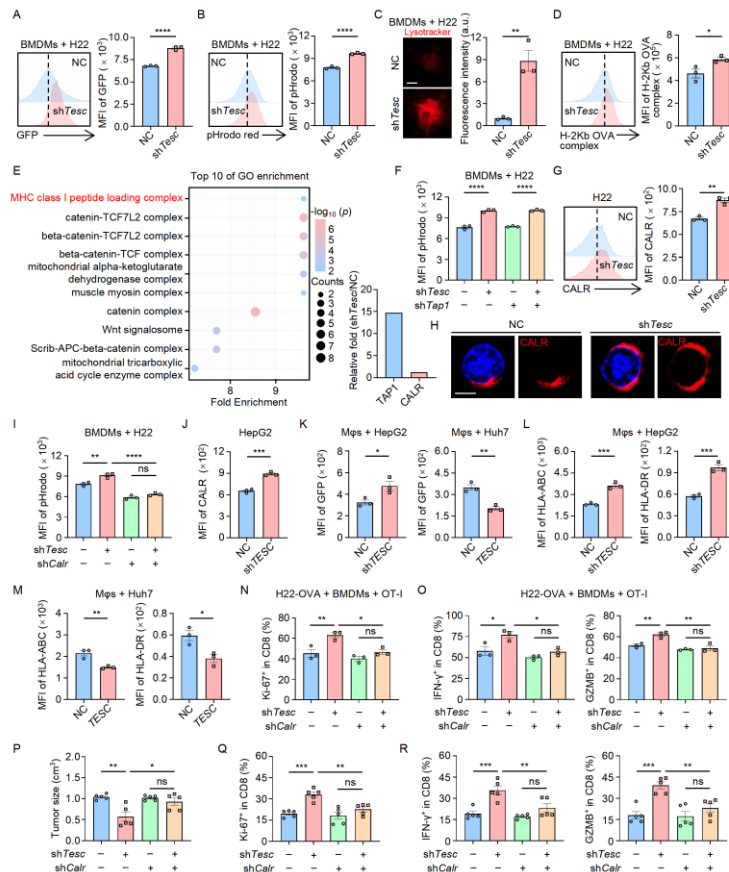
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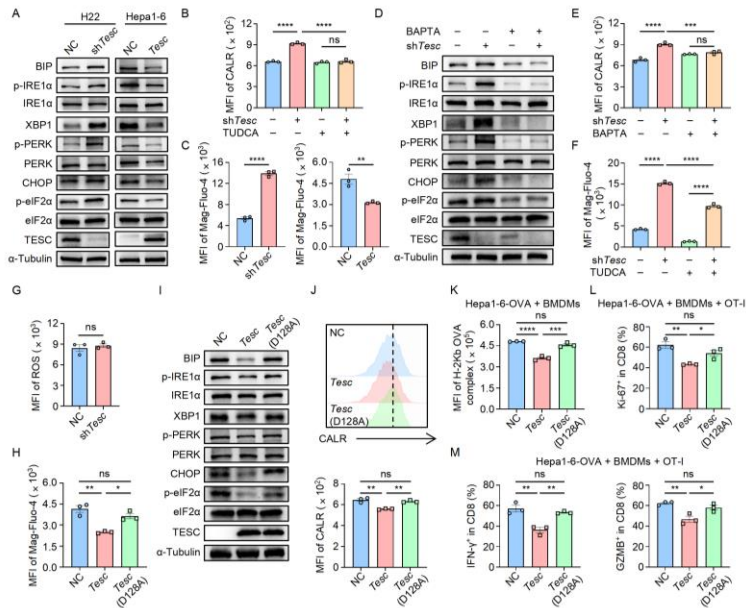
949 **Figure 2.** Tumor TESC impairs CD8<sup>+</sup> T-cell-mediated antitumor immunity. (A–D) Sh*Tesc* H22  
950 cells were inoculated into mouse livers ( $n = 5-7$ ). The infiltration of CTLs (CD8<sup>+</sup>), T helper  
951 cells (CD4<sup>+</sup>), NK cells (NKp46<sup>+</sup>), B cells (B220<sup>+</sup>), neutrophils (Ly-6G<sup>+</sup>), and macrophages  
952 (F4/80<sup>+</sup>) (A) and CTL functions (B–D) were analyzed. Scale bar, 50  $\mu$ m. 2-tailed Student's t  
953 test. (E and F) *Tesc* Hepa1-6 cells were inoculated into mouse livers ( $n = 6$ ). The infiltration of  
954 CTLs (CD8<sup>+</sup>) (E) and CTL functions (F) were analyzed. Scale bar, 50  $\mu$ m. 2-tailed Student's t  
955 test. (G) Effects of CD8<sup>+</sup> T-cell depletion on sh*Tesc* in mouse hepatoma tissues ( $n = 5$ ). 1-way  
956 ANOVA analysis. (H) OVA-loaded *Tesc* Hepa1-6 cells were inoculated into the liver of  
957 C57BL/6 mice ( $n = 5$ ). Surface expression of SIINFEKL–H-2Kb tetramer in CTLs and the  
958 functions of tetramer<sup>+</sup> CTLs were analyzed. 2-tailed Student's t test. (I) IFN- $\gamma$  ELISPOT in  
