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GARP-mediated active TGF- β 1 induces bone marrow NK cell dysfunction in AML patients with early relapse post-allo-HSCT

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Abstract:

Relapse is a leading cause of death after allogeneic hematopoietic stem cell transplantation (allo-HSCT) for acute myeloid leukemia (AML). However, the underlying mechanisms remain poorly understood. Natural killer (NK) cells play a crucial role in tumor surveillance and cancer immunotherapy, and NK cell dysfunction has been observed in various tumors. Here, we performed ex vivo experiments to systematically characterize the mechanisms underlying the dysfunction of bone marrow-derived NK (BMNK) cells isolated from AML patients experiencing early relapse after allo-HSCT. We demonstrated that higher levels of active transforming growth factor- β 1 (TGF- β 1) were associated with impaired effector function of BMNK cells in these AML patients. TGF- β 1 activation was induced by the overexpression of glycoprotein A repetitions predominant (GARP) on the surface of CD4 $^{+}$ T cells. Active TGF- β 1 significantly suppressed mTORC1 activity, mitochondrial oxidative phosphorylation, the proliferation, and cytotoxicity of BMNK cells. Furthermore, pretreatment with the clinical stage TGF- β 1 pathway inhibitor, galunisertib, significantly restored mTORC1 activity, mitochondrial homeostasis, and cytotoxicity. Importantly, the blockade of the TGF- β 1 signaling improved the anti-tumor activity of NK cells in a leukemia xenograft mouse model. Thus, our findings reveal a mechanism explaining BMNK cell dysfunction and suggest that targeted inhibition of TGF- β 1 signaling may represent a potential therapeutic intervention to improve outcomes in AML patients undergoing allo-HSCT or NK cell-based immunotherapy.

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3

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56 **Key Points:**

57 (1) GARP-induced activation of TGF- β 1 attenuates effector functions of BMNK
58 cells *ex vivo*.

59 (2) Pharmacologic inhibition of TGF- β 1 signaling restores NK cell-mediated
60 anti-leukemic responses in leukemia xenograft mouse models.

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89 **Abstract**

90 Relapse is a leading cause of death after allogeneic hematopoietic stem cell
91 transplantation (allo-HSCT) for acute myeloid leukemia (AML). However, the
92 underlying mechanisms remain poorly understood. Natural killer (NK) cells play a
93 crucial role in tumor surveillance and cancer immunotherapy, and NK cell
94 dysfunction has been observed in various tumors. Here, we performed *ex vivo*
95 experiments to systematically characterize the mechanisms underlying the
96 dysfunction of bone marrow-derived NK (BMNK) cells isolated from AML patients
97 experiencing early relapse after allo-HSCT. We demonstrated that higher levels of
98 active transforming growth factor- β 1 (TGF- β 1) were associated with impaired
99 effector function of BMNK cells in these AML patients. TGF- β 1 activation was
100 induced by the overexpression of glycoprotein A repetitions predominant (GARP) on
101 the surface of CD4 $^{+}$ T cells. Active TGF- β 1 significantly suppressed mTORC1
102 activity, mitochondrial oxidative phosphorylation, the proliferation, and cytotoxicity
103 of BMNK cells. Furthermore, pretreatment with the clinical stage TGF- β 1 pathway
104 inhibitor, galunisertib, significantly restored mTORC1 activity, mitochondrial
105 homeostasis, and cytotoxicity. Importantly, the blockade of the TGF- β 1 signaling
106 improved the anti-tumor activity of NK cells in a leukemia xenograft mouse model.
107 Thus, our findings reveal a mechanism explaining BMNK cell dysfunction and
108 suggest that targeted inhibition of TGF- β 1 signaling may represent a potential
109 therapeutic intervention to improve outcomes in AML patients undergoing allo-HSCT
110 or NK cell-based immunotherapy.

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112 **Keywords:** NK cells; AML; allo-HSCT; active TGF- β 1; GARP.

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119 **Introduction**

120 Acute myeloid leukemia (AML) is an aggressive hematological malignancy with
121 higher incidence in older adults, and has presented challenges for hematologists over
122 the last decades^{1, 2}. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is
123 an effective therapy and the only curative option for majority of the patients with
124 AML^{1, 3, 4}. Even with current treatments, relapse of the original disease after
125 transplantation, remains frequent and is associated with particularly poor outcome⁴⁻⁷.
126 Especially, after day 100 post-allo-HSCT, relapse becomes a primary cause of death^{4,}
127 ⁸. However, the underlying mechanisms leading to early relapses is poorly understood
128 and requires further investigation.

129 AML relapse is associated with the ability of AML cells to escape from immune
130 surveillance⁷. Natural killer (NK) cells have crucial roles in the immunosurveillance
131 of cancer. Recently, several studies have shown that down-regulation of AML cell
132 killing by NK cells after allo-HSCT may help AML cells to evade immune
133 surveillance^{4, 9}. NK cells are the first reconstituting lymphocytes and may represent up
134 to 80% of peripheral blood lymphocytes during the first 100 days after
135 transplantation¹⁰⁻¹⁴. NK cells kill leukemic cells via the exocytosis of granules
136 containing cytolysis-related proteins such as Granzymes, the secretion of effector
137 cytokines such as interferon- γ (IFN- γ), and tumor necrosis factor α (TNF- α)^{13, 15}.
138 However, tumor cells often evade NK cell-mediated cytotoxicity and immune
139 surveillance in the tumor microenvironment due to the action of immunosuppressive
140 factors such as TGF- β 1¹⁶⁻¹⁹. Functionally impaired NK cells in AML patients show
141 reduced expression of activating receptors and increased expression of inhibitory
142 receptors⁹.

143 TGF- β 1 plays an integral role in regulating immune responses and triggers
144 signaling mainly through binding to the TGF- β receptor (TGF- β R) complex. This is
145 composed of two type I TGF- β (TGF- β RI, also known as ALK5) and two type II
146 TGF- β (TGF- β RII) receptor subunits. Both receptors are serine/threonine kinases^{20, 21}.
147 TGF- β 1 is produced as an inactive dimeric complex that undergoes activation and
148 processing before exerting its functional effects^{22, 23}. Glycoprotein-A repetitions

149 predominant (GARP), a type I transmembrane cell surface docking receptor, is
150 abundantly expressed on platelets and regulatory T (Treg) lymphocytes²³⁻²⁶, and
151 regulates activation of latent TGF- β 1²⁴. Active TGF- β 1 could inhibit effector
152 functions of NK cells and CD8⁺ T cells^{18, 27-30}. Blockade of the TGF- β 1 pathway has
153 become an attractive approach to restore anti-tumor immunity²⁹. Galunisertib is an
154 orally administered small molecule inhibitor of TGF- β R1 kinase. It abrogates the
155 activation of the canonical TGF- β 1 pathway by specifically downregulating the
156 phosphorylation of SMAD2. Its ability to inhibit the proliferation of tumor cells was
157 shown in several mouse models^{20, 31}. However, the mechanisms leading to the
158 dysfunction of bone marrow NK (BMNK) cells have not been fully characterized in
159 AML patients. Thus, whether the inhibition of TGF- β 1 signaling could restore
160 anti-tumor activity in patients with relapsing AML remains unclear.

161 In this study, we performed *ex vivo* experiments to investigate the mechanisms of
162 TGF- β 1 activation in the bone marrow of AML patients who relapse after allo-HSCT.
163 We also investigated the mechanisms by which TGF- β 1 induced dysfunction in
164 BMNK cells. Furthermore, we wanted to ascertain whether the *in vitro* and *in vivo*
165 anti-tumor activity of NK cells could be restored by inhibiting TGF- β 1 signaling.

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179 **Methods**

180 **Human samples**

181 Bone marrow mononuclear cells (BMMCs) were isolated from residual bone
182 marrow samples after laboratory tests of AML patients with ($n = 20$) or without ($n =$
183 50) early relapse after allo-HSCT. Early relapse was referred to by relapse within six
184 months after achieving complete remission (CR)³² post-allo-HSCT. NK cells applied
185 to *in vivo* assays were purified from blood of healthy donors. All human samples used
186 were obtained under the approval of the Ethics Committee of the First Affiliated
187 Hospital of the University of Science and Technology of China (2021-N(H)-120;
188 Hefei, China). Written informed consent was obtained from all patients. The clinical
189 characteristics of patients are shown in **Supplementary Tables 1-3**.

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191 **Mice**

192 Female NOD/ShiLtJGpt-Prkdc^{em26Cd52}IL-2rg^{em26Cd22}/Gpt (NCG) mice were
193 purchased from GemPharmatech³³. All animals were kept in specific pathogen-free
194 conditions. All experimental procedures involving mice were carried out as prescribed
195 by the National Guidelines for Animal Usage in Research (China) and were approved
196 by the Ethics Committee of the University of Science and Technology of China
197 (USTCACUC1701038).

