

1 **A novel anti-PD-L1/IL-15 immunocytokine overcomes resistance to**
2 **PD-L1 blockade and elicits potent antitumor immunity**

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14 **Abstract**

15 Despite the demonstrated immense potential of immune checkpoint inhibitors in
16 various types of cancers, only a minority of patients respond to these therapies.
17 Immunocytokines designed to deliver an immune-activating cytokine directly to the
18 immunosuppressive tumor microenvironment (TME) and block the immune
19 checkpoint simultaneously may provide a strategic advantage over the combination of
20 two single agents. To increase response rate to checkpoint blockade, in this study we
21 developed a novel immunocytokine (LH01) composed of the antibody against
22 programmed death-ligand 1 (PD-L1) fused to IL-15 receptor alpha-sushi

23 domain/IL-15 complex. We demonstrate that LH01 efficiently binds mouse or human
24 PD-L1 and maintains IL-15 stimulatory activity. In syngeneic mouse models, LH01
25 showed improved antitumor efficacy and safety versus anti-PD-L1 plus LH02
26 (Fc-Sushi-IL15) combination and overcame resistance to anti-PD-L1 treatment.
27 Mechanistically, the dual anti-immunosuppressive function of LH01 led to activation
28 of both the innate and adaptive immune response and decreased levels of transforming
29 growth factor- β 1 (TGF- β 1) within the TME. Furthermore, combination therapy with
30 LH01 and bevacizumab exerts synergistic antitumor effects in HT29 colorectal
31 xenograft model. Collectively, our results provide supporting evidence that fusion of
32 anti-PD-L1 and IL-15 might be a potent strategy to treat patients with cold tumors or
33 resistance to checkpoint blockade.

34 **Keywords:** Immunocytokines; PD-L1 blockade; Resistance; Combination therapy

35 **1. Introduction**

36 Therapeutic antibodies that block programmed death-1 (PD-1)/programmed
37 death-ligand 1 (PD-L1) pathway demonstrate cure-like benefits in patients with
38 various types of cancers, but a large proportion of patients experienced a low response
39 rate or rapidly developed resistance to these therapies with relapsed disease ^[1]. One of
40 the main reasons may be the existence of an immunosuppressive tumor
41 microenvironment (TME), which is caused by altering the immune checkpoint
42 molecule expression, immunosuppressive cytokine secretion, oxygen nutrition status,
43 etc ^[2]. Cytokines play an indispensable role in regulating immune response, including
44 innate and adaptive immunity, and are the cornerstone of cancer immunotherapy. A

45 variety of immune activating cytokines such as IL-15 have potent anti-tumor efficacy
46 and can markedly prolong the survival periods of patients, which can be combined
47 practically with immune checkpoint inhibitors (ICIs) to address the issue of resistance
48 and increase response rate ^[3, 4].

49 Recombinant human IL-15 was at the top of the National Cancer Institute's list of
50 potential biopharmaceuticals for tumor immunotherapy in 2008 ^[5]. IL-15 has a unique
51 mechanism of action in which it binds to IL-15R α expressed by antigen-presenting
52 cells, then the IL-15/IL-15R α complex is trans-presented to neighboring NK or CD8⁺
53 T cells expressing only the IL-15R β/γ receptor ^[6]. In addition to inhibiting
54 IL-2-induced activation-induced cell death, a process that leads to the elimination of
55 stimulated T cells and induction of T-cell tolerance, IL-15 can support long lasting
56 CD8⁺ T cell memory and effector responses against diseased cells ^[7, 8]. Recombinant
57 IL-15 has demonstrated clinical activity in the treatment of certain cancers, including
58 advanced renal cell carcinoma and metastatic melanoma, and significant increases in
59 the number of memory CD8⁺ T and NK cells were observed in patients' peripheral
60 blood ^[9, 10]. There is evidence that increased PD-L1 expression in tumors and
61 decreased IL-15 levels in the TME are correlating with poor clinical outcomes ^[11, 12].

62 A clinical trial showed that an IL-15 superagonist, ALT-803, can re-induce
63 immunotherapy response in PD-1-relapsed and refractory non-small cell lung cancer
64 (NSCLC) ^[13]. Unfortunately, the short half-life and the systemic toxicities of
65 high-dose administration, which can cause fever, fall of blood pressure, flu-like
66 symptoms due to lack of target activity, restrict the further clinical applications of

67 IL-15^[14].

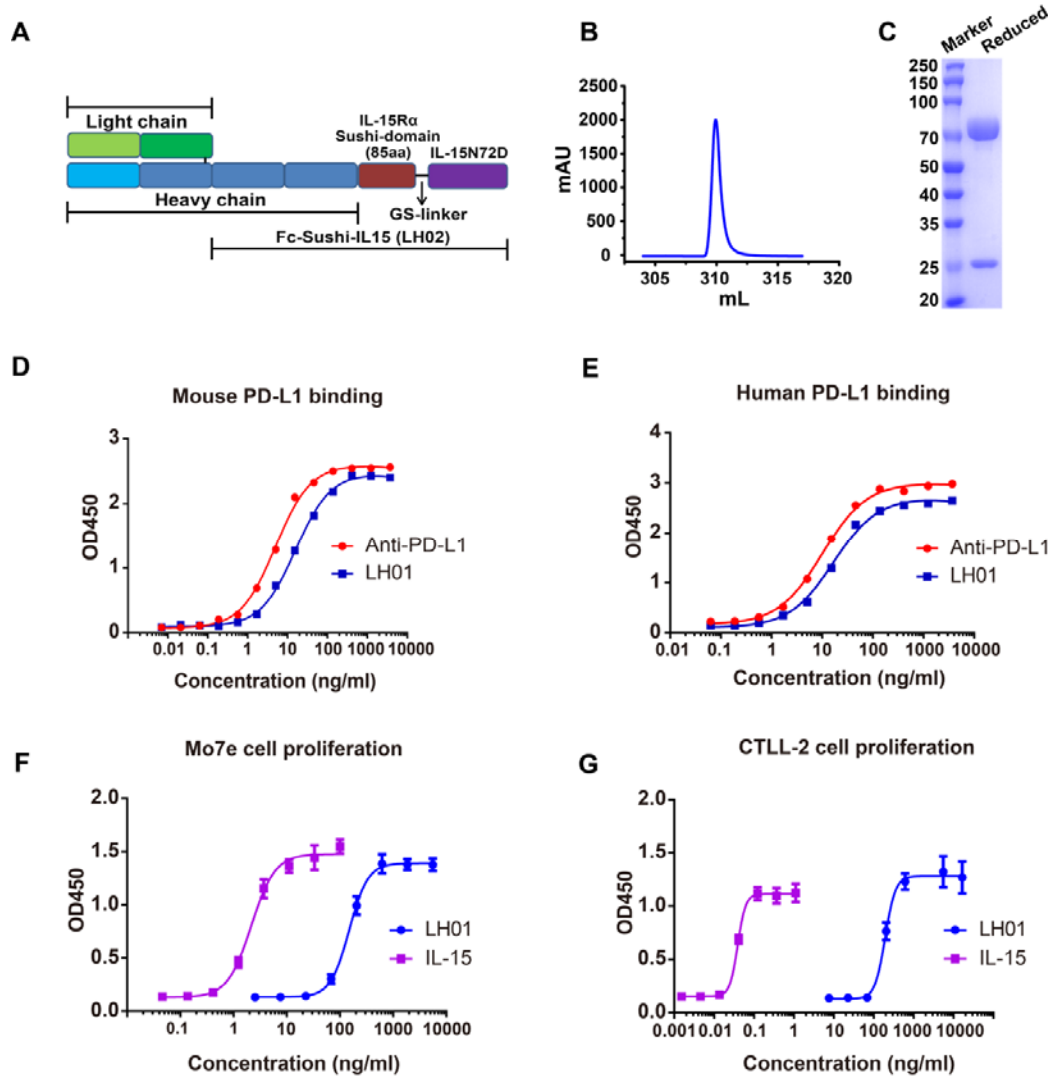
68 Prolonging half-life and increasing targeting ability at the tumor site of this
69 pro-inflammatory cytokine are feasible solutions to the above problems. It has been
70 reported that complexation with the IL-15R α -sushi domain can improve IL-15
71 half-life and bioavailability *in vivo* and is effective in mimicking IL-15
72 trans-presentation^[15, 16]. Additionally, the IL-15R α -sushi domain is a selective and
73 potent agonist of IL-15 action through IL-2/15R $\beta\gamma$ ^[17]. Immunocytokines are also
74 known as antibody-cytokine fusion proteins, which can utilize the targeting ability of
75 antibody to enrich cytokines at the tumor site. On the one hand, it can enhance tumor
76 targeting capability and reduce the side-effects of cytokines caused by systemic
77 administration. On the other hand, this allows antibodies and cytokines to generate
78 synergistic antitumor effects^[18, 19]. Hence, it is a practical strategy to generate an
79 immunocytokine composed of anti-PD-L1 and the IL-15R α -sushi domain/IL-15
80 complex to enhance antitumor activity.