959 tumor-infiltrating lymphocytes from mice bearing OVA-loaded NC or *Tesc* Hepa1-6 hepatoma  
960 ( $n = 5$ ). 2-tailed Student's t test. (J) OT-I splenocytes were cultured with dead OVA-loaded *Tesc*  
961 Hepa1-6 cells for 3 days. IFN- $\gamma$  production in the supernatant was measured by ELISA ( $n = 3$ ).  
962 2-tailed Student's t test. (K) *Tesc* Hepa1-6 cells were cultured with T cells in the presence of  
963 anti-CD3 and anti-CD28 antibodies. CTL functions were analyzed ( $n = 3$ ). 2-tailed Student's t  
964 test. (L) Comparison of the T-cell constitution between two groups (GSE151530). Patients were  
965 grouped by the median tumor TESC expression in tumor cells. (M) The top 10 downregulated  
966 KEGG pathways enriched in T cells from tumor samples with high TESC expression. ns, not  
967 significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ . CTL, cytotoxic T  
968 lymphocyte; GZMB, granzyme B; OVA, Ovalbumin.  
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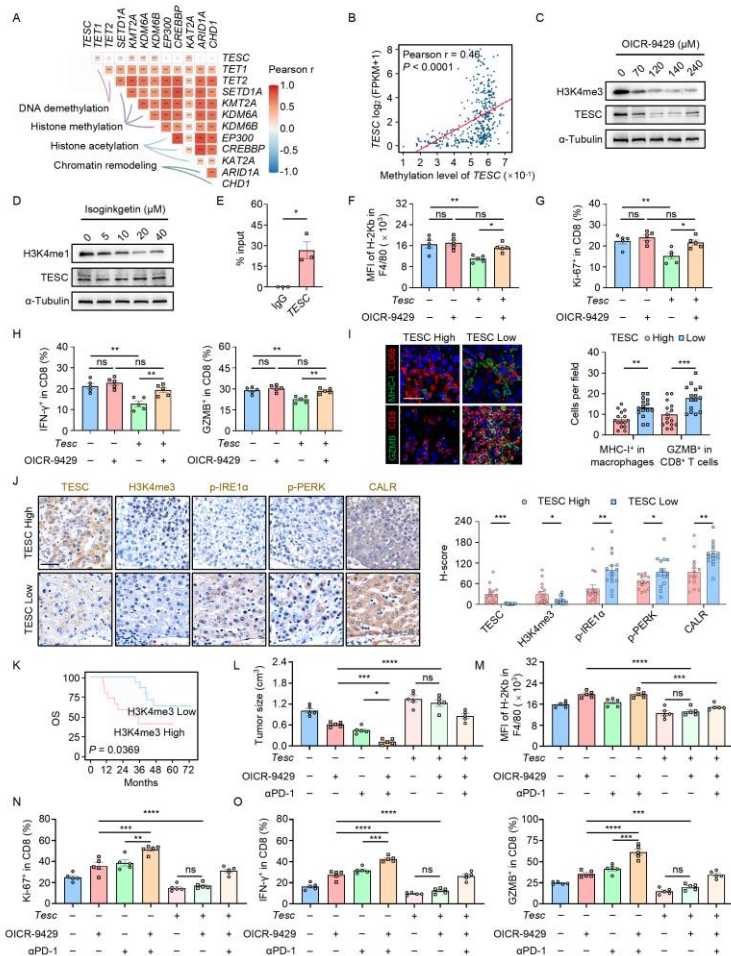
971  
 972 **Figure 3.** TESC attenuates tumor immunogenicity by disrupting antigen presentation by APCs.  