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199 **Xenograft mouse models and treatment**

200 HL60 cells (5×10^5), labeled with luciferase, were injected into female NCG mice (6
201 weeks) via the tail vein to establish a leukemia xenograft model. Tumor formation
202 was confirmed after 1 week, and then 2.5×10^6 NK cells were transfused into every
203 mouse. To support NK cell survival *in vivo*, each mice was injected with 50,000 U
204 IL-2 (Jiangsu Kingsley Pharmaceuticals) intraperitoneally every 2 days³⁴.

205 In the first set of experiments such tumor carrying NK cells infused mice were
206 randomized into 4 groups ($n = 6$ mice per group). Group 1 received 50 μ l PBS by
207 intraperitoneal injection (i.p.) (QW). Group 2 received 5×10^5 GARP⁺CD4⁺ T cells
208 alone. Group 3 received 50 μ l latent TGF- β 1, at 5 ng/ml (R&D, Cat# 299-LT; i.p.,

209 QW), while group 4 received the same dose of latent TGF- β 1 in the presence of 5×10^5
210 GARP $^+$ CD4 $^+$ T cells.

211 Galunisertib was obtained from Selleck (Cat# LY2157299) and dissolved in 1%
212 sodium carboxymethyl cellulose (CMC-Na) (as the drug vehicle). During the second
213 set of experiments, the mice were randomized into six groups ($n = 6$ mice per group):
214 Group 1 was administered 50 μ l of PBS (i.p.; QW) and received 150 μ l of 1%
215 CMC-Na (oral gavage; BID; vehicle control). Group 2 received 5×10^5 GARP $^+$ CD4 $^+$ T
216 cells alone. Group 3 received 5×10^5 GARP $^+$ CD4 $^+$ T cells in the presence of 50 μ l
217 latent TGF- β 1 at 5 ng/ml. Group 4 received 50 μ l of active TGF- β 1 (R&D, Cat#
218 240-B) at 5 ng/ml (i.p.; QW), in combination with 150 μ l of 1% CMC-Na. Group 5
219 received 5×10^5 GARP $^+$ CD4 $^+$ T cells in the presence of 50 μ l latent TGF- β 1 at 5 ng/ml
220 and galunisertib at 75 mg/kg twice daily (BID) by oral gavage for 21 days²⁰. Group 6
221 received 50 μ l of active TGF- β 1 (5 ng/ml) in combination with galunisertib (75 mg/kg,
222 BID) for 21 days.

223 AML burden was monitored through bioluminescence imaging using the IVIS
224 Spectrum Imaging System (Perkin Elmer) at the indicated time points. Quantitative
225 image data were analyzed using Living Image Software (Perkin Elmer).

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227 **Survival analysis**

228 We analyzed the survival rates of 173 AML patients (**Supplementary Table 4**)
229 from the Gene Expression Profiling Interactive Analysis (GEPIA) database
230 (<http://gepia.cancer-pku.cn>)³⁵. The patients were classified into high- and low-risk
231 groups based on the cut-off risk score, and median values were used as cutoffs³⁴.
232 Kaplan–Meier survival curve analyses were performed, and the significance of overall
233 survival rates was estimated using log-rank tests.

234

235 **Statistical analysis**

236 We used two-tailed unpaired or paired Student’s *t*-tests between two groups, one-way
237 analysis of variance (ANOVA) across multiple groups, the Mann–Whitney U test for
238 continuous variables, and Chi-Squared and Fisher exact tests for categorical variables,

239 and the Wilcoxon signed rank test. We employed Prism 8 (GraphPad) software to
240 determine statistical significance. Statistical parameters were indicated in the figure
241 legends of each figure. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$). Data
242 are presented as mean \pm SD.

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244 **Data Sharing Statement**

245 For original data, please contact ustcwhm@ustc.edu.cn. Microarray data are
246 available at GEO under accession number GSE190546.

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248 Other detailed methods are available in the Supplementary materials.

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269 **Results**

270 **Active TGF- β 1 in the bone marrow of patients with relapsed AML**

271 TGF- β 1 is one of the most studied cytokines with an immunosuppressive effects on
272 various immune cells, including NK cells¹⁴. Recent studies have demonstrated that
273 TGF- β 1 causes poor responses to cancer immunotherapy¹⁸. Here, we investigated the
274 role of TGF- β 1 in the relapse of AML. Kaplan–Meier survival analysis showed that
275 high expression of TGF- β 1 in bone marrow correlated with poorer survival rates in
276 patients with AML, whereas TGF- β 2 and TGF- β 3 expression levels were not
277 associated with poor outcome (**Figure 1A; Supplementary Figure 1A, B**).
278 Immunofluorescence analysis demonstrated that active TGF- β 1 expression was
279 considerably increased in of AML patients with early relapse, compared to those with
280 non-relapsing AML (**Figure 1B, C**). Western blotting detected a similar difference,
281 with elevated active TGF- β 1 levels in the BMMCs of relapsing AML patients (**Figure**
282 **1D**). However, despite these differences in the levels of active TGF- β 1, total TGF- β 1
283 levels in the bone marrow did not show statistically significant differences between
284 the two groups. ELISA results showed significantly higher levels of active TGF- β 1 in
285 the bone marrow of patients with relapsed AML compared to those without relapse
286 (**Figure 1E**). Next, we performed immunohistochemical (IHC) analysis using samples
287 of bone marrow biopsies. Again, significantly higher levels of active TGF- β 1 were
288 found in the bone marrow of AML patients with early relapse (**Figure 1F**).

289 TGF- β 1 inhibits host immunosurveillance mechanisms^{36,37}. Therefore, we
290 investigated whether the increased activity of TGF- β 1 contributed to the dysfunction
291 of BMNK cells. BMNK cells were isolated from patients who did not relapse after
292 allo-HSCT and stimulated with either latent or active TGF- β 1, and whole-genome
293 transcriptome microarray analysis was performed on these samples. Functional
294 enrichment analyses of the differentially expressed genes, using the Gene Ontology
295 (GO) database demonstrated significant changes in the genes that regulated NK
296 cell-mediated cytotoxicity and NK cell activation in cells treated with active TGF- β 1
297 (**Figure 1G**). Moreover, gene set enrichment analyses (GSEA) showed reduced
298 expression of genes regulating NK cell-mediated cytotoxicity and NK cell activation

299 in cells treated with active TGF- β 1 (**Supplementary Figure 1C, D**). We then
300 compared the differentially expressed genes related to the regulation of NK cell
301 mediated cytotoxicity and activation of NK cells. The results showed downregulation
302 of *GZMB*, *GZMA*, *TNF*, *NCR3*, *TBX21* and *MTOR* in the NK cells stimulated with
303 active TGF- β 1 compared to those stimulated with latent TGF- β 1 or unstimulated
304 controls (**Figure 1H**). These results suggested that higher active TGF- β 1 levels in the
305 bone marrow microenvironment could be responsible for the suppressed effector
306 function of NK cells in AML patients with early relapse.

307

308 Active TGF- β 1 impairs the effector function of BMNK cells

309 We then performed experiments to determine whether active TGF- β 1 suppressed
310 the effector function of BMNK cells. The co-culture experiments with HL60 target
311 cells showed reduced cytotoxicity of BMNK cells stimulated with active TGF- β 1
312 compared to BMNK cells stimulated with latent (**Figure 2A**). Additionally, the
313 proportion of polyfunctional effector NK cells, including TNF- α ⁺IFN- γ ⁺ NK cells,
314 Granzyme B⁺IFN- γ ⁺ NK cells, CD107a⁺IFN- γ ⁺ NK cells, and Granzyme B⁺CD107a⁺
315 NK cells, were significantly reduced when BMNK cells were stimulated with active
316 rather than latent TGF- β 1 (**Figure 2B, C**). Furthermore, NKG2D expression was also
317 significantly reduced in BMNK cells treated with active TGF- β 1 (**Supplementary**
318 **Figure 2A, B**).

319 Smad2/3-dependent signaling axis is referred to as the canonical signaling pathway
320 of TGF- β 1³⁸. Flow cytometry analysis showed that stimulation of BMNK cells with
321 active TGF- β 1 resulted in a higher proportion of pSmad2/3⁺ NK cells, thereby
322 suggesting increased activation of Smad2/3 (**Figure 2D, E**). Walzer et al. reported that
323 TGF- β 1 inhibited both the activation and effector function of NK cells by repressing
324 the mammalian target of rapamycin (mTOR) signaling pathway³⁸. MTOR activity is
325 an important regulator of metabolism in NK cells and can be measured by estimating
326 the expression of phosphorylated S6 ribosomal protein (pS6)³⁴. As the expression of
327 pS6 was significantly reduced in NK cells stimulated with active TGF- β 1 in our
328 experiments (**Figure 2D, E**), it was likely that active TGF- β 1 inhibited the activation

329 of BMNK cells via suppressing mTOR activity.