81 In this study, we characterized the biochemical activity of LH01, a bifunctional fusion
82 protein designed to overcome resistance to PD-1/PD-L1 blockade via improving the
83 target activity of IL-15 and blocking the PD-L1 pathway concurrently. The
84 anti-PD-L1 moiety of LH01 is based on atezolizumab, which has been approved to
85 treat different types of cancers^[20-22]. We compared the antitumor efficacy of LH01
86 versus anti-PD-L1+LH02 in murine carcinoma models and preliminarily investigated
87 the mechanism that LH01 overcame resistance to anti-PD-L1 treatment. For the first
88 time, we evaluated the synergistic antitumor effect of combinative administration of

89 LH01 and bevacizumab.

90 Results

91 Biochemical characterization of the bifunctional fusion protein: LH01



92

93 Figure 1. Design, preparation and biochemical characterization of LH01

94 (A) Schematic representation of fusion proteins: IL-15R α sushi-domain/IL-15 was

95 directly fused to the C terminus of the Atezolizumab (LH01) or Atezolizumab's Fc

96 portion (LH02). (B) LH01 was purified by protein A affinity chromatography. (C)

97 Purified LH01 was characterized by reduced SDS-PAGE. (D and E) Binding of LH01

98 to plate-bound human or mouse PD-L1. Data was analyzed using the one site-total to

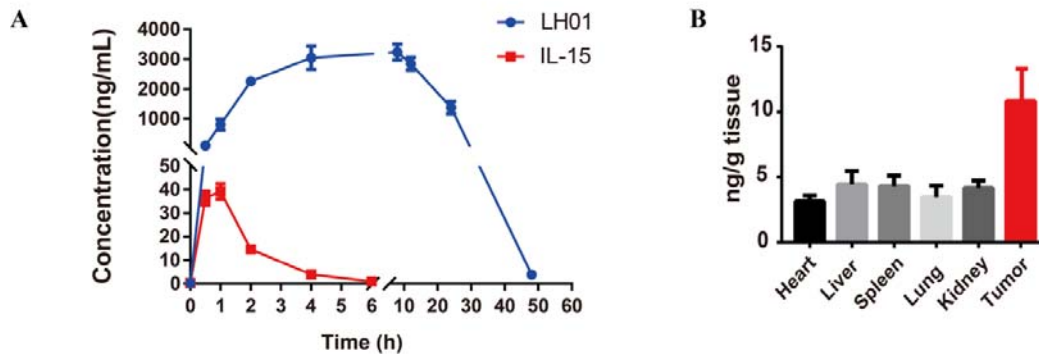
99 calculate the EC₅₀ values. (F and G) The biological activity was compared to IL-15
100 monomer at different concentrations by determining the proliferative potential in
101 human Mo7e cells and murine CTLL-2 cells. Data was analyzed using the four
102 parameter fit logistic equation to calculate the EC₅₀ values, and graphs were shown as
103 mean ± SD.

104 The fusion of IL15R α -sushi domain (Ile 31 to Val 115) and human IL15 mutant
105 (IL-15N72D) to the C-terminus of the anti-PD-L1 monoclonal antibody was expected
106 to improve the target activity and reduce untoward effects of IL-15 (Figure 1A; Figure
107 S1). A new molecular called Fc-Sushi-IL15 (LH02) was also designed as a
108 non-targeting control (Figure 1A). The mature LH01 protein, whose light chain and
109 heavy chain migrate as approximately 25 kDa and 70 kDa proteins under reducing
110 conditions on SDS-PAGE respectively (Figure 1B), was purified by one-step protein
111 A affinity chromatography (Figure 1C). The purification process of secreted
112 anti-PD-L1 and LH02 protein are the same as that of LH01. Light and heavy chain of
113 anti-PD-L1 migrate as an approximately 25 kDa and 50 kDa protein, and LH02
114 migrates as an approximately 50 kDa protein under reducing conditions on
115 SDS-PAGE (Figure S2).

116 In ELISAs, LH01 bound human or mouse PD-L1 with a profile similar to that of the
117 anti-PD-L1 antibody (EC₅₀ = 16.8 and 10.2 ng/mL (or 84.1 and 70.5 pM), 15.9 and
118 5.0 ng/mL (79.5 and 34.7 pM), respectively), indicating that the binding of the
119 anti-PD-L1 moiety was not affected (Figure 1D and 1E). As shown in Figure 1F and
120 1G, LH01 exhibited weaker proliferative capacity than IL-15 in human Mo7e cells

121 (EC₅₀ = 149.5 and 2.2 ng/mL (or 0.74 and 0.17 nM)), whereas markedly reduced
122 proliferative activities of LH01 were observed compared to IL-15 in mouse CTLL-2
123 cells (EC₅₀ = 194.5 and 0.039 ng/mL (or 970.4 and 3.03 pM)), which may be
124 explained by the finding that the IL-15R α -Sushi domain was able to bind IL-15 with
125 high affinity and inhibited proliferation driven through the high affinity IL-15R α / β / γ
126 signaling complex of the CTLL-2 cells. In a word, LH01 retained strong proliferative
127 capacity in both human Mo7e and mouse CTLL-2 cells.

128 **Prolonged half-life and improved tumor-targeting distribution of LH01**



129
130 **Figure 2. Prolonged half-life and improved tumor-targeting distribution of LH01**
131 (A) Male Balb/c mice aged 9 weeks were intraperitoneally injected with 24.0 μ g of
132 LH01 or 3.6 μ g of IL-15 (equimolar of IL-15 molecules). The pharmacokinetics
133 curves of LH01 and IL-15 monomer were plotted (n = 5). (B) MC38 tumor-bearing
134 mice (n = 4) were i.p. injected with LH01 at a dose of 1 mg/kg. Tissues were collected
135 at 24 h after injection. The concentrations of LH01 were measured by ELISA. Both
136 graphs show mean \pm SEM

137 **Table 1. Pharmacokinetic parameters of IL-15 and LH01**

Parameters	IL-15	LH01
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Half-life (T _{1/2}), h	1.02	12.52
T _{max} , h	1	8
C _{max} , ng/mL	39.26	3231.91
AUC (0→∞), ng×h/mL	79.58	81819.12
MRT, h	1.62	20.27

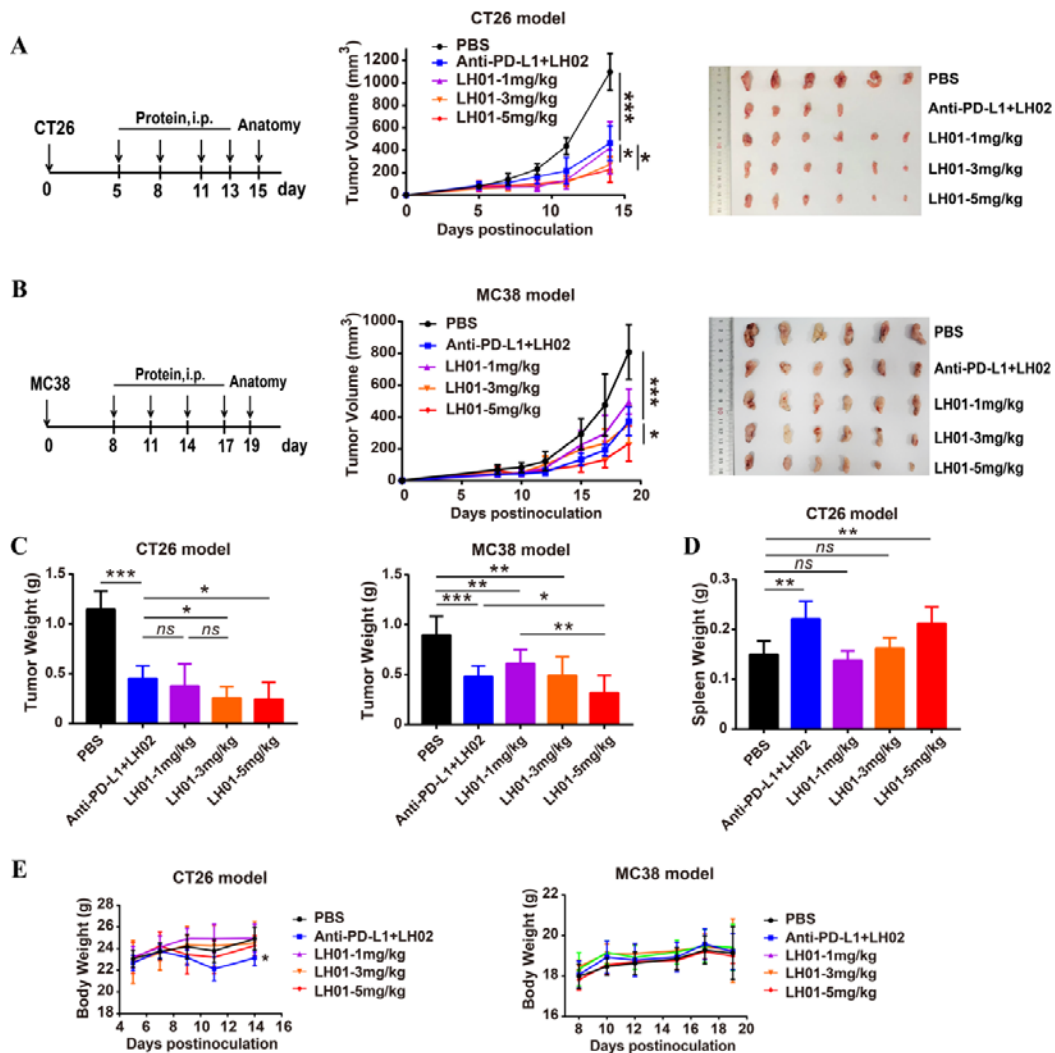
138 Calculated with PK Solver 2.0 for a noncompartmental model.

139 AUC, area under the curve; MRT, mean resident time

140 In order to provide medication guidance for the following animal experiments, we
141 explored the pharmacokinetic properties of LH01. Plasma concentrations of LH01
142 and IL-15 climbed to peaks and then decreased over time, but that of LH01 decreased
143 markedly slower than IL-15 (Figure 2A). LH01 peaked around 8 h at a concentration
144 of 3231 ng/mL, whereas IL-15 peaked about 1h at a concentration of 39 ng/mL (Table.
145 1). The half-lives were calculated to be about 12.52 h for LH01 and 1.02 h for IL-15
146 monomer, indicating that the fusion of IL15 and the sushi domain of the IL15
147 receptor-alpha to the C-terminus of the anti-PD-L1 monoclonal antibody have
148 markedly prolonged the half-life of IL-15 by more than 12 folds (Table. 1). To further
149 trace LH01 distribution, we collected various tissues 24 h after mice received
150 treatment of LH01. The *in vivo* biodistribution of LH01 displayed a certain specificity,
151 and the concentration of LH01 in tumor tissues was 2 folds higher than that in normal
152 tissues (Figure 2B).

153 **LH01 improves antitumor efficacy and safety versus anti-PD-L1+LH02**

154 **combination**



155

156 **Figure 3. LH01 reduces CT26 and MC38 tumor burden and ameliorates safety**

157 **versus LH02+anti-PD-L1**

158 (A and B) CT26 tumor cells (1×10^6 , subcutaneously) and MC38 tumor cells (5×10^5 ,

159 subcutaneously) were implanted into the right flank of female Balb/c and C57BL/6

160 mice, respectively. Mice were randomized into 5 groups based on tumor size and

161 treatment initiated when tumors reached 50-100mm³. Mice were treated with PBS,

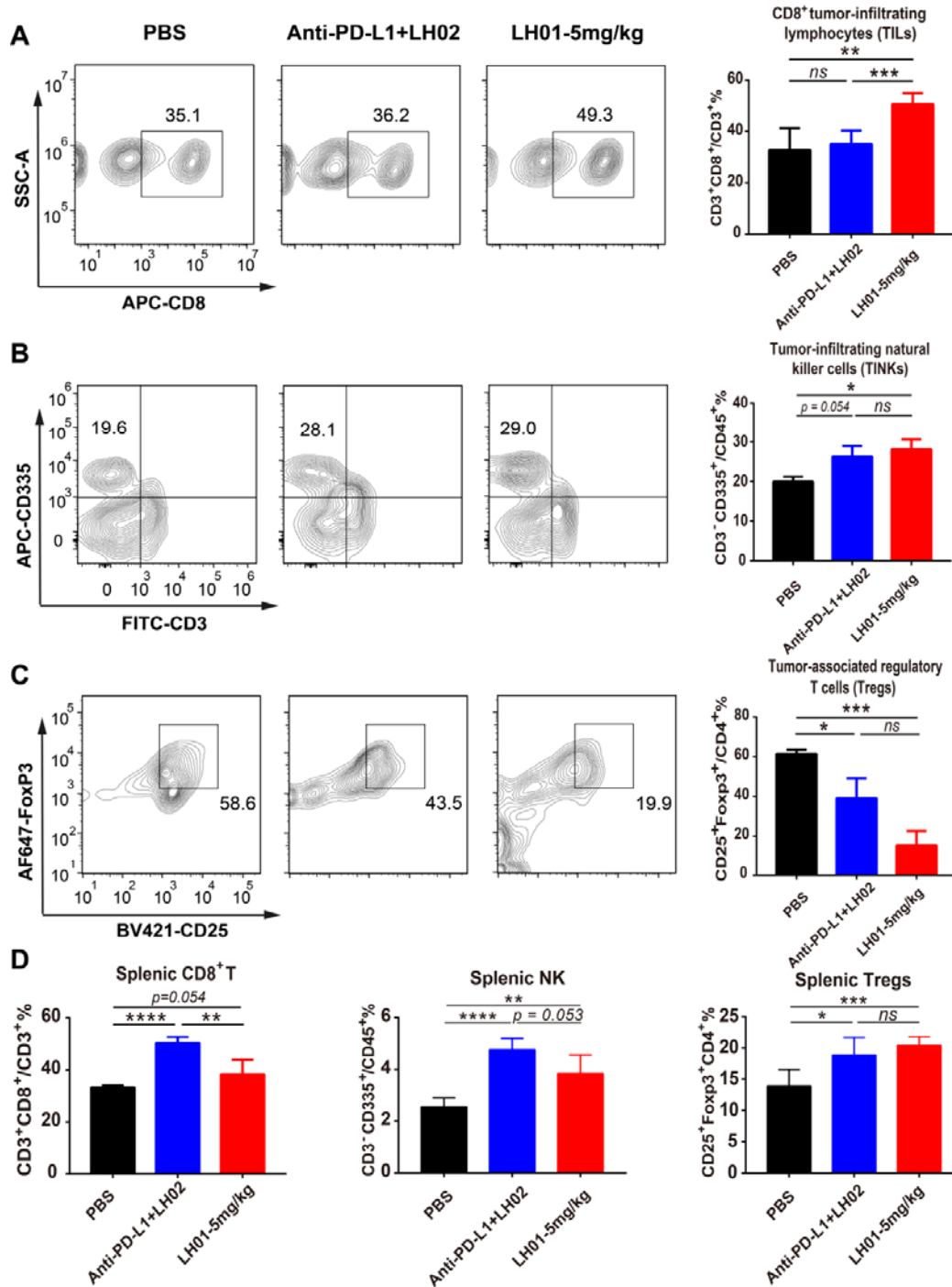
162 LH02+anti-PD-L1 or LH01, and the progression curves of tumor volumes were

163 depicted (n = 6). Tumors were removed and photographed after euthanasia. C, Tumors
164 were weighed. D, Spleens of Balb/c mice were removed and weighed after euthanasia.
165 F, Body weights of mice was recorded. All graphs show mean \pm SD.

166 We explored the antitumor effects of LH01 among different doses and meanwhile, we
167 compared the antitumor efficacy of LH01 versus anti-PD-L1 plus LH02 in murine
168 CT26 and MC38 tumor models. Considering that 5 of 8 CT26 tumor-bearing mice
169 died at day 9 after receiving two intraperitoneal treatments of LH02 (1 mg/kg), we
170 decided against using equimolar doses of LH01 and anti-PD-L1+LH02. Instead,
171 LH01 was compared with anti-PD-L1 (5 mg/kg) and LH02 (0.5 mg/kg). LH01 at 3
172 mg/kg induced similar reduction in CT26 tumor burden compared with 5 mg/kg (TGI:
173 61.6% (1 mg/kg), 75.3% (3 mg/kg), 79.4% (5 mg/kg)) (Figure 3A), and showed
174 greater decrease in tumor weight than anti-PD-L1+LH02 (Figure 3C). In MC38 tumor
175 models, the immunocytokine demonstrated the antitumor activity in a dose-dependent
176 manner (TGI: 38.5% (1 mg/kg), 56.4% (3 mg/kg), 71.1% (5 mg/kg)), and LH01 (3
177 mg/kg) exhibited similar antitumor efficacy versus anti-PD-L1+LH02 (Figure 3B and
178 3C). Notably, in CT26 tumor-bearing mice, anti-PD-L1+LH02 significantly increased
179 the spleen weight compared to PBS group, while there was no obvious spleen weight
180 gain in LH01 group at dose of 1 mg/kg or 3 mg/kg, indicating that LH01 exerted good
181 tumor targeting capability (Figure 3D). LH01 was well tolerated in both tumor models,
182 as neither CT26 nor MC38 tumor-bearing mice obviously lost weight after treatment
183 (Figure 3E). anti-PD-L1+LH02 showed good tolerability in MC38 tumor models, but
184 in CT26 tumor models 2 of 6 mice died after receiving two intraperitoneal treatments

185 due to systemic toxicity. Collectively, these data illustrate that LH01 has greater
 186 antitumor activity than anti-PD-L1+LH02 and a favorable tolerability.

187 **LH01 induces both innate and adaptive immune cell activation in tumors**



188

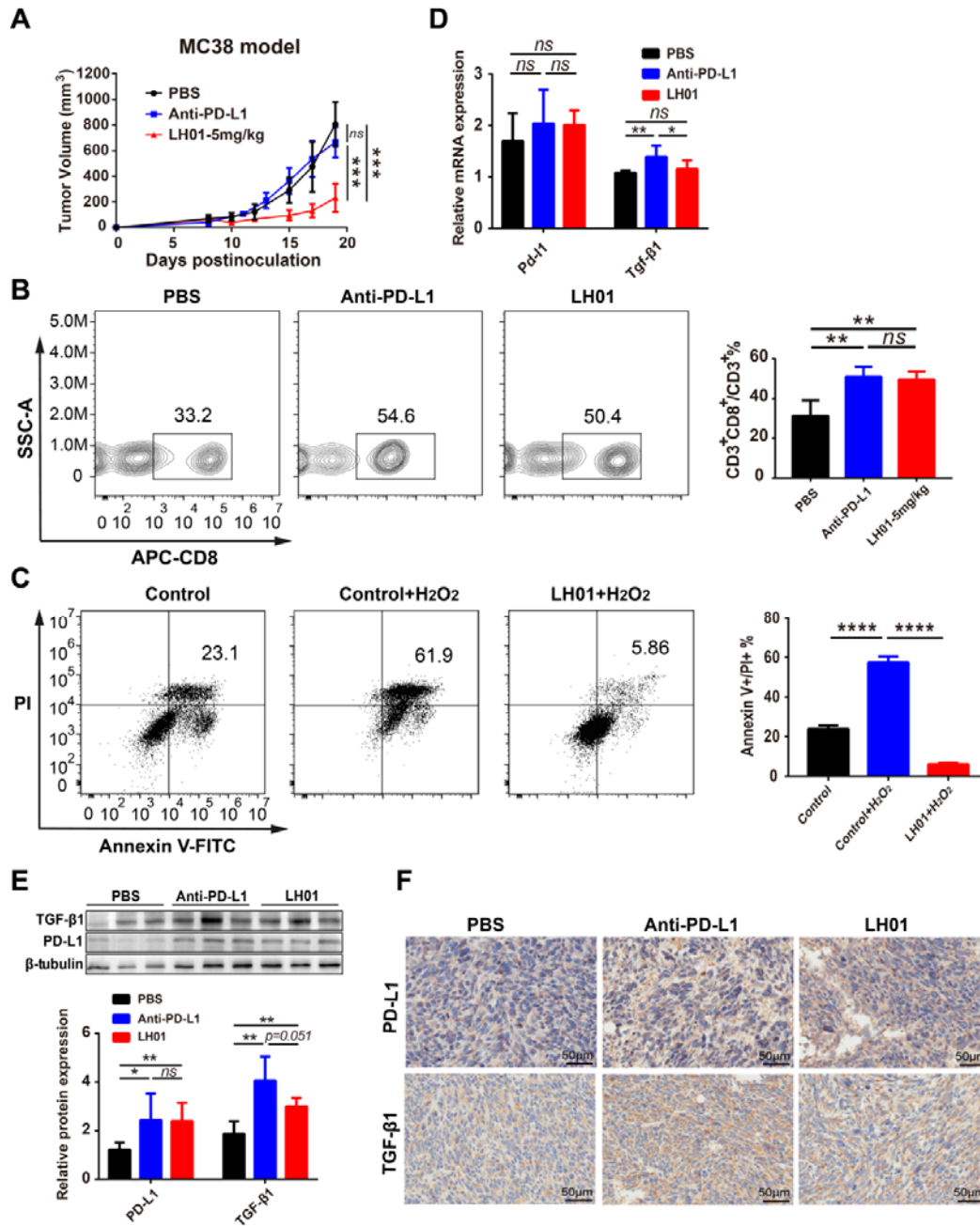
189 **Figure 4. LH01 increases both adaptive and innate immune cell activation in**

190 **tumors and spleens**

191 Flow cytometry analysis of dissociated tumors and spleens from CT26 tumor-bearing
192 mice treated as described in Figure 3. (A-D) The percentages of intratumor (A-C) and
193 splenic (D) CD8⁺ T cells, NK cells and Tregs were shown for populations of CD3⁺,
194 CD45⁺ and CD4⁺ lymphocytes. Data are reported as the mean \pm SEM.

195 IL-15 is a pleiotropic cytokine that plays a vital role in regulating innate and adaptive
196 immunity and can strongly expand CD8⁺ T and NK cells with much weaker
197 regulatory T cells (Tregs)-stimulating activity ^[23]. To explore the changes in splenic
198 and intratumoral CD8⁺ T, NK and Tregs populations, we performed flow cytometry
199 analysis of dissociated tumors and spleens from CT26 tumor-bearing mice. LH01
200 markedly increased the CD8⁺ tumor-infiltrating lymphocytes (TILs) compared with
201 PBS or LH02+anti-PD-L1, which suggested that LH01 can selectively activate CD8⁺
202 T cells in the tumor (Figure 4A). Besides its effects on CD8⁺ T cells, LH01 treatment
203 also increased the tumor-infiltrating natural killer cells (TINKs) and dramatically
204 decreased the tumor-associated Tregs (Figure 4B and 4C). In comparison with PBS
205 group, LH01 treatment only elicited slight increase in splenic CD8⁺ T cells, while
206 anti-PD-L1+LH02 treatment markedly increased splenic CD8⁺ T and NK cells, which
207 indicated good tumor targeting activity of LH01 (Figure 4D, Figure S3). To our
208 surprise, we found that LH01 treatment remarkably increased splenic Tregs versus
209 PBS group, which may be beneficial to reduce systemic toxicity (Figure 4D).

210 **LH01 overcomes resistance to PD-1/PD-L1 blockade in MC38 model correlated**
 211 **to inhibition of TGF- β 1**



212
 213 **Figure 5. LH01 overcomes resistance to anti-PD-L1 treatment related to**
 214 **suppression of TGF- β 1**

215 (A) C57BL/6 mice transplanted s.c. with MC38 cells (right) were treated with PBS,

216 anti-PD-L1 or LH01 as described in Figure 3, and the progression curves of tumor
217 volumes were depicted (n = 6 mice/group). (B) The percentage of CD8⁺ TILs was
218 shown for populations of CD3⁺ lymphocytes. (C) Well-grown CTLL-2 cells were
219 planked with 2*10⁵ cells per well on 12-well cell culture plates. Flow cytometric
220 analysis of CTLL-2 cells untreated (left), treated with 50 μM H₂O₂ (middle) or 50 μM
221 H₂O₂ + LH01 (right) for 18 hours. The proportion of late apoptotic cells is statistically
222 analyzed (n = 4). (D and E) Quantitative real-time PCR analysis and western blot
223 were performed to measure the expression levels of PD-L1 and TGF-β1 in tumor
224 tissues. (F) tumor tissues were fixed, followed by immunohistochemical staining for
225 PD-L1 and TGF-β1. Data are reported as the mean ± SD.

226 Our results showed that mice did not respond to anti-PD-L1 treatment at a dose of 10
227 mg/kg with primary resistance to therapy, while LH01 displayed obvious therapeutic
228 improvements (Figure 5A). Both anti-PD-L1 and LH01 treatments relieved inhibition
229 of T cell via PD-1/PD-L1 axis, and showed a significant increase in CD8⁺ TILs than
230 control group (Figure 5B). The above meant that impairment of T cell function caused
231 by immunosuppressive TME may contribute to resistance to PD-L1 blockade. As an
232 important feature of TME, high reactive oxygen species (ROS) is detrimental to the
233 survival and function of T lymphocytes [24]. We explored whether LH01 can inhibit
234 the apoptosis induced by oxidative stress. The results of flow cytometry demonstrated
235 that the apoptosis rate was significantly higher than that of the control group (57.4 ±
236 3.0 % VS 23.9 ± 1.7 %) after T lymphocyte cell line CTLL-2 was incubated with
237 50 μM H₂O₂ for 18h, whereas the addition of LH01 could dramatically reverse the

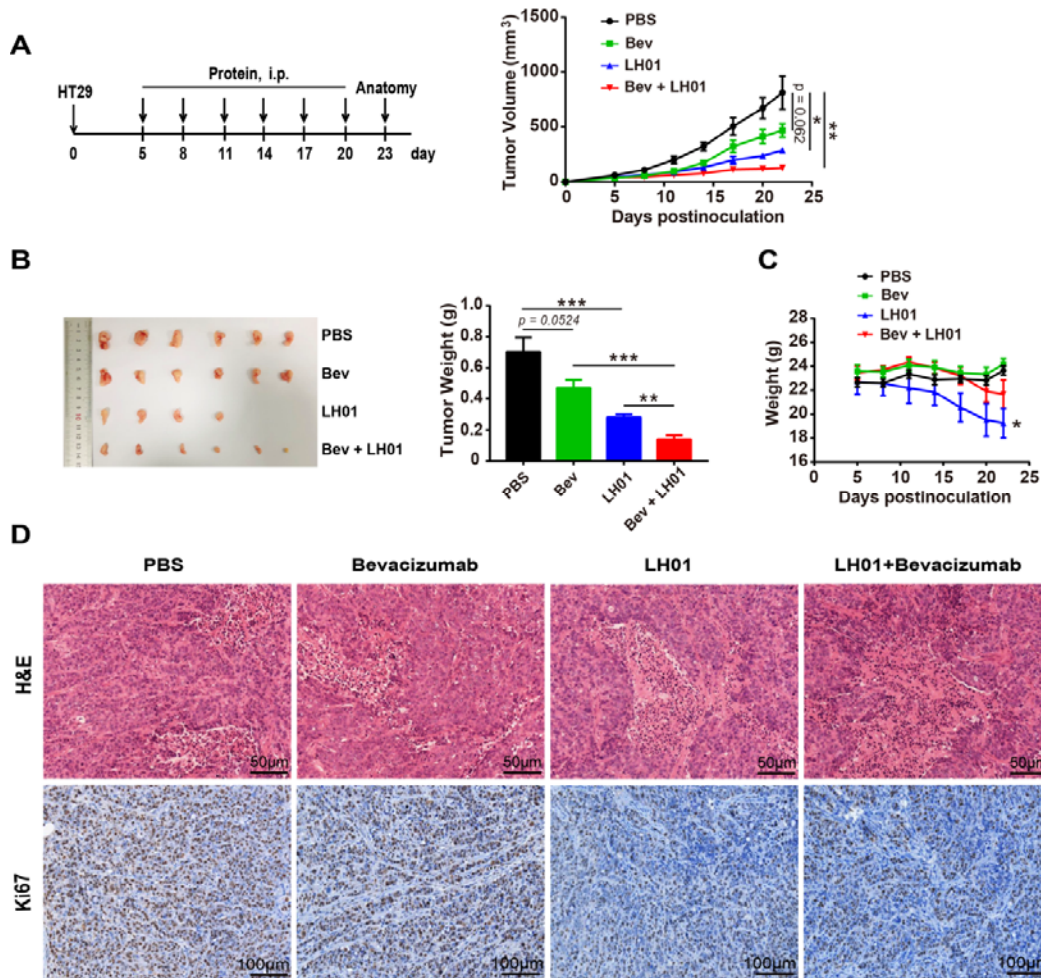
238 apoptosis induced by H₂O₂ (57.4 ± 3.0 % VS 6.2 ± 0.6 %) (Figure 5C).

239 Transforming growth factor-β (TGF-β) exerts diverse effects in tumorigenesis and
240 progression. The pleiotropic nature of TGF-β signaling within the TME facilitates
241 tumor immune escape and promotes tumor progression via induction of
242 epithelial-mesenchymal transition, angiogenesis, and stromal modification ^[25, 26]. It
243 has revealed that TGF-β participates in the resistance to PD-1/PD-L1 blockade ^[27, 28].

244 Anti-PD-L1 treatment markedly increased expression levels of PD-L1 and TGF-β1,
245 which partly explained the resistance to its treatment (Figure 5D-5F). Intriguingly,
246 compared to anti-PD-L1 group, LH01 treatment did not significantly alter PD-L1
247 expression levels, but remarkably reduced TGF-β1 levels (Figure 5D-5F). The results
248 suggested that inhibition of TGF-β signaling may target mechanisms of resistance and
249 sensitize tumors to immunotherapy.

250 **Combination therapy with LH01 and bevacizumab exerts synergistic antitumor**

251 **effect**



252 **Figure 6. Combining LH01 with bevacizumab enhances antitumor activity**

253 (A and B) NOD-SCID mice were inoculated subcutaneously with 3×10^6 HT29 cells,
254 and subsequently received 3×10^6 fresh human PBMCs intravenously on the same
255 day. Mice were randomized into 4 groups and treatment initiated when tumors
256 reached 40-80mm³. Mice were treated intraperitoneally with LH01 (3 mg/kg),
257 bevacizumab (10 mg/kg) or LH01 (3.0 mg/kg) + bevacizumab (10 mg/kg) at days 5, 8,
258 11, 14, 17 and 20 (n = 6). (A) Tumor volumes were measured every 3 days. (B)
259 Tumor weight (day 23). (C) Body weights of mice. (D) tumor tissues were fixed,
260 followed by H&E staining and immunohistochemical staining for Ki67. CI was
261 calculated based on the formula $Ea+b/(Ea + Eb - Ea \times Eb)$. Data are reported as the
262

263 mean \pm SEM.

264 Previous studies have displayed that angiogenesis is an essential process for the
265 proliferation of solid tumor and VEGF can elicit immunosuppressive effects in the
266 TME, suggesting that anti-angiogenic agents and LH01 could generate synergistic
267 antitumor efficacy ^[29, 30]. Bevacizumab is a molecularly targeted drug that can inhibit
268 tumor angiogenesis by binding to vascular endothelial growth factor A (VEGF-A)
269 around tumor ^[31]. In a HT29 xenograft model, mice experienced a slight and
270 significant reduction in tumor volume and tumor weight after receiving bevacizumab
271 and LH01, respectively (Figure 6A and 6B). Two mice died after receiving four
272 intraperitoneal treatments of LH01, possibly due to graft-versus-host disease caused
273 by infused human peripheral lymphocytes (Figure 6C). Combination therapy of LH01
274 and bevacizumab markedly reduced tumor volume and tumor weight (Figure 6A and
275 6B) versus LH01 or bevacizumab and showed synergistic antitumor activities (CI =
276 1.09). Larger areas of necrosis were observed in the combination regimen than the
277 other three groups (Figure 6D). Both LH01 monotherapy and combination therapy
278 obviously reduced the expression levels of Ki67 compared to PBS or anti-VEGF
279 treatment, which demonstrated a poor proliferative and metastatic ability of tumor
280 cells (Figure 6D). Our results indicate that LH01 is a promising candidate to exert
281 enhanced antitumor activities in combination with angiogenesis inhibitors.

282 **Discussion**

283 The antibodies targeting the immune checkpoint have become the protagonist of
284 immunocytokines with the breakthrough progress of immune checkpoint inhibitors

285 for tumor treatment in the past few years. Recently, Martomo et al. preliminarily
286 explored the antitumor activity of anti-PD-L1/IL-15 fusion protein KD033 on various
287 solid tumor models in mice, whereas they did not provide further rationale for KD033
288 to treat patients with cold tumors or resistance to ICIs ^[32]. LH01 is different in
289 structure compared to KD033: the antibody part is atezolizumab and the sushi domain
290 is 85 amino acids in length with higher binding affinity to IL-15 than the 65 amino
291 acids of KD033 ^[15]. Interestingly, our results show that LH01 can overcome primary
292 resistance to PD-1/PD-L1 blockade by down-regulating TGF- β 1 levels within the
293 TME without markedly affecting PD-L1 expression. Additionally, we demonstrate
294 that LH01 can induce the development of an inflamed TME through enhancing the
295 populations of CD8⁺ TILs and TINKs with a decrease in Tregs populations.
296 Resistance is a major obstacle to cancer immunotherapy, and its mechanisms are
297 varied and complicated. Amelioration of primary resistance to anti-PD-L1 therapy by
298 using LH01 may be related to converting inherently immunosuppressive TME to
299 immunosupportive one.

300 The format of immunocytokine has a significant impact on its targeting activity. The
301 homodimeric format usually possesses a high binding avidity to the target and a long
302 residence time at the tumor site. In our study, we found that the LH01 did not
303 substantially increase weight of the spleen of tumor-bearing mice at the dose of 1
304 mg/kg and 3 mg/kg compared to that of control group, which indicated that the LH01,
305 a homodimeric fusion protein, had good tumor targeting activity. It should be noted
306 that LH01 has a favorable safety at a high dose (5 mg/kg) in both CT26 and MC38

307 models, with no difference observed in mice body weights. On the other hand, fast
308 blood clearance profiles may be beneficial to reduce untoward effects associated with
309 the use of potent pro-inflammatory cytokine payloads, which perhaps partially
310 explains the good safety of LH01 in mice.

311 In principle, certain immunocytokine products could mimic the action of bispecific
312 antibodies (BsAbs). The cytokine moiety can engage in a binding interaction with its
313 cognate receptor on the surface of T cells, thus creating an immunological synapse
314 with the tumor cells ^[33]. Bispecific T cell engagers (BiTEs), one kind of BsAbs, have
315 been attracting a great deal of attention due to its unique mechanism of action and
316 significant antitumor activity. BiTEs demonstrated remarkable efficacy in B cell
317 hematologic malignancies, but the use of such new drugs to treat solid tumors is
318 unsatisfactory ^[34]. Although BiTEs can redirect T cells to specific tumor antigens and
319 activate T cells directly, the immunosuppressive factors in the TME, including high
320 levels of ROS, hypoxia, TGF- β , etc, are not conducive to the proliferation and
321 survival of T and NK cells in solid tumors, which can importantly reduce its antitumor
322 activity ^[35, 36]. However, the pro-inflammatory cytokine moiety of immunocytokine
323 can convert the tumor immunosuppressive microenvironment to a certain extent, and
324 promotes the activation and proliferation of T and NK cells, which is supported by our
325 results that LH01 can inhibit the apoptosis of CTLL-2 under high levels of ROS and
326 down-regulate the TGF- β 1 levels in TME. In terms of BiTEs, a major restriction of
327 tumor-associated antigen selection in solid tumors is that low-level expression is often
328 found in normal tissue exposing the patients to a risk of “on-target, off-tumor”

329 toxicity^[36]. In the case of immunocytokines, it seems not so demanding for antigen
330 specificity, and the adverse effects of immunocytokines are mainly caused by
331 cytokine moiety.

332 Given that LH01 is well tolerated in preclinical models, we believe that this
333 bifunctional fusion protein represents a promising candidate for inclusion in
334 combination therapy regimens. We have validated this in our murine models, in which
335 combining LH01 with a VEGF-A inhibitor bevacizumab elicits enhanced and superior
336 antitumor activity over that of either agent alone. Vascular abnormalities resulting
337 from elevated levels of proangiogenic factors (e.g. VEGF and angiopoietin 2) are a
338 hallmark of most solid tumor^[37]. Additionally, proangiogenic factors have been
339 reported to play a vital role in immunosuppressive TME^[38]. For instance, VEGF can
340 directly elevate PD-L1 expression on dendritic cells resulting in impaired function of
341 T cells, and VEGF can also directly binds to VEGFR2 on regulatory T cells (Tregs)
342 and myeloid-derived suppressor cells (MDSCs), which increases these
343 immunosuppressive cells into TME^[39, 40]. Our results further indicate that the
344 combination of the other inhibitors of VEGF signaling pathway including small
345 molecule receptor tyrosine kinases inhibitors (sunitinib, sorafenib, and pazopanib)
346 with LH01 has the potential to generate greater antitumor effects.

347 Our study has some limitations. First of all, the mechanisms that LH01 overcomes
348 resistance to anti-PD-L1 remains to be further studied because a variety of factors
349 including other immune checkpoints, cancer neoantigens, soluble MHC related
350 molecules, and cytokines in the TME also affect anti-cancer immune response^[41]. In

351 addition, we noted that the CT26 tumor-bearing mice showed slightly ungroomed hair
352 without weight loss after third administration of LH01 at a dose of 5 mg/kg, which
353 was associated with side effects caused by cytokine IL-15. Given that most of
354 immunocytokines still produce the same adverse effects as cytokines in clinical trials
355 ^[42], further efforts should be made to improve safety by structure-based design.

356 In conclusion, LH01 elicits superior antitumor efficacy and a good safety profile in
357 preclinical models. LH01 possesses the potential to help T cells resist damage from
358 unfavorable factors and overcome primary resistance to PD-1/PD-L1 blockade by
359 inhibiting TGF- β 1 within the TME, which offers supporting evidence for clinical use
360 of LH01 for treatment of patients with resistance to ICIs or cold tumors. LH01 can be
361 combined more practically with other therapies to target even more pathways to
362 improve clinical benefit. Altogether, LH01 represents a potential candidate for further
363 clinical investigation.

364 **Materials and Methods**

365 **Cloning, expression, and purification**

366 The plasmids encoding LH01, LH02, and anti-PD-L1 were constructed as shown in
367 Figure S1. The DNA sequences of IL-15 mutant (IL-15N72D) and IL-15R α
368 sushi-domain (Ile 31 to Val 115) were amplified by polymerase chain reaction using
369 the pIL-15 and psIL-15R α /Fc we reported previously as template ^[43]. All the plasmids
370 were constructed by inserting the DNA fragments into the vector we used before ^[43].
371 The light and heavy chain expression plasmids of LH01 or anti-PD-L1 were mixed at
372 2:1 and co-transfected using liner polyethylenimine (PEI) with a molecular weight of

373 25 kDa (Polysciences, Warrington, PA, USA). LH02 was produced by transfecting
374 HEK293E cells with Fc-Sushi-IL15-expression plasmid alone. LH01, anti-PD-L1 and
375 LH02 were all purified by affinity chromatography using a protein A affinity column
376 (GE Healthcare, Piscataway, NJ, USA) and analyzed in reducing condition on sodium
377 dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

378 **Cell lines**

379 HEK293E, CTLL-2 cell lines was kept in our laboratory and cultured as previous
380 descriptions ^[43]. Mo7e, MC38 and CT26 murine colon carcinoma cell lines were
381 obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).
382 Mo7e cells were grown in RPMI 1640 (Gibco, Waltham, MA, USA) containing 10%
383 FBS (Gibco) and 10ng/mL human GM-CSF (Sino Biological, Beijing, China). MC38
384 and CT26 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM)
385 containing 10% FBS. All cells above were maintained under aseptic conditions and
386 incubated at 37°C with 5% CO₂.

387 **Measurement of LH01 binding and pharmacokinetics by enzyme-linked** 388 **immunosorbent assays (ELISAs)**

389 *ELISAs for PD-L1 binding*

390 ELISAs were performed using standard methods. Briefly, 96-well ELISA plates
391 (Corning, Corning, NY, USA) were coated by incubating with 1.0 µg/mL of
392 recombinant human or mouse PD-L1 (Novoprotein, Nanjing, China) at 4°C overnight,
393 then washed four times with PBST (PBS, 0.05% Tween-20) and blocked with 5%
394 bovine serum albumin for 2 hours at room temperature. After washing the plates,

395 serial dilutions (1:3) of LH01 and anti-PD-L1 were added to the plates in duplicate
396 and incubated at room temperature for 2 hours. Plates were washed four times and
397 incubated with Peroxidase AffiniPure Goat Anti-Human IgG (H+L) (Jackson
398 ImmunoResearch, West Grove, PA, USA, 1:10,000 dilution) at room temperature for
399 1 hour. After being washed, TMB single component substrate solution (Solarbio,
400 Beijing, China) was added to the plates and incubated in the dark for 3-5min. After
401 terminating the reaction with 2 M sulfuric acid, absorbance was read at 450 nm.

402 *Pharmacokinetic evaluation of LH01 and IL-15 by ELISAs*

403 Plasma samples were drawn from mice 0.5, 1, 2, 4, 8, 12, 24, and 48 hours after
404 treatment with LH01, and 0.5, 1, 2, 4, and 6 hours after treatment with IL-15
405 monomer. A 96-well ELISA plate, previously coated overnight at 4°C with 1.0 µg/mL
406 of recombinant human PD-L1, was incubated with plasma samples for 2 hours from
407 mice treated with LH01. The following experimental procedure was the same as
408 described above. The human IL-15 ELISA Pair Set (Sino Biological) was used for the
409 quantitative determination of IL-15 monomer.

410 **Cell Proliferation Assay**

411 Mo7e cells were wash with human GM-CSF free medium (RPMI1640 + 10% FBS)
412 and seeded into 96-well plate with 2×10^4 cells in a volume of 50 µL per well. After 4
413 hours' starvation, serial dilutions (1:3) of LH01 or IL-15 was added to the plate in
414 sextuplicate at 50µL per well to achieve a final density of 2×10^4 cells/100 µL/well.
415 After being incubated for 96 hours at 37°C with 5% CO₂, the cell viability was
416 measured using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Absorbance was

417 read at 450 nm, and the final OD450 value was calculated as the reading of sample
418 well minus the reading of blank well containing medium. The method used in
419 CTLL-2 cell proliferation assay was identical to that of Mo7e, except that the number
420 of cells used was 1×10^4 and the incubation time was 72 hours.

421 **Animal experiments**

422 Female Balb/c, C57BL/6 and NOD-SCID mice aged 6-8 weeks were purchased from
423 Shanghai SLAC Laboratory Animal Co., Ltd and reared under specific pathogen-free
424 conditions. All experiments were approved by the Animal Care and Use Committee of
425 Shanghai Jiao Tong University. All mice were treated humanely throughout the
426 experimental period. Human peripheral blood mononuclear cells (PBMCs) were
427 isolated by Ficoll density gradient centrifugation to serve human T lymphocytes with
428 procedures previously we described ^[45]. For antitumor studies, tumors were measured
429 every two days using digital caliper, and volumes were calculated as $(\text{length} \times$
430 $\text{width}^2)/2$. Tumor Growth Inhibition (TGI): $\text{TGI}(\%) = 100 \times (1 - T/C)$. T and C were the
431 mean tumor volume of the treated and control groups, respectively.

432 **Flow cytometric analysis of splenic and intra-tumoral CD8⁺ T, NK and** 433 **regulatory T cells**

434 150 mg tumor tissues were finely minced and digested with 4 mL lysis solution (2
435 mg/mL collagenase IV and 1.2 mg/mL hyaluronidase). The digested tumor tissues
436 were filtered through 200-mesh nylon net to obtain the cell suspension. Centrifuge,
437 then discard the supernatant, and wash the cells once with 6 mL FACS buffer (PBS +
438 2% FBS). The cells were re-suspended in 6 mL FACS buffer, and filtrated through

439 200-mesh nylon net again to obtain pre-treated single cell suspension. The spleens
440 were gently grinded and lymphocytes were isolated with lymphocyte separation
441 medium (Dakewe, Beijing, China).

442 Cell samples were blocked with anti-mouse CD16/CD32 mAb 2.4G2 (BD
443 Biosciences, San Jose, CA, USA) at 4°C for 15 min and incubated with surface
444 marker antibodies at 4°C for 25 min. For the detection of Tregs, cell samples would be
445 further incubated with FOXP3 Fix/Perm buffer (BioLegend, San Diego, CA, USA)
446 for 20 min and FOXP3 Perm Buffer for 15 min at room temperature before
447 anti-FOXP3 was added.

448 The antibodies and reagents were used as follows: anti-mouse CD45-Percp/Cyanine
449 5.5 (BioLegend), anti-mouse CD45.2-PE (BioLegend), hamster anti-mouse
450 CD3e-FITC (BD Biosciences), rat anti-mouse CD4-PE (BD Biosciences), rat
451 anti-mouse CD8a-APC (BD Biosciences), rat anti-mouse Nkp46-Alexa Flour 647
452 (BD Biosciences), anti-mouse CD25-Brilliant Violet 421 (BioLegend),
453 anti-mouse/rat/human FOXP3-Alexa Fluor 647 (BioLegend). Flow cytometry was
454 performed on a CytoFLEX cytometer (Beckman Coulter) and analyzed by FlowJo 10
455 (TreeStar, Ashland, OR, USA).

456 **Flow cytometric analysis of cell apoptosis**

457 Flow cytometry was performed to detect the apoptosis of CTLL-2 cells by using
458 Annexin V-FITC/PI Apoptosis Detection Kit (Vazyme) and analyzed by FlowJo 10
459 (TreeStar).

460 **RNA isolation and qRT-PCR analysis of mRNA expression**

461 Total RNAs of pretreated tumor tissues were extracted by using Ultrapure RNA Kit
462 (Cwbio, Beijing, China). cDNA was synthesized using a PrimeScript RT Master Mix
463 (Takara, Tokyo, Japan), and quantitative real-time polymerase chain reactions
464 (qRT-PCR) were analyzed on an Applied Biosystems 7500 Fast Real-Time PCR
465 System (ThermoFisher Scientific, Eugene, OR, USA) using Hieff[®] qPCR SYBR[®]
466 Green Master Mix (Yeasen, Shanghai, China). The primer sequences are listed in
467 table S1. All results were normalized to GAPDH expression and calculated using the
468 $2^{-(\Delta\Delta C_t)}$ method.

469 **Western blotting**

470 MC38 tumor tissues were lysed using radio immunoprecipitation assay buffer
471 (Beyotime, Shanghai, China). Protein lysates were separated on 10% SDS-PAGE gels
472 and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). The
473 membranes were blocked with 5% nonfat dry milk at room temperature for 2 hours
474 and then incubated at 4°C overnight with primary antibodies against β -tubulin
475 (Abcam, Cambridge, MA, USA), PD-L1 (ABclonal, Wuhan, China) or TGF- β 1
476 (Abcam). Membranes were washed three times and incubated with HRP-conjugated
477 secondary antibodies. Target proteins were visualized using ECL (ThermoFisher
478 Scientific). The autoradiograms were analyzed with Image J software to quantify the
479 band densities.

480 **Histopathological and IHC analysis**

481 The tumor tissues were fixed in 4% paraformaldehyde, and then embedded in paraffin,

482 sectioned (4 μm), and stained with hematoxylin and eosin. After dewaxing and
483 hydration, the tumor sections were treated with heat induced epitope retrieval and 3%
484 hydrogen peroxide for 15 minutes to block the endogenous peroxidase activity. Next,
485 the tumor sections were blocked with 5% BSA for 30 min and incubated with
486 anti-mouse PD-L1 rabbit antibody (ABclonal), anti-mouse TGF- β 1 rabbit antibody
487 (Abcam), or anti-human Ki67 rabbit antibody (Servicebio, Wuhan, China) at 4°C
488 overnight. Afterward, the sections were incubated with the HRP-conjugated goat
489 anti-rabbit secondary antibody (Servicebio) for 50 minutes. Finally, the sections were
490 stained with DAB detection kit (Dako, Copenhagen, Denmark) and hematoxylin.
491 Then the slides were observed under the OLYMPUS BX53 Microscope and
492 photographed.

493 **Statistical analysis**

494 The statistical significance of differences between experimental groups was
495 determined with two-tailed Student t test and analysis of variance using Prism 7.0
496 (GraphPad, San Diego, CA, USA). (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, P
497 < 0.0001).

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504 **Authors' Contributions**

505 **W. Shi:** Conceptualization, methodology, investigation, data curation, formal analysis,
506 writing-original draft. **L. Lv:** Validation, investigation. **N. Liu:** Resources,
507 methodology, discussion. **H. Wang:** Supervision, investigation. **Y. Wang:**
508 Investigation, formal analysis. **W. Zhu:** Validation, supervision. **Z. Liu:** Statistical
509 analysis. **J. Zhu:** Resources. **H. Lu:** Conceptualization, supervision, funding
510 acquisition, writing-review & editing. All the authors read and approved the final
511 manuscript.

512 **Declaration of interests**

513 The authors declare that they have no known competing financial interests or personal
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