 973 (A–C) CTV-labeled OT-I cells were cultured with different numbers of dead OVA-loaded *Tesc*  
 974 Hepa1-6 cells in the presence of BMDMs for 3 days. CTV dilution (A) and OT-I cell functions  
 975 were analyzed (B and C) ( $n = 3$ ). 2-tailed Student's t test. (D–G) OT-I cells were cultured with  
 976 dead OVA-loaded sh*Tesc* H22 cells for 3 days, with macrophages (D and E) or with/without  
 977 DCs (F and G). OT-I cell functions were analyzed ( $n = 3$ ). 2-tailed Student's t test (D and E)  
 978 and 1-way ANOVA analysis (F and G). (H–J) Effects of macrophage depletion on the tumor  
 979 size (H) and CTL function (I and J) in sh*Tesc* H22 hepatoma-bearing mice ( $n = 4$ ). 1-way  
 980 ANOVA analysis. ns, not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ .  
 981 APC, antigen-presenting cell; BMDM, marrow-derived macrophage; DC, dendritic cell.  
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**Figure 4.** TESC restrains CALR membrane translocation to inhibit macrophage function. (A) FACS analysis of GFP in BMDMs cultured with dead GFP-labeled sh*Tesc* H22 cells ( $n = 3$ ). 2-tailed Student's t test. (B and C) BMDMs were cultured with dead sh*Tesc* H22 cells for 20 hours, followed by pHrodo Red FACS analysis and LysoTracker confocal imaging ( $n = 3$ ). Scale bar, 10  $\mu$ m. 2-tailed Student's t test. (D) FACS analysis of H-2Kb–OVA complexes in BMDMs cultured with dead OVA-loaded sh*Tesc* H22 cells for 48 hours ( $n = 3$ ). 2-tailed Student's t test. (E) Top 10 GO biological processes enriched in sh*Tesc* H22 cells by DIA-MS proteomics, with the top pathway involving CALR and TAP1. (F) FACS analysis of pHrodo Red in BMDMs cultured with dead TAP1-silenced (sh*Tap1*)-transfected sh*Tesc* H22 cells ( $n = 3$ ). 1-way ANOVA analysis. (G and H) FACS and confocal analysis of CALR expression in sh*Tesc* H22 cells ( $n = 3$ ). Scale bar, 10  $\mu$ m. 2-tailed Student's t test. (I) FACS analysis of pHrodo Red in BMDMs cultured with dead CALR-silenced (sh*Calr*)-transfected sh*Tesc* H22 cells ( $n = 3$ ). 1-way ANOVA analysis. (J) FACS analysis of CALR expression in sh*TESC* HepG2 cells ( $n = 3$ ). 2-tailed Student's t test. (K–M) FACS analysis of GFP, HLA-ABC, and HLA-DR in macrophages cultured with dead GFP-labeled sh*TESC* HepG2 or *TESC* Huh7 cells. ( $n = 3$ ). 2-tailed Student's t test. (N and O) Functional analysis of OT-I cells cultured with dead OVA-loaded sh*Calr*-transfected sh*Tesc* H22 cells and macrophages for 3 days ( $n = 3$ ). 1-way ANOVA analysis. (P–R) Tumor size and CTL function were analyzed after inoculation of sh*Calr*-transfected sh*Tesc* H22 cells ( $n = 5$ ). 1-way ANOVA analysis. ns, not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ . FACS, flow cytometry.