330 Abnormal glucose metabolism suppresses the normal effector functions of human
331 NK cells^{36, 39, 40}. Therefore, we examined the metabolic output of BMNK cells using
332 the Seahorse XF Cell Mito Stress Test Kit³⁴. BMNK cells pre-treated with active
333 TGF-β1 demonstrated reduced oxygen consumption rate (OCR), an indicator of
334 oxidative phosphorylation (OXPHOS), compared to the control NK cells in the basal
335 state (**Figure 2F, G**). Maximum respiratory capacity of the mitochondria represents
336 the maximum ATP-generating ability of the mitochondria under conditions of high
337 energy demand, and is essential for cellular functions^{34, 41}. We tested the maximum
338 respiratory capacity of active TGF-β1 treated and control NK cells by using the
339 mitochondrial decoupler, carbonylcyanide-4-(trifluoromethoxy) phenylhydrazone
340 (FCCP). Treatment with active TGF-β1 significantly reduced maximum respiratory
341 capacity relative to that seen in control NK cells (**Figure 2F, G**). Mitochondrial
342 membrane potential can be analyzed by staining cells with the mitochondrial
343 membrane potential-sensitive dye, tetramethylrhodamine methyl ester (TMRM)³⁴.
344 TMRM staining was significantly decreased in NK cells treated with active TGF-β1
345 compared to the control NK cells (**Figure 2H**). MitoTracker Green staining also
346 showed that this coincided with a significantly reduced mitochondrial mass (**Figure**
347 **2I**). Flow cytometry analysis, using the Ki-67 antibody, showed that proliferation of
348 the NK cells treated with active TGF-β1 was significantly reduced (**Supplementary**
349 **Figure 2C**). However, Annexin V/7-AAD staining demonstrated that rate of
350 apoptosis did not differ between control NK cells and those treated with active
351 TGF-β1 (**Supplementary Figure 2D**). Taken together, these results showed that
352 active TGF-β1 impairs the effector function, proliferation, mTORC1 activity, and
353 mitochondrial respiration of BMNK cells.

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355 **GARP expressed on the surface of CD4⁺ T cells activates TGF-β1**

356 GARP could regulate the bioavailability and activation of latent TGF-β1²⁴. Next,
357 we analyzed the role of GARP in the activation of TGF-β1 in the context of AML
358 survival. A Kaplan-Meier analysis showed that overall survival of AML patients with

359 high GARP expression was significantly lower than those patients with low GARP
360 expression (**Figure 3A**). The overexpression of GARP was associated with a poor
361 prognosis in patients with other tumors, such as lung squamous cell carcinoma and
362 mesothelioma (**Supplementary Figure 3**). GARP is expressed on activated CD4⁺
363 Tregs and is involved in the release of activated TGF-β1^{29,42}. Several studies reported
364 that more than 25% of CD4⁺ T cells in the human bone marrow are phenotypically
365 and functionally Treg cells⁴³. The percentage of GARP⁺ BMMCs was significantly
366 higher in AML patients with early relapse (**Figure 3B, C**). Furthermore, GARP
367 expression was significantly higher on the surface of CD4⁺ T cells and CD41a⁺
368 platelets of relapsing AML patients (**Figure 3D, E**). At the same time, differences in
369 GARP expression on BMNK cells and CD8⁺ T cells were statistically insignificant
370 between patients with or without relapse (**Figure 3F, G**). Furthermore, active TGF-β1
371 levels in the bone marrow positively correlated with GARP expression by bone
372 marrow CD4⁺ T cells (**Figure 3H-L**).

373 We then investigated whether GARP-induced activation of latent TGF-β1 had an
374 effect on the anti-tumor activity of NK cells. We purified GARP⁻CD4⁺ and
375 GARP⁺CD4⁺ T cells, as well as NK cells from the bone marrow of AML patients
376 without relapse (**Supplementary Figure 4A, B**). The expression of FOXP3, a marker
377 of Treg cells, was significantly higher on the GARP⁺ population of CD4⁺ T cells
378 (**Supplementary Figure 4C, D**). NK cells express a variety of activating and
379 inhibitory receptors, and the balance of signals received simultaneously via these is
380 critical for NK cell function⁴⁴. NKG2D is a widely studied activating NK cell receptor,
381 signaling via DAP10. Its ligands, ULBPs and MICA/B, are found to be generally
382 expressed on AML cells⁴⁴. The engagement of another key activating pathway, the
383 CD94/NKG2C complex, triggers NK cell effector function via DAP12⁴⁵. On the other
384 hand, two inhibitory receptors, NKG2A and KLRG1, which bind HLA-E and
385 members of the classical cadherin family, respectively, have been verified to potently
386 suppress NK cell cytotoxicity^{46,47}. To assess the role of GARP⁺CD4⁺ lymphocytes in
387 activating latent TGF-β1, we performed co-culture experiments. BMNK cells
388 pre-incubated in the presence of both latent TGF-β1 and GARP⁺CD4⁺ T cells showed

389 significantly reduced cytotoxicity against HL 60 target cells, compared BMNK cells
390 co-cultured with GARP⁺CD4⁺ T cells alone, or to NK cells pre-incubated with latent
391 TGF-β1 and GARP⁺CD4⁺ T cells (**Figure 4A-C**). Furthermore, the proportion of
392 CD107a⁺IFN-γ⁺ NK cells, Granzyme B⁺CD107a⁺ NK cells, and Granzyme B⁺IFN-γ⁺
393 NK cells decreased significantly in the latent TGF-β1 plus GARP⁺CD4⁺ T cell
394 co-cultures compared to NK cells pre-incubated with GARP⁺CD4⁺ T cells in the
395 absence of TGF-β1, or to NK cells cultures containing latent TGF-β1 and
396 GARP⁺CD4⁺ T cells (**Figure 4D-G**). This suggested that GARP impaired the
397 anti-leukemic responses of NK cells via the activation of latent TGF-β1. The culture
398 supernatant of NK cells pre-incubated with latent TGF-β1 and GARP⁺CD4⁺ T cells
399 showed significantly higher levels of active TGF-β1 than any other combined culture
400 (**Figure 4H**). This strongly supported the notion that GARP⁺CD4⁺ T cells induced the
401 activation of latent TGF-β1. Furthermore, NK cells co-cultured with latent TGF-β1
402 plus GARP⁺CD4⁺ T cells showed substantial down-regulation of NKp30 and NKG2D
403 compared to the control NK cells, NK cells co-cultured with GARP⁺CD4⁺ T cells, and
404 NK cells co-cultured with latent TGF-β1 plus GARP⁺CD4⁺ T cells (**Figure 4I, J**). The
405 proportion of NKG2A⁺ NK cells was higher and the proportion of Ki-67⁺ NK cells
406 was decreased when NK cells were pre-incubated with latent TGF-β1 and
407 GARP⁺CD4⁺ T cells compared to any other combined culture (**Supplementary**
408 **Figure 4E-H**).

409 To investigate whether GARP-induced activation of latent TGF-β1 could impair the
410 anti-leukemia effect of NK cells *in vivo*, we injected luciferase-labeled HL60 cells
411 into NCG (NOD/ShiLtJGpt-Prkdc^{em26Cd52}IL-2rg^{em26Cd22}/Gpt) mice by tail vein to
412 establish a leukemia xenograft model ³³. After confirming engraftment by BLI, NK
413 cells were transferred into these mice in the presence of either PBS, GARP⁺CD4⁺ T
414 cells, latent TGF-β1, or GARP⁺CD4⁺ T cells plus latent TGF-β1 (**Figure 4K**).
415 Compared with the PBS treated control group, the GARP⁺CD4⁺ T cells alone group,
416 or the latent TGF-β1 alone treatment group, the GARP⁺CD4⁺ T cells plus latent
417 TGF-β1 group exhibited a significantly higher AML burden and shorter survival time
418 (**Figure 4L-N**). Taken together, these findings demonstrated that GARP expressing T

419 cells induced NK cell dysfunction by activating TGF- β 1.

420

421 **Active TGF- β 1 levels during relapse correlate with impaired anti-leukemic
422 responses of BMNK cells *ex vivo***

423 Next, we examined whether BMNK cell-mediated anti-leukemic responses were
424 impaired and wanted to establish if active TGF- β 1 impaired anti-leukemic responses
425 in patients with relapsing AML. Firstly, we analyzed the proportion and number of
426 BMNK cells in our cohort. We observed that the overall percentage and number of
427 NK cells were significantly reduced in the bone marrow of AML patients with early
428 relapse compared to those without relapse (**Supplementary Figure 5A, B**).
429 Furthermore, the proportions of Ki-67 $^+$ BMNK cells were significantly lower in
430 patients with relapsed AML compared to those without AML relapse, but the
431 percentage of apoptotic NK cells was comparable between the two groups
432 (**Supplementary Figure 5C**). Moreover, the proportion of NK cells expressing
433 inhibitory NKG2A $^+$ and KLRG1 $^+$ receptors increased while those carrying the
434 activating NKG2C $^+$ CD94 $^+$ NK receptors decreased in relapsing AML patients
435 (**Supplementary Figure 5D, E**). In addition, TMRM $^+$ and MitoTracker Green $^+$
436 BMNK cells, as well as NKp30 expression were obviously lower in patients with
437 relapsed AML compared to those without relapse (**Supplementary Figure 5F-H**).

438 In co-culture experiments with HL60 target cells, the cytotoxicity of BMNK cells
439 purified from AML patients with early relapse was significantly decreased when
440 compared to NK cells from patients without relapse (**Figure 5A, B**). Moreover, the
441 proportion of polyfunctional effector NK cells all showed significant reductions in
442 relapsing patients (**Figure 5C-G**). This suggested that the anti-tumor effector function
443 of NK cells was compromised in AML patients with early relapse. The expression of
444 NKG2D by BMNK cells also decreased significantly in this group (**Figure 5H**).
445 Moreover, the proportion of polyfunctional effector NK cells showed a negative
446 correlation with the levels of active TGF- β 1 in the bone marrow (**Figure 5I**). We
447 performed IHC analysis using serial sections prepared from bone marrow biopsy
448 samples and found reduced numbers of NK cells and decreased levels of Granzyme B

449 in the bone marrow of AML patients with poor outcomes (**Figure 5J and K**).

450 Next, we analyzed the intracellular IFN- γ and Granzyme B levels by
451 immunofluorescence in purified BMNK cells after stimulation with IL-2 and IL-12.
452 Both Granzyme B and IFN- γ expression levels were significantly lower in the BMNK
453 cells from AML patients with early relapse compared to those from AML patients
454 without relapse (**Supplementary Figure 5I, J**). Taken together, these findings
455 showed that the anti-leukemic responses of NK cells were significantly impaired in
456 AML patients with early relapse and negatively correlated with the levels of activated
457 TGF- β 1 in the bone marrow microenvironment.

458

459 **Inhibition of active TGF- β 1 signaling restores anti-leukemic activity of NK 460 cells**

461 Next, we investigated if the inhibition of TGF- β 1/TGF- β R1 signaling could restore
462 the anti-leukemic activity of BMNK cells. A study by Homgaard et al. reported that
463 targeting the TGF- β 1 pathway with galunisertib, an inhibitor of TGF- β R1 in clinical
464 studies, promoted anti-tumor immunity of hepatocellular cancer (HCC) or pancreatic
465 cancer patients to immunotherapy²⁰. Therefore, we tested if treatment with this
466 compound could reverse the observed impairments in the anti-leukemic function of
467 BMNK cells from AML patients with early relapse. The cytotoxicity of BMNK cells
468 against primary AML blast cells was significantly improved, when galunisertib was
469 added to NK cells isolated from AML patients with early relapse (**Figure 6A-C**).
470 Specifically, *ex vivo* pretreatment with galunisertib restored both degranulation and
471 cytokine expression in the NK cells isolated from these AML patients (**Figure 6D, E**).
472 Galunisertib pretreatment significantly downregulated pSMAD2/3 expression while
473 substantially upregulating pS6 expression by BMNK cells of relapsing AML patients
474 (**Figure 6F, G**). Moreover, pretreatment with the compound significantly increased
475 mitochondrial membrane potential and mitochondrial mass (**Figure 6 F, G**). The
476 obtained data clearly indicates that galunisertib has the ability to increase mTOR
477 activity, restore mitochondrial homeostasis, and improve the effector function of
478 BMNK cells isolated from AML patients with early relapse.

479 We then verified whether the inhibition of TGF- β 1/TGF- β R1 signaling restored the
480 effector function of BMNK cells by abrogating the effects of active TGF- β 1.
481 Therefore, we pretreated naive NK cells with (1) latent TGF- β 1 or active TGF- β 1 plus
482 anti-TGF- β 1 antibody/galunisertib or (2) latent TGF- β 1 in the presence of
483 GARP $^+$ CD4 $^+$ T cells or GARP $^+$ CD4 $^+$ T cells plus anti-TGF- β 1 or galunisertib. Then,
484 the NK cells were co-cultured with primary AML blasts. We observed that anti-tumor
485 functions of NK cells were significantly reduced by active TGF- β 1, but restored by
486 treatment with galunisertib, which blocked TGF- β 1/TGF- β R1 signaling
487 (**Supplementary Figure 6A and B**).

488 To directly assess the ability of galunisertib to restore the anti-tumor activity of NK
489 cells *in vivo*, we used a leukemia xenograft model based on injecting
490 luciferase-labeled HL60 cells into NCG mice. After confirming engraftment, NK cells
491 were transferred into the mice and the mice were injected with GARP $^+$ CD4 $^+$ T cells,
492 GARP $^+$ CD4 $^+$ T cells in the presence of latent TGF- β 1, active TGF- β 1 or vehicle
493 (**Figure 6H**). These experiments demonstrated that both groups of mice receiving
494 GARP $^+$ CD4 $^+$ T cells in the presence of latent TGF- β 1 and receiving active TGF- β 1,
495 had significantly higher AML burden and shorter survival time (**Figure 6I-K**). In
496 addition, BLI indicated that in mice injected with active TGF- β 1, NK cells failed to
497 control tumor growth in the later stages of observation (day 35) (**Figure 6I-K**).
498 Importantly, we found that if the injection of GARP $^+$ CD4 $^+$ T cells in the presence of
499 latent TGF- β 1 or the injection of active TGF- β 1 was followed by the administration
500 of galunisertib, this significantly reduced tumor burden and prolonged survival
501 (**Figure 6H-K**). Our results indicated that the inhibition of TGF- β 1/TGF- β R1
502 signaling effectively restored impaired anti-leukemic responses of NK cells caused by
503 active TGF- β 1. Taken together, our findings demonstrated that the effector function
504 of NK cells was impaired by the presence of active TGF- β 1 in the bone marrow
505 microenvironment of AML patients with early relapse. We also showed that inhibition
506 of TGF- β 1 signaling by galunisertib could restore the anti-leukemic functions of NK
507 cells both *in vitro* and *in vivo*.

509 **Discussion**

510 Post-transplantation relapse is very frequent in AML patients and represents the
511 main cause of treatment failure and death. In this study, we demonstrated that active
512 TGF- β 1 levels are significantly increased in the bone marrow of AML patients
513 experiencing early relapse. Furthermore, we showed that active TGF- β 1 suppressed
514 the anti-leukemic action of BMNK cells by downregulating mTORC1 activity and
515 reducing mitochondrial respiration. We also demonstrated that inhibition of TGF- β 1
516 signaling at least partially restored the anti-leukemic effect of NK cells. Taken
517 together, these findings highlight a novel immune escape mechanism in the relapse of
518 AML following allogeneic transplantation.

519 The causes of immune cell dysfunction are dependent on the tumor type and tumor
520 microenvironment that can exhibit considerable individuality and multiformity. We
521 previously demonstrated that the aberrant expression of fructose-1,6-bisphosphatase
522 in NK cells lead to their dysfunction that was dependent on increased TGF- β 1
523 expression in the lung cancer microenvironment³⁶. Here, we found that active TGF- β 1
524 levels were significantly increased in the bone marrow of relapsing patients. The
525 impaired effector function of BMNK cells showed a correlation with the levels of
526 active TGF- β 1. Furthermore, BMNK cells isolated from patients with relapsed AML
527 showed dysfunctional mitochondrial metabolism. Additionally, treatment with active
528 TGF- β 1 significantly inhibited the mTOR activity, mitochondrial OXPHOS and
529 anti-tumor activity of BMNK cells.

530 Recently, GARP has been identified as a latent TGF- β 1-binding protein playing a
531 key role in regulating the bioavailability of TGF- β 1^{24,48}. In latent TGF- β 1 the mature
532 TGF- β 1 dimer remains noncovalently associated with the latency associated peptide
533 (LAP), preventing its binding to the TGF- β 1 receptor¹⁸. Activation of TGF- β 1
534 requires the release of mature TGF- β 1 from LAP, a critical step that can be mediated
535 by multiple mechanisms. The binding of latent TGF- β 1 to GARP requires the
536 formation of a disulfide bond with a key cysteine residue in the LAP. The association
537 of this complex with alpha-beta integrins ultimately releases mature, active TGF- β 1^{48,}
538 ⁴⁹. Our study showed an increased expression of GARP by bone marrow CD4 $^{+}$ T cells

539 in AML patients with early relapse. Furthermore, incubation of GARP⁺CD4⁺ T cells
540 with latent TGF-β1 significantly increased active TGF-β1 levels in the culture
541 supernatant, inhibiting mTOR and the anti-tumor activity of NK cells. Previous
542 reports indicated that the bone marrow is a reservoir for Treg cells, and the abundance
543 of these cells was linked to inadequate cytotoxic activity of immune cells, causing
544 poor survival in many cancer types^{43, 48, 50}. Activated TGF-β1 plays a crucial role in
545 Treg cell development and biology. The TGF-β1-triggered activation of Smad3
546 results in the formation of Smad3/Smad4 heterodimers that translocate to the nucleus
547 and bind to FOXP3 enhancer. This activation of Smad3 is essential for inducing
548 FOXP3 expression in naive CD4⁺ T cells, thus promoting their differentiation into
549 induced Treg cells with suppressive function^{48, 51}. Despite accumulating into GARP
550 activation on CD4⁺ T cells, and its possible role in Treg activation by activated
551 TGF-β1, more preclinical work is needed to clarify how GARP⁺ Treg cells may
552 regulate the anti-tumor effects of NK cells in the bone marrow microenvironment.

553 Furthermore, we observed that pre-treatment with galunisertib restored
554 mitochondrial function and the cytotoxic effector function of BMNK cells from
555 patients with relapsed AML. Importantly, we found that treatment with galunisertib
556 improved the anti-tumor capacity of NK cells in a leukemia xenograft mouse model.
557 Several studies have demonstrated that NK cell-based immunotherapy is a promising
558 treatment option in several cancers^{10, 52-54}. Our results suggested that enhancing the
559 function of BMNK cells by inhibiting TGF-β1/ TGF-βR1 may improve outcomes
560 after AML therapy and potentially prevent relapse following allo-HSCT.

561 The presented study has a few limitations. An increased proportion of
562 TGF-β1-producing NK cells have been reported in patients with breast cancer²⁹.
563 However, in our study, we did not investigate the role of autocrine TGF-β1 signaling
564 in relapse. Slattery et al. reported that GARP was constitutively overexpressed by NK
565 cells of some metastatic breast cancer patients. Moreover, targeting GARP/TGF-β1
566 complexes on NK cells isolated from such patients replicated the effects of TGF-β1
567 neutralization²⁹. However, in this study, we did not explore the relevance of GARP⁺
568 NK cells in the bone marrow of patients with AML relapse. Furthermore, the main

569 source of TGF- β 1 in the bone marrow of patients with relapsing AML currently
570 remains unknown, although several immune and non-immune cells can produce
571 TGF- β 1.

572 In conclusion, our results demonstrate that GARP-mediated active TGF- β 1 release
573 induces BMNK cell dysfunction in AML patients suffering early relapse
574 post-allo-HSCT, and that the inhibition of TGF- β 1 signaling by galunisertib could
575 restore the anti-tumor activity of NK cells both *in vitro* and *in vivo*. Thus, TGF- β 1
576 signaling inhibitor may have therapeutic application against tumors by restoring NK
577 cells. However, further studies will be required to ascertain the dosage, safety, and
578 efficacy of galunisertib in the treatment of AML patients.

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604

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606 data, and wrote the manuscript. D.W., X.Z., and Y.Z. performed the experiments and
607 interpreted the data. Y.L. and P.Y. helped to analyze the data. H.W., H.L., J.J. and H.Z.
608 helped to collected whole-blood samples and patient information. R.S., Y.W., B.F. and
609 Z.T established techniques of flow cytometry and interpreted the data. Z.S., X.Z. and
610 H.W. designed the study, supervised the research, and revised the manuscript.

611

612 **Disclosure of Conflicts of Interest:** The authors have declared that no conflict of
613 interest exists.

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758 **Figure Legends**

759 **Figure 1. Active TGF- β 1 levels are upregulated significantly in the bone marrow**
760 **of AML patients with early relapse after allo-HSCT.** (A) Kaplan-Meier analyses of
761 overall survival rates of AML patients from the Gene Expression Profiling Interactive
762 Analysis (GEPIA) dataset according to TGF- β 1 expression levels. High TGF- β 1
763 expression ($n = 87$); Low TGF- β 1 expression ($n = 86$). Median value was used as the
764 cutoff. P value was calculated by log-rank test. (B) Representative confocal
765 microscopy images showing the levels of active TGF- β 1 in bone marrow
766 mononuclear cells (BMMCs) isolated from AML patients with early relapse (left) or
767 without relapse (right) after allo-HSCT. Scale bar, 50 μ m. (C) Mean fluorescence
768 intensity (MFI) of active TGF- β 1 in randomly selected single BMMCs from AML
769 patients with early relapse (red; $n = 5$ patients) or without relapse (blue; $n = 7$
770 patients). Each dot represents MFI of active TGF- β 1 in a single cell of the two groups;
771 the number of cells were 65 and 31, respectively. (D) Western blotting analysis
772 showing the levels of active TGF- β 1 in the BMMCs of AML patients with early
773 relapse ($n = 3$) or without relapse ($n = 3$). (E) ELISA or cytometric bead array (CBA)
774 results showing the levels of total TGF- β 1, active TGF- β 1, IL-10, IL-4, and IFN- γ in
775 the bone marrow of AML patients with early relapse ($n = 16$) or without relapse ($n =$
776 22). (F) Representative immunohistochemical (IHC) images showing the staining
777 intensity for active TGF- β 1 in bone marrow biopsy samples from AML patients with
778 early relapse or without relapse. Scale bars, 100 μ m. (G) Functional enrichment
779 analyses of differentially expressed genes (DEGs) between control and active
780 TGF- β 1-treated NK cells indicating the most enriched biological processes. (H) Heat
781 maps show normalized expression levels of genes regulating NK cell-mediated
782 cytotoxicity (left) and NK cell activation (right) in purified BMNK cells pre-treated
783 with DMSO (solvent control), latent TGF- β 1 (10 ng/mL), or active TGF- β 1 (10
784 ng/mL). Each column depicts one sample. The data in C and E were analyzed by
785 two-tailed unpaired Student's t-test; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$;
786 **** $P < 0.0001$. Data are represented as means \pm SD.

788 **Figure 2. Active TGF- β 1 inhibits effector function, mTORC1 activity, and**
789 **mitochondrial respiration of bone marrow derived NK cells *ex vivo*.** (A) Flow
790 cytometry analysis showing the percentage of 7-AAD⁺ HL60 cells (target cells) when
791 co-cultured for 5 h with control (DMSO) NK cells, 10 ng/ml latent TGF- β 1-treated
792 NK cells, and 10 ng/ml active TGF- β 1-treated NK cells to estimate cytotoxicity. NK
793 cells: target cells ratio = 5:1; $n = 20$. (B and C) Flow cytometry data indicating the
794 proportion of IFN- γ ⁺TNF- α ⁺ NK cells, IFN- γ ⁺Granzyme B⁺ NK cells, IFN- γ ⁺
795 CD107a⁺ NK cells, and Granzyme B⁺ CD107a⁺ NK cells within the total population
796 of control NK cells, latent TGF- β 1-treated NK cells, and active TGF- β 1-treated NK
797 cells that were co-cultured with HL60 cells for 5 h. $n = 20$. (D and E) Flow cytometry
798 analysis showing the proportion of (D) pSMAD2/3⁺ and (E) pS6⁺ NK cell populations
799 in the control (black) and active TGF- β 1-treated (red) BMNK cells. $n = 15$. (F and G)
800 Oxygen consumption rates (OCR) of control and active TGF- β 1-stimulated NK cells
801 under basal conditions and in response to oligomycin (Oligo), the mitochondrial
802 decoupler FCCP, and rotenone + antimycin (R + A). (G) Estimation of OCR values
803 (OXPHOS activity) under basal conditions (left) and maximum respiration rates after
804 FCCP uncoupling (right). OCR were analyzed for 9 donors per group. (H and I) Flow
805 cytometry analysis indicating the proportion of (H) TMRM⁺ and (I) MitoTracker
806 Green⁺ NK cells in the control (black) and active TGF- β 1-treated (red) groups of
807 BMNK cells. $n = 15$. Data were analyzed by one-way ANOVA with Tukey's multiple
808 comparisons test (A, C) or two-tailed paired Student's t-test (E, I); * $P < 0.05$;
809 ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. The data are represented as means \pm SD.
810

811 **Figure 3. Surface expression levels of GARP on CD4⁺ T cells correlate positively**
812 **with active TGF- β 1 levels in the bone marrow of relapsing AML patients.** (A)
813 Kaplan-Meier analyses of overall survival rates of AML patients from the GEPIA
814 dataset according to GARP expression levels. High GARP expression ($n = 87$); Low
815 GARP expression ($n = 86$). Median value was used as the cutoff. P value was
816 calculated by log-rank test. (B and C) Flow cytometry analysis indicating GARP
817 expression on BMMCs isolated from AML patients with early relapse (red; $n = 17$) or

818 without relapse (blue; $n = 22$). (D and E) Flow cytometry analysis showing GARP
819 expression levels on CD4 $^{+}$ T cells (left) and platelets (right) from the bone marrow of
820 AML patients with early relapse (red; $n = 9$) or without relapse (blue; $n = 19$). (F and
821 G) Flow cytometry analysis showing GARP expression levels on NK cells (left) and
822 CD8 $^{+}$ T cells (right) from the bone marrow of AML patients with early relapse (red; n
823 = 9) or without relapse (blue; $n = 19$). (H-L) Spearman's rank correlation analysis
824 showing the relationship between active TGF- β 1 levels and the proportion of GARP $^{+}$
825 lymphocytes or the MFI corresponding to the density of GARP as shown by the
826 Spearman correlation coefficients (r) and P values. The data in C, E, and G were
827 analyzed by two tailed unpaired Student's t-test; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$;
828 **** $P < 0.0001$. The data are represented as means \pm SD.

829

830 **Figure 4. GARP $^{+}$ CD4 $^{+}$ T cells induce TGF- β 1 activation that inhibits NK
831 cell-mediated anti-tumor effects both *in vitro* and *in vivo*.** (A-C) Flow cytometry
832 data indicating the percentage of 7-AAD $^{+}$ HL60 cells (target cells) co-cultured for 5 h
833 with BMNK cells pre-treated with DMSO (solvent control; black), latent TGF- β 1 (10
834 ng/mL; blue), latent TGF- β 1 (10 ng/mL) plus GARP $^{-}$ CD4 $^{+}$ T cells (purple),
835 GARP $^{+}$ CD4 $^{+}$ T cells (green), or latent TGF- β 1 (10 ng/mL) plus GARP $^{+}$ CD4 $^{+}$ T cells
836 (red) to estimate cytotoxicity. The NK cells: target cells ratio = 5:1; the ratio of NK
837 cells: GARP $^{-}$ CD4 $^{+}$ T cells or GARP $^{+}$ CD4 $^{+}$ T cells was 5:1. $n = 10$. (D-G) Flow
838 cytometry analysis showing the proportion of (D) CD107a $^{+}$ IFN- γ $^{+}$ NK cells, (E)
839 Granzyme B $^{+}$ CD107a $^{+}$ NK cells, and (F) Granzyme B $^{+}$ IFN- γ $^{+}$ NK cells within the
840 total BMNK cell population that was pre-treated with either DMSO (solvent control;
841 black), latent TGF- β 1 (10 ng/mL; blue), latent TGF- β 1 (10 ng/mL) plus GARP $^{-}$ CD4 $^{+}$
842 T cells (purple), GARP $^{+}$ CD4 $^{+}$ T cells (green), or latent TGF- β 1 (10 ng/mL) plus
843 GARP $^{+}$ CD4 $^{+}$ T cells (red) and co-cultured with HL60 cells for 5 hours. $n = 10$. (H)
844 ELISA results indicating the levels of active TGF- β 1 in the supernatants of co-culture
845 experiments described in D-G. (I and J) Flow cytometry analysis showing NKp30
846 (Left, I) and NKG2D (Right, I) expression levels on BMNK cells pre-treated with
847 DMSO (solvent control), latent TGF- β 1 (10 ng/mL), latent TGF- β 1 (10 ng/mL) plus

848 GARP⁻CD4⁺ T cells, GARP⁺CD4⁺ T cells, or latent TGF- β 1 (10 ng/mL) plus
849 GARP⁺CD4⁺ T cells. $n = 10$. (K) Experimental design: NCG mice were injected into
850 the tail vein with 5×10^5 HL60 cells stably expressing luciferase. After confirmation of
851 engraftment by bioluminescence imaging (BLI) on day 7, 2.5×10^6 NK cells were
852 transferred to all the mice via tail vein in combination with the injection of PBS
853 control (50 μ L, i.p.; QW), 5×10^5 GARP⁺CD4⁺ T cells, latent TGF- β 1 (50 μ L, 5 ng/ml;
854 i.p.; QW), or 5×10^5 GARP⁺CD4⁺ T cells in the presence of latent TGF- β 1. AML
855 burden was monitored by BLI at the indicated time points. (L) BLI of AML burden.
856 (M) AML burden was quantified as the average value of the total flux (p/s). $n = 6$
857 mice per group. (N) Kaplan–Meier survival curve of mice bearing HL60 cell-derived
858 tumors. Statistical significance was determined by log-rank Mantel–Cox test. $n = 6$
859 mice per group. The data in B, C, G, H, J and M were analyzed by one-way ANOVA
860 with Tukey’s multiple comparisons test; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$;
861 **** $P < 0.0001$. The data are represented as means \pm SD.

862

863 **Figure 5. Higher levels of active TGF- β 1 are associated with impaired**
864 **anti-leukemic responses of NK cells in the bone marrow of patients with relapsed**
865 **AML.** (A and B) Flow cytometry analysis indicating the percentage of 7-AAD⁺ HL60
866 cells (target cells) co-cultured for 5 h with NK cells isolated from the bone marrow of
867 AML patients with early relapse (red; $n = 13$) or without relapse (blue; $n = 21$). NK
868 cell: target cell ratio = 5:1. (C-G) Flow cytometry data of the proportion of (C) IFN- γ ⁺
869 TNF- α ⁺ NK cells, (D) IFN- γ ⁺ CD107a⁺ NK cells, (E) IFN- γ ⁺ Granzyme B⁺ NK cells,
870 and (F) Granzyme B⁺ CD107a⁺ NK cells among the total NK cells purified from the
871 bone marrow of AML patients with early relapse (red; $n = 13$) or without relapse (blue;
872 $n = 21$) after co-culture with HL60 cells for 5 h. (H) Flow cytometry analysis showing
873 NKG2D expression on NK cells isolated from the bone marrow of AML patients with
874 early relapse (red; $n = 22$) or without relapse (blue; $n = 28$). (I) Spearman’s rank
875 correlation analysis shows the relationship between active TGF- β 1 levels and the
876 proportion of different immune cell types and indicated molecules as shown by the
877 Spearman correlation coefficients (r) and P values. The data in B, G, and H were

878 analyzed by two-tailed unpaired Student's t-test; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$;
879 **** $P < 0.0001$. The data are represented as means \pm SD. (J and K) Representative
880 IHC images show the staining intensity for CD56, Granzyme B, and IFN- γ in the
881 bone marrow biopsy samples from AML patients with early relapse (J) or without
882 relapse (K). Scale bars, 100 μ m.

883

884 **Figure 6. Inhibition of TGF- β 1 signaling restores anti-leukemic activity of NK**
885 **cells.** (A-C) Flow cytometry analysis showing the percentage of 7-AAD $^+$ primary
886 AML blasts and Annexin V $^+$ primary AML blasts (target cells) when co-cultured for
887 5 h with NK cells purified from the bone marrow of AML patients with early relapse.
888 The BMNK cells were pretreated with DMSO (solvent control) or galunisertib (10
889 μ M) for 24 h before co-culture. NK cell: target cell ratio = 5:1. (D and E) Flow
890 cytometry analysis indicating the proportions of IFN- γ $^+$ Granzyme B $^+$ NK cells,
891 IFN- γ $^+$ TNF- α $^+$ NK cells, Granzyme B $^+$ CD107a $^+$ NK cells, IFN- γ $^+$ CD107a $^+$ NK
892 cells among control and galunisertib-treated NK cells isolated from the bone marrow
893 of AML patients with early relapse. $n = 15$. (F and G) Flow cytometry analysis
894 showing the proportion of pSMAD2/3 $^+$ NK cells (upper left), pS6 $^+$ NK cells (upper
895 right), TMRM $^+$ NK cells (lower left), and MitoTracker Green $^+$ NK cells (lower right)
896 among control and galunisertib-treated NK cells isolated from the bone marrow of
897 AML patients with early relapse. $n = 15$. (H) Experimental scheme: NCG mice were
898 injected with 5×10^5 HL60 cells stably expressing luciferase into the tail vein. After
899 confirmation of engraftment by BLI on day 7, 2.5×10^6 NK cells were transferred to all
900 the mice via tail vein in combination with vehicle control, 5×10^5 GARP $^+$ CD4 $^+$ T cells,
901 5×10^5 GARP $^+$ CD4 $^+$ T cells in the presence of latent TGF- β 1 (50 μ l, 5 ng/ml; i.p.; QW),
902 active TGF- β 1 (50 μ l, 5 ng/ml; i.p.; QW) in combination with vehicle control, 5×10^5
903 GARP $^+$ CD4 $^+$ T cells in the presence of latent TGF- β 1 and galunisertib at 75 mg/kg
904 twice daily (BID) by oral gavage for 21 days, or active TGF- β 1 in combination with
905 galunisertib (75 mg/kg; BID for 21 days) for treatment. AML burden was monitored
906 by BLI at the indicated time points. (I) BLI of AML burden. (J) AML burden was
907 quantified as the average value of the total flux (p/s). $n = 6$ mice per group. (K)

908 Kaplan–Meier survival curve of mice bearing HL60 cell-derived tumors. Statistical
909 significance was determined by log-rank Mantel–Cox test. $n = 6$ mice per group. The
910 data in B, C, E and G were analyzed by two-tailed paired Student’s t-test; The data in
911 J were analyzed by one-way ANOVA with Tukey’s multiple comparisons test.
912 * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. The data are represented as
913 means \pm SD.

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Figure 1

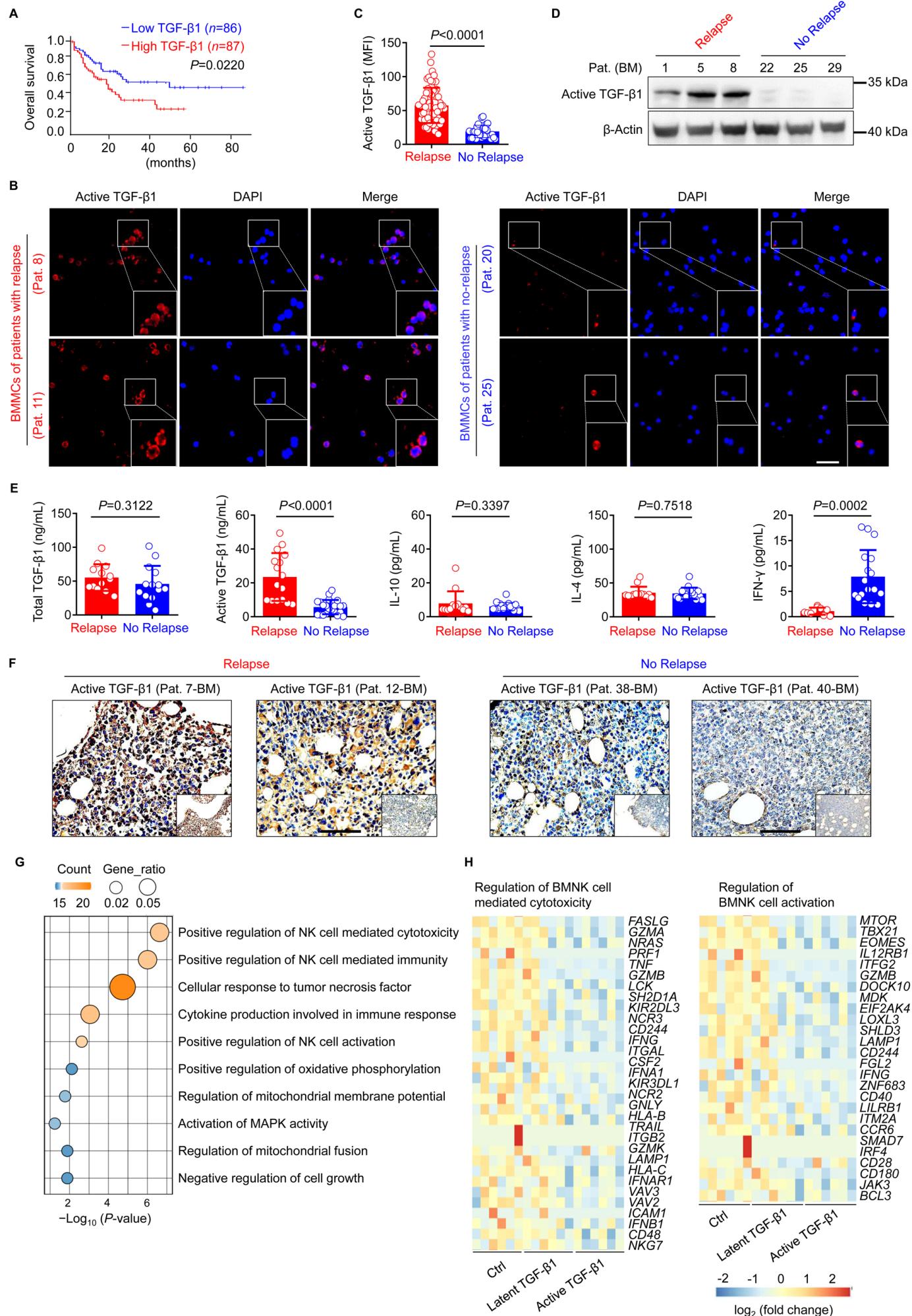


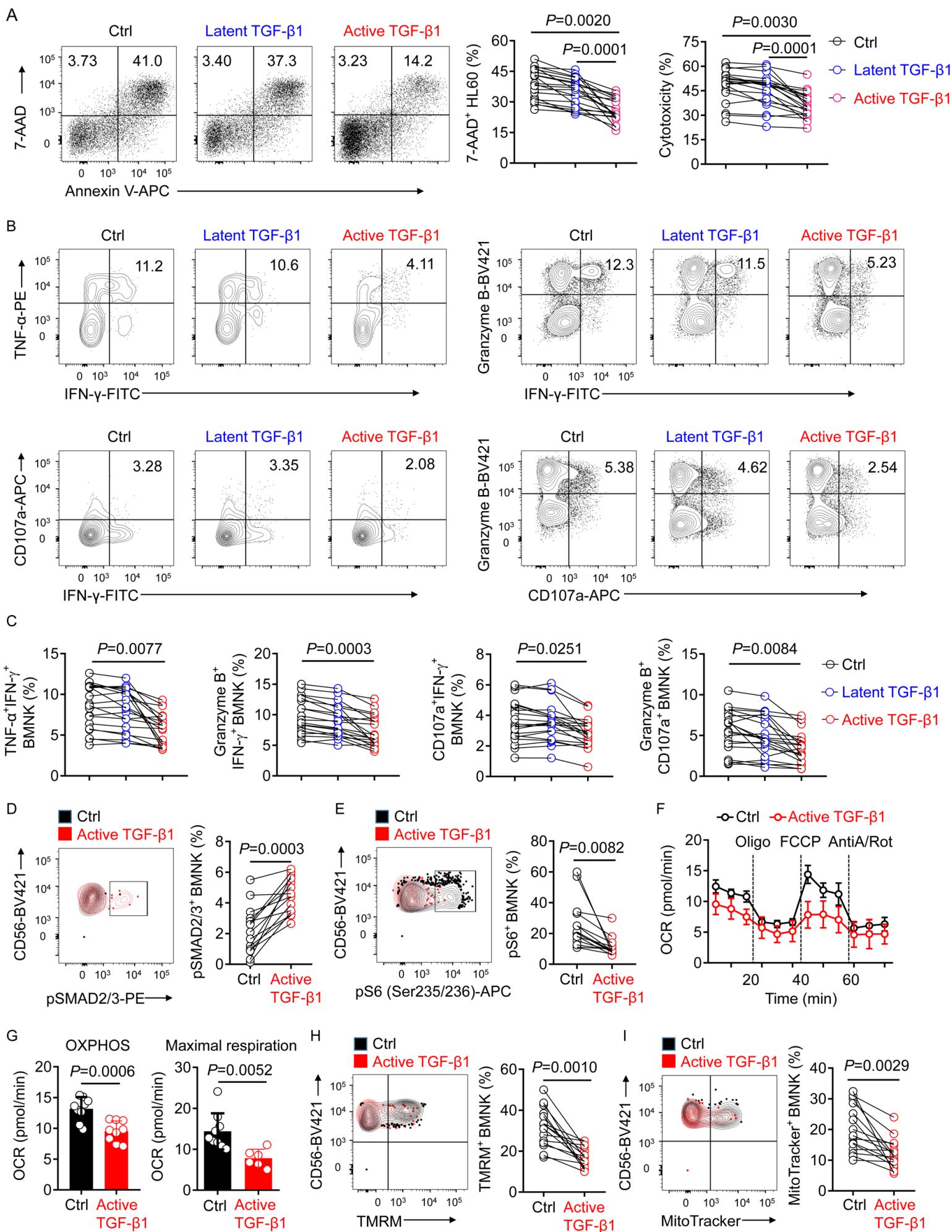
Figure 2

Figure 3

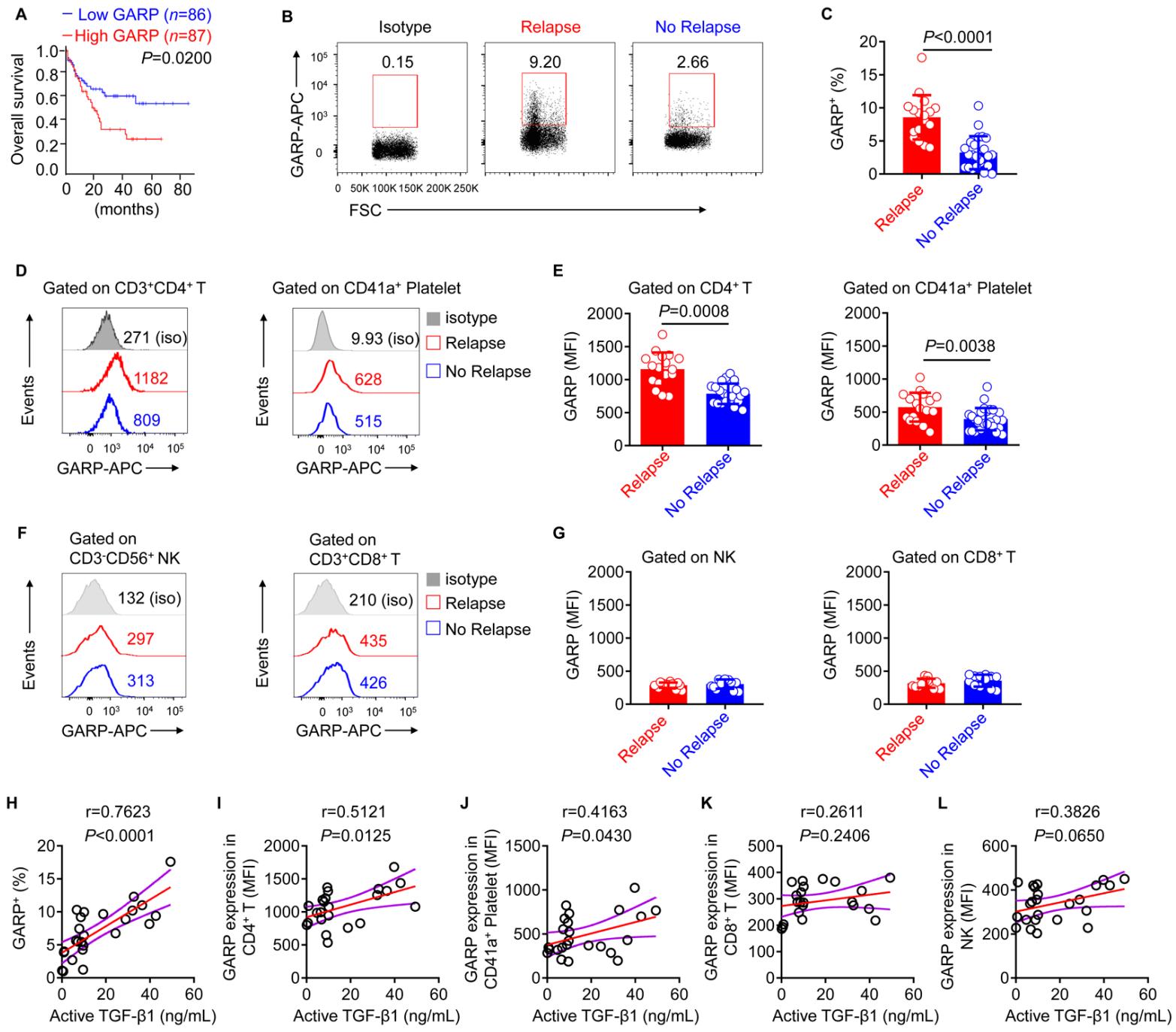


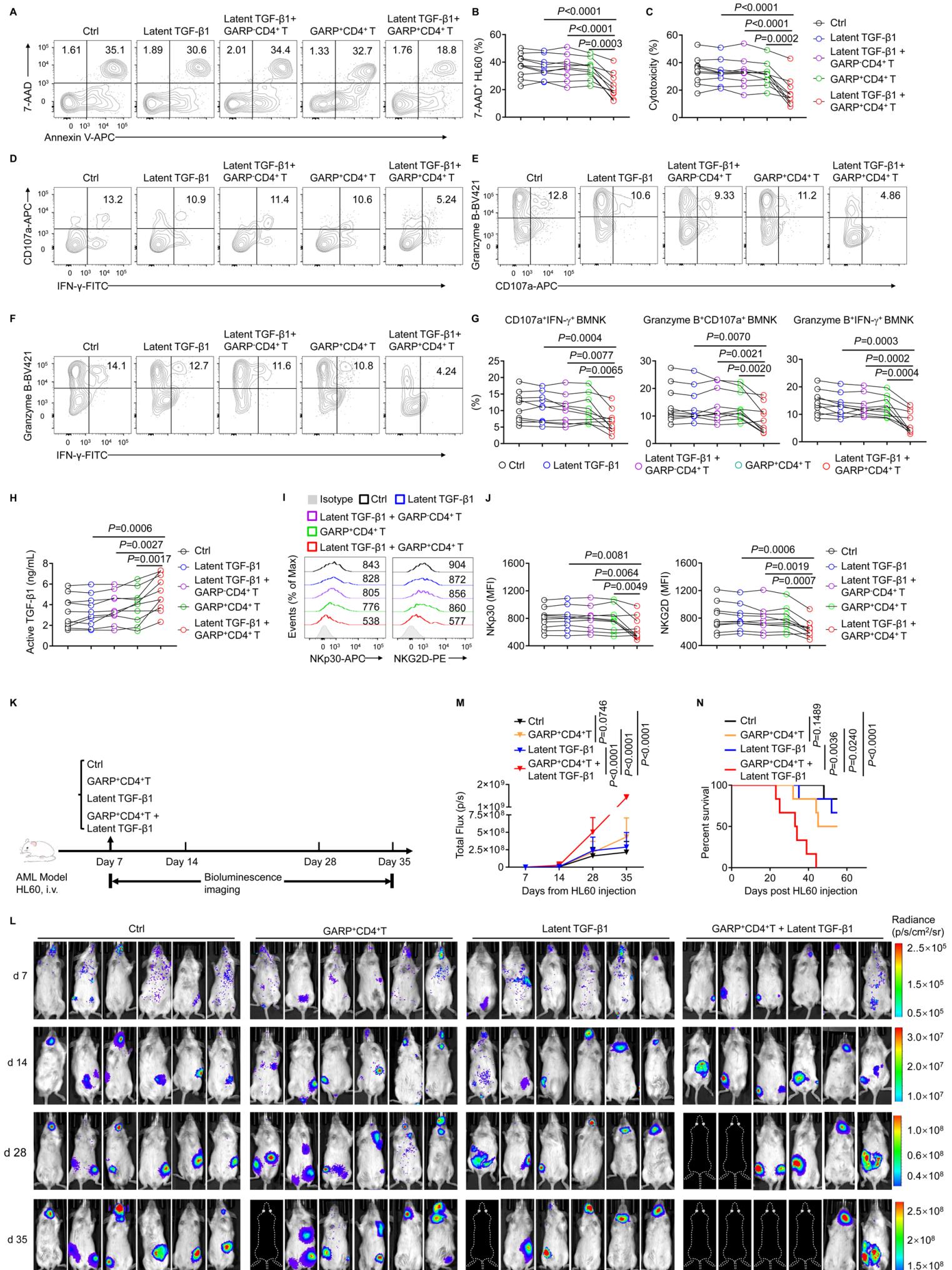
Figure 4

Figure 5

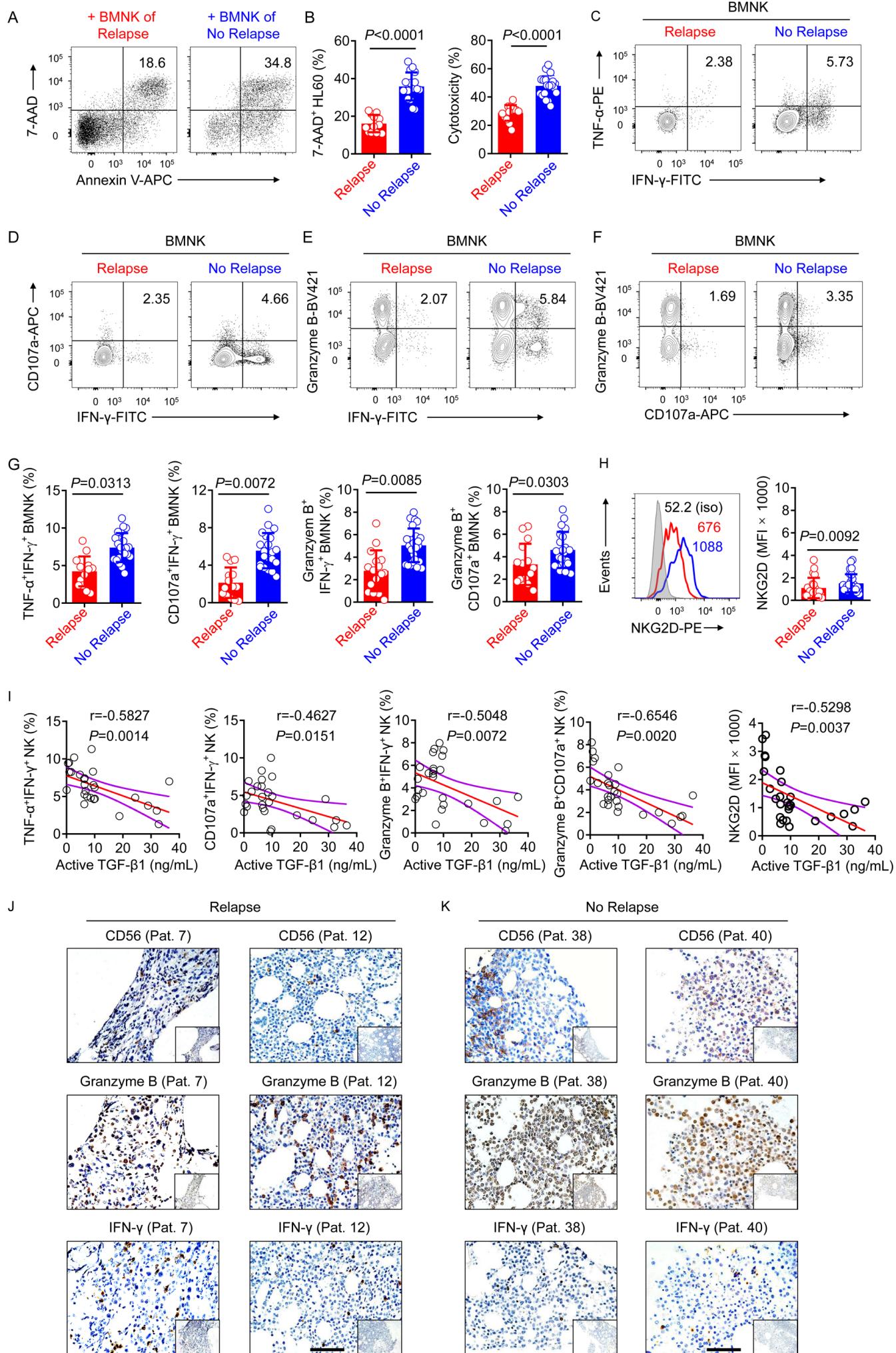


Figure 6