**Figure 5.** TESC regulates CALR surface exposure by modulating calcium homeostasis and attenuating ER stress. (A) Immunoblot analysis of ER stress signaling activation in sh*Tesc* H22 or *Tesc* Hepa1-6 cells. (B) FACS analysis of CALR expression in sh*Tesc* H22 cells treated with TUDCA for 24 hours ( $n = 3$ ). 1-way ANOVA analysis. (C) FACS analysis of ER calcium level in sh*Tesc* H22 or *Tesc* Hepa1-6 cells stained with Mag-Fluo-4-AM ( $n = 3$ ). 2-tailed Student's *t* test. (D) Immunoblot analysis of ER stress signaling activation in sh*Tesc* H22 cells left untreated or incubated with BAPTA for 1 hour. (E) FACS analysis of CALR expression in sh*Tesc* H22 cells treated with BAPTA for 1 hour ( $n = 3$ ). 1-way ANOVA analysis. (F) FACS analysis of ER calcium levels in sh*Tesc* H22 cells left untreated or incubated with TUDCA for 24 hours ( $n = 3$ ). 1-way ANOVA analysis. (G) FACS analysis of ROS level in sh*Tesc* H22 cells ( $n = 3$ ). 2-tailed Student's *t* test. (H) FACS analysis of ER calcium level in *Tesc*- or D128A point mutant TESC-overexpressing [*Tesc* (D128A)] Hepa1-6 cells ( $n = 3$ ). 1-way ANOVA analysis. (I) Immunoblot analysis of ER stress signaling activation in *Tesc*- or *Tesc* (D128A) Hepa1-6 cells. (J) FACS analysis of CALR expression in *Tesc* or *Tesc* (D128A) Hepa1-6 cells ( $n = 3$ ). 1-way ANOVA analysis. (K) FACS analysis of H-2Kb–OVA complexes in BMDMs cultured with dead OVA-loaded *Tesc* or *Tesc* (D128A) Hepa1-6 cells for 48 hours ( $n = 3$ ). 1-way ANOVA analysis. (L and M) Functional analysis of OT-I cells cultured with dead OVA-loaded *Tesc* or *Tesc* (D128A) Hepa1-6 cells and macrophages for 3 days ( $n = 3$ ). 1-way ANOVA analysis. ns, not significant,  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ , and  $****P < 0.0001$ . ER, endoplasmic reticulum; ROS, reactive oxygen species.



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**Figure 6.** Targeting H3K4me3-induced TESC overcomes immune suppression and potentiates the efficacy of PD-1 combination therapy in HCC. (A) Heatmap of correlations between TESC expression and epigenetic regulator-related genes (TCGA-HCC). (B) Scatter plot from MethMarkerDB database showing the relationship between TESC methylation and expression. (C and D) Immunoblot analysis of TESC and H3K4me3 or H3K4me1 expression in H22 cells treated with OICR-9429 or isoginkgetin for 24 hours. (E) ChIP-qPCR showing the binding of H3K4me3 to TESC promoter in H22 cells ( $n = 3$ ). 2-tailed Student's t test. (F–H) *Tesc* Hepa1-6 hepatoma-bearing mice were untreated or injected with OICR-9429 ( $n = 5$ ). H-2Kb levels in macrophages (F) and CTL functions (G and H) were analyzed. 1-way ANOVA analysis. (I and J) Cohort 3 HCC patients were grouped by median TESC expression. Tumor TESC, H3K4me3, p-IRE1 $\alpha$ , p-PERK, and CALR were analyzed by immunohistochemistry. MHC-I $^+$  macrophages and GZMB $^+$  CTLs were analyzed by immunofluorescence. Scale bar, 50  $\mu$ m. 2-tailed Student's t test. (K) Correlation of H3K4me3 levels with OS of cohort 3 HCC patients grouped by median H3K4me3 expression. (L–O) *Tesc* H22 hepatoma-bearing mice were untreated or injected with OICR-9429, the  $\alpha$ PD-1 antibody or their combination ( $n = 5$ ). The tumor size (L), H-2Kb level in macrophages (M) and CTL function (N and O) were analyzed. 1-way ANOVA analysis. ns, not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ .