



Title	Tumor-derived interleukin-34 creates an immunosuppressive and chemoresistant tumor microenvironment by modulating myeloid-derived suppressor cells in triple-negative breast cancer
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1 **Tumor-derived interleukin-34 creates an immunosuppressive and**
2 **chemoresistant tumor microenvironment by modulating myeloid-**
3 **derived suppressor cells in triple-negative breast cancer**

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28

29 **ABSTRACT**

30 Triple-negative breast cancer (TNBC) is an aggressive breast cancer subtype
31 characterized by a lack of therapeutic targets. The paucity of effective treatment options
32 motivated a number of studies to tackle this problem. Immunosuppressive cells infiltrated
33 into the tumor microenvironment (TME) of TNBC are currently considered as candidates
34 for new therapeutic targets. Myeloid-derived suppressor cells (MDSCs) have been
35 reported to populate in the TME of TNBC, but their roles in the clinical and biological
36 features of TNBC have not been clarified. This study identified that interleukin-34 (IL-
37 34) released by TNBC cells is a crucial immunomodulator to regulate MDSCs
38 accumulation in the TME. We provide evidence that IL-34 induces a differentiation of
39 myeloid stem cells into monocytic MDSCs (M-MDSCs) that recruits regulatory T (Treg)
40 cells, while suppressing a differentiation into polymorphonuclear MDSCs (PMN-
41 MDSCs). As a result, the increase in M-MDSCs contributes to the creation of an
42 immunosuppressive TME, and the decrease in PMN-MDSCs suppresses angiogenesis,
43 leading to an acquisition of resistance to chemotherapy. Accordingly, blockade of M-
44 MDSC differentiation with an estrogen receptor inhibitor or anti-IL-34 monoclonal
45 antibody suppressed M-MDSCs accumulation causing retardation of tumor growth and
46 restores chemosensitivity of the tumor by promoting PMN-MDSCs accumulation. This

47 study demonstrates previously poorly understood mechanisms of MDSCs-mediated
48 chemoresistance in the TME of TNBC, which is originated from the existence of IL-34,
49 suggesting a new rationale for TNBC treatment.

50

51 **KEYWORDS**

52 Interleukin-34, Triple-negative breast cancer, Myeloid-derived suppressor cells, 4T1,
53 Tumor microenvironment, Chemotherapy

54

55 **STATEMENTS AND DECLARATIONS**

56 **Competing Interests**

57 The authors declare no potential conflicts of interest

58

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65

66 **Ethics approval and consent to participate**

67 All animal procedures were approved by the Animal Care Committee of Hokkaido
68 University (Approval number: 19-0094). All human clinical research was performed
69 under the Declaration of Helsinki and was approved by the Clinical Research
70 Administration Center of Hokkaido University Hospital (Registration number: 022-0009)
71 and the Institutional Review Board of Osaka Rosai Hospital (Registration number: 31-18,
72 2020-41, 20210125). Informed consent was obtained from all individual subjects before
73 tissue acquisition.

74

75 **Authors' contributions**

76 NK and KS designed the study. NK, TK, AH, SI, and JNK performed experiments. SI,
77 TO, and MT obtained clinical samples. All authors analyzed data and discussed the results.
78 NK, RO, and KS contributed to manuscript preparation. All authors approved the final
79 version of this manuscript for publication.

80

81 INTRODUCTION

82 Breast cancer is the most common cancer and a major cause of cancer-related death
83 among women worldwide.[1] It is reported that the average risk to develop breast cancer
84 in normal women's life is as many as 12%.[2] According to Global Cancer Statistics 2020:
85 GLOBOCAN 2020, the incidence and mortality of female breast cancer in 2020
86 worldwide were 2,261,419 new cases and 684,996 deaths, respectively.[1] Breast cancer
87 is a highly heterogeneous disease with varying biological and clinical characteristics,
88 which therefore makes treatment methods and disease prognosis diverse among subtypes.
89 To distinguish the subtype of breast cancer, immunohistochemical (IHC) staining of
90 breast cancer tumors is commonly used and can classify into four subtypes based on the
91 expression of estrogen receptor (ER), progesterone receptor (PgR), and human epidermal
92 growth factor receptor-related 2 (HER2). Among the subtypes, triple-negative breast
93 cancer (TNBC) is characterized by the lack of these three receptors and accounts for 15-
94 20% of all breast cancer.[3] TNBC typically shows a high degree of aggressiveness, poor
95 prognosis, and a high rate of relapse.[4] Furthermore, it does not respond towards targeted
96 treatment option except for surgery.[5] However, most TNBC patients are less susceptible
97 to chemotherapy or become resistant to it during treatment.[6] Thus, chemoresistance is
98 a critical problem that needs to be addressed for successful TNBC treatment.

99 In the past few decades, many studies have attempted to reveal the mechanism
100 of inducing chemoresistance.[7][8][9] Most of them have emphasized the intrinsic ones,
101 which occur inside cancer cells, such as apoptotic avoidance machinery or DNA repair
102 mechanisms.[10][11] On the other hand, a lot of papers with recent advances have shown
103 that extrinsic mechanisms are also important.[12][13] Its representative example is an
104 interaction between tumor and immune cells within the tumor microenvironment (TME).
105 The TME contains various immune system elements including myeloid cells,
106 lymphocytes, and blood vessels. In particular, tumor-associated macrophages (TAMs)
107 and myeloid-derived suppressor cells (MDSCs) constitute the dominant immune cell
108 population in various tumors and play critical roles in multiple aspects of TME.[5][14]
109 Importantly, an increase of TAMs and MDSCs infiltration into tumors is a hallmark of
110 developing chemoresistance and correlates with poor prognosis.[15][16][17] Indeed,
111 these myeloid cells in the TME are involved in many parts of pro-tumorigenic processes,
112 including angiogenesis, immunosuppression, and tumor progression, and it is known that
113 they are frequently observed in TNBC tumors.[5] However, the mechanism of TAMs- or
114 MDSCs-mediated chemoresistance acquisition in TNBC has not been elucidated in detail.

115 Accumulating evidence has indicated that tumor-derived interleukin-34 (IL-34)
116 can be a key molecule that causes chemoresistance.[4][18] Indeed, the importance of IL-

117 34 in chemoresistance is supported by the findings in a lung cancer study indicating that
118 exposure of chemotherapeutic agents to cancer cells increases their production of IL-
119 34.[19] In addition, IL-34 has been shown to induce immunosuppressive myeloid cells
120 like TAMs and thus to be a possible linker between immunosuppressive TME and
121 chemoresistance.[18][19][20] On the other hand, we have previously reported that IL-34
122 was expressed significantly higher and more frequently in TNBC than other breast cancer
123 subtypes.[4] IL-34 was an independent poor prognosis factor in TNBC, and patients
124 bearing IL-34 high-expressing TNBC showed a low survival rate.[4] From these data
125 described above, it is anticipated that IL-34 may play an important role to induce
126 chemoresistance in TNBC.

127 Based on these backgrounds, we have investigated the relationship between IL-
128 34 and chemoresistance in TNBC. We firstly identified that, in TNBC tumors, IL-34
129 preferably induces MDSCs but not TAMs, and the IL-34-MDSCs axis contributes to
130 chemoresistance. It is notable that blocking of IL-34 or MDSCs had a therapeutic
131 potential to remedy the chemoresistance in TNBC. We here propose IL-34 as a molecular
132 driver of chemoresistance in TNBC and suggest a novel therapeutic strategy.

133

134 **MATERIALS AND METHODS**

135 **Cell lines**

136 The TNBC cell line 4T1 was obtained from the American Type Culture Collection
137 (ATCC) and cultured in RPMI-1640 (Fujifilm Wako Pure Chemical). Culture media was
138 supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich), 100 U/ml penicillin
139 (Nacalai Tesque), 100 µg/ml streptomycin (Nacalai Tesque), 0.1 mM MEM nonessential
140 amino acids (Nacalai Tesque), and maintained at 37°C with 5% CO₂. Mycoplasma
141 contamination of cell lines was checked using the MycoAlert™ Mycoplasma Detection
142 Kit (Lonza), according to the manufacturer's instructions; no evidence of contamination
143 was found.

144

145 **Generation of IL-34 knockout (KO) cell line**

146 IL-34 KO 4T1 cell line was generated using mL-34 CRISPR/Cas9 KO plasmid (Santa
147 Cruz Biotechnology). The plasmid was transfected into 2×10⁶ cells using the Neon®
148 Transfection System (Thermo Fisher Scientific), and the cells were incubated for 24 hours.
149 After incubation, successful transfection of the plasmid was visually confirmed by the
150 detection of green fluorescent protein (GFP) via fluorescent microscopy ObserverZ1
151 (Carl Zeiss AG). Plasmid-incorporated cells were selected by the GFP expression using

152 FACS Aria cell sorter (BD Biosciences) 48 hours after transfection. Then, puromycin (2
153 $\mu\text{g/ml}$) was added to the medium and cultured for 48 hours to select the transfected cells.

154

155 **Cell proliferation assay**

156 Ctrl and IL-34 KO 4T1 cells (3×10^5) were seeded in 10 cm culture dishes. The number
157 of cells was measured using a hemocytometer from day 1 to 3. When the cells were
158 counted, they were stained with trypan blue, and the blue-stained cells were excluded
159 from the total cell count.

160

161 **Mouse experiments**

162 Six to seven weeks old female BALB/c and MMTV-PyMT (FVB/N-Tg (MMTV-PyVT)
163 634Mul/J) mice were purchased from Japan SLC and The Jackson Laboratory,
164 respectively. The mice were maintained under specific pathogen-free conditions and
165 housed in 12 hours light/12 hours dark cycle in the animal facility at Hokkaido University.
166 For *in vivo* assay, 1×10^5 4T1 cells were inoculated subcutaneously into the right flank of
167 syngeneic female mice. Anti-IL-34 antibody (clone: C054-35) ($200 \mu\text{g/mouse}$), paclitaxel
168 (PTX) (1.3 mg/kg), and/or methylpiperidino pyrazole (MPP) (1.0 mg/kg) were
169 intraperitoneally (i.p.) administered when tumor size reached 5 mm in diameter. The anti-

170 IL-34 antibody, PTX, and MPP were purchased from BioLegend, Nippon Kayaku, and
171 Cayman Chemical, respectively. Tumor size was measured by caliper three times a week.
172 The 4T1 tumors were collected 14 days after the cell inoculation. Before the tumor
173 recovery, for histological analysis and immunohistochemical (IHC) staining, mice
174 underwent perfusion fixation. All animal experiments were approved by the Animal Care
175 Committee of Hokkaido University (Approval number: 19-0094).

176

177 **Isolation of tumor-infiltrating immune cells from solid tumor**

178 According to the manufacturer's instructions, the isolation of tumor-infiltrating immune
179 cells from solid tumors was performed by using BD Horizon Dri Tumor and Tissue
180 Dissociation Reagent (BD Biosciences). The recovered tumor-infiltrating immune cells
181 were analyzed by flow cytometry.

182

183 ***In vitro* differentiation of bone marrow-derived MDSCs**

184 Bone marrow cells were isolated from the femora of female BALB/c mice and depleted
185 of red blood cells with ACK lysis buffer. 1×10^6 bone marrow cells were cultured in 2 ml
186 of RPMI-1640 culture media supplemented with 10% FBS, 100 U/ml penicillin, 100
187 $\mu\text{g/ml}$ streptomycin, 50 ng/ml recombinant GM-CSF (BioLegend), with/without 50

188 ng/ml recombinant IL-34 (BioLegend). Cell cultures in 6-well plates were maintained at
189 37°C with 5% CO₂. Cells were collected on day 3 and analyzed by flow cytometry.

190

191 **Collection of breast cancer patient-derived samples**

192 For constructing human breast cancer organoids, we prepared breast cancer tissues
193 obtained from breast cancer patients who underwent core needle biopsy under
194 ultrasonographic guidance at Hokkaido University Hospital. For IHC examination of
195 human TNBC tissues, 35 patients with Stages I–III TNBC were recruited retrospectively
196 at Osaka Rosai Hospital, as previously described.[21] These patients underwent core
197 needle biopsy under ultrasonographic guidance. Tumor biopsy samples were fixed in 10%
198 buffered formaldehyde for histological analysis. Informed consent was obtained from all
199 subjects before tissue acquisition, and organoid experiments and IHC examination
200 utilizing patient-derived breast cancer tissues were approved by the Clinical Research
201 Administration Center of Hokkaido University Hospital (Registration number: 022-0009)
202 and the Institutional Review Board of Osaka Rosai Hospital (Registration number: 31-18,
203 2020-41, 20210125), respectively. All experiments utilizing human material were
204 conducted according to the Declaration of Helsinki.

205

206 **Histological analysis**

207 The tumors were fixed in 4-10% Paraformaldehyde (Fujifilm Wako Pure Chemical) for
208 24 hours at 4°C. Tumors were dehydrated through 70-100% ethanol and immersed into
209 xylene, then embedded in paraffin. The embedded samples were sliced, and 5 µm thick
210 sections were obtained. For hematoxylin and eosin (H&E) staining, sections were
211 deparaffinized, and immersed into H&E, then observed with a light microscope BX53F
212 (Olympus).

213

214 **IHC staining**

215 Sections were deparaffinized, and endogenous peroxidase was blocked by 0.3 % H₂O₂ in
216 distilled water for 20 minutes. For FOXP3 staining, antigen retrieval was performed for
217 1 minute by boiling the sections in citrate buffer. Sections were then incubated with
218 BlockAce (DS Pharma Biomedical) in PBS for 1 hour to block non-specific reactions.
219 After protein blocking, the mouse sections were incubated with rat anti-mouse CD4
220 (1:100, #13-9766-80, Thermo Fisher Scientific), FOXP3 (1:100, #14-5773-80, Thermo
221 Fisher Scientific), CD31 (1:500, #550274, BD Pharmingen), or Gr-1 (1:200, #108401,
222 BioLegend) antibody in PBS overnight at room temperature. Regarding the human
223 sections, incubated with mouse anti-human IL-34 (1:4000, #MABT493, Sigma Aldrich),

224 FOXP3 (1:200, #14-4777-82, Thermo Fisher Scientific), CD14 (1:500, #75181, Cell
225 Signaling Technology), CD15 (1:200, #555400, BD Pharmingen), or CD31 (1:1000,
226 #11265-1-AP, Proteintech) antibody in PBS overnight at room temperature. Then, the
227 sections were incubated with biotinylated donkey anti-rat/mouse IgG (Jackson
228 ImmunoResearch) in PBS as the secondary antibody for 1 hour at room temperature,
229 followed by incubation using an ABC Elite kit (Vector Laboratories) for 1 hour. Finally,
230 the sections were incubated with 3, 3'-diaminobenzidine tetrahydrochloride (Fujifilm
231 Wako Pure Chemical) in Tris-HCl containing H₂O₂ for 5-20 minutes and counterstained
232 with hematoxylin. Sections were then enclosed using toluene. The observation was
233 performed using a light microscope BX53F (Olympus).

234

235 **Generation of human breast cancer organoids and IL-34 inhibitory experiment**

236 Within 30 minutes of acquisition, breast tissue was minced, followed according to the
237 manufacturer's instructions, and the isolation of tumor-infiltrating immune and cancer
238 cells from solid tumors was performed by using BD Horizon Dri Tumor and Tissue
239 Dissociation Reagent (BD Biosciences). Then, 1×10^5 cells were resuspended in 50 μ l of
240 Matrigel, plated on the bottom of a 96-well plate, and solidified Matrigel. After Matrigel
241 solidified, 200 μ l of the organoid medium was added to each well and supplemented with

242 50 ng/ml recombinant human GM-CSF, IL-2, and IL-6 (BioLegend). Organoids in a 96-
243 well plate were maintained at 37°C with 5% CO₂. Twelve hours after these manipulations,
244 anti-IL-34 antibodies or control IgG were added to organoids, and MDSCs within the
245 organoids were analyzed by flow cytometry on day 3.

246

247 **Flow cytometry analysis and fluorescence-activated cell sorting**

248 For extracellular staining, cells (2×10^5 cells/well) were blocked with FcR Blocking
249 Reagent (TONBO biosciences) and stained the following molecules with antibodies or
250 dye; CD3, CD4, CD14, CD15, CD45, CD86, CD115, CD11b, CD33, F4/80, Ly6C, and
251 Ly6G (BioLegend), and 4', 6-diamidino-2-phenylindole (Cayman Chemical). For
252 intracellular cytokine staining, cells were fixed and permeabilized with
253 Cytofix/Cytoperm™ Buffer (BD Biosciences) after extracellular staining, then stained
254 FOXP3 (BioLegend). Data were acquired using the FACSCelesta flow cytometer (BD
255 Biosciences) and analyzed using FlowJo software (BD Biosciences). The gating
256 strategies of immune cells are shown in Fig. S1. Cell sorting of tumor-infiltrating M-
257 MDSCs and PMN-MDSCs was performed by FACS Aria cell sorter (BD Biosciences).
258 The antibodies used are summarized in Supplementary Table 1.

259

260 **Quantitative polymerase chain reaction (qPCR)**

261 Total RNA was extracted by using the TriPure Isolation Reagent (Roche). ReverTra Ace[®]
262 qPCR RT Master Mix (Toyobo) was used to transcribe mRNA to cDNA. qPCR was
263 performed on cDNA using specific primers and KAPA SYBR[®] FAST qPCR Master Mix
264 (2x) ABI Prism[®] (Kapa Biosystems) on a StepOnePlus Real-Time PCR System (Thermo
265 Fisher Scientific). Values were normalized to the expression of *Gapdh*. Relative
266 expression levels were calculated using the 2-ddCt method. The primers are listed in
267 Supplementary Table 2.

268

269 **Clinical data analyses**

270 Gene expression data and clinical data of IL-34 in the “Breast Invasive Carcinoma
271 (TCGA, PanCancer Atlas)” dataset (N=1083) were obtained from www.cbioportal.org.
272 We assigned these samples to breast cancer subtypes based on the PAM50 classification,
273 and compared gene expression levels of IL-34, M-MDSC signature (CD274, NOS2, IL-
274 10, VEGFA, CD14, TNF), PMN-MDSC signature (STAT1, STAT6, IRF1, ANXA1, LYZ,
275 CXCL1, CXCL2, CXCR2, IL-8, LILRA3, TREM1, PTGS2, IL-4R, VEGFB, VEGFC,
276 OLR1, CD244, LOX, SLC27A2), Treg cell signature (CD25), and vascular endothelial
277 cell signature (CD31). Then we calculated the Spearman correlation coefficient and

278 generated the Kaplan-Meier survival curve. Data with missing survival information were
279 excluded from this analysis.

280

281 **Statistical analysis**

282 Sample size and statistical tests are described in the figure legends. Every series of data
283 are shown as mean \pm SEM. A two-sided Student's *t*-test was used to compare between 2
284 groups. One-way ANOVA with Tukey's test was used to compare between 3 or more
285 groups. The survival curves of the mice were determined using the Kaplan-Meier method,
286 and the log-rank test was used for statistical evaluation. Pearson or Spearman correlation
287 coefficient was used to examine the correlation between the two indicators. The p-value
288 of < 0.05 was considered to be statistically significant. All statistical analyses were
289 performed using JMP[®] 14 software (SAS Institute).

290

291 **RESULTS**

292 **Tumor-derived IL-34 promotes tumor growth and orchestrates TME**

293 We previously reported that IL-34 expression is elevated in human TNBC tumors and
294 identified IL-34 as an independent poor prognostic factor for TNBC;[4] however, how
295 IL-34 affects the TNBC's TME remains unknown. As an approach to identify the
296 functions of IL-34 in the TME, we used a murine TNBC cell line 4T1. We first confirmed
297 the effects of IL-34 on tumor growth using IL-34-producing and -deficient 4T1
298 (hereinafter referred to as control (Ctrl) and knockout (KO), respectively). Interestingly,
299 Ctrl and KO cells proliferated equally *in vitro* (Fig. S2a), while KO cells showed
300 significantly slower tumor growth compared to Ctrl cells in a subcutaneous
301 transplantation model (Fig. 1a). At the time of tumor harvest, we confirmed that KO
302 tumors weight showed marked reduction compared to Ctrl tumors (Fig. 1b). Furthermore,
303 survival was prolonged in mice bearing KO tumors compared to Ctrl tumors (Fig. S2b).
304 We then investigated how IL-34 promotes tumor growth *in vivo*. Based on previous
305 reports that showed IL-34 enhances tumor growth by inducing TAMs,[18]-[20] we
306 performed flow cytometry analysis of tumor-infiltrating immune cells to examine
307 whether tumor-derived IL-34 increased them. However, there was no significant
308 difference in the number of TAMs infiltrated into Ctrl and KO tumors, nor was a

309 difference in the number of T cells (Fig. 1c). On the other hand, the expression level of
310 CD86 on macrophages was significantly increased in KO tumors (Fig. 1d). It has been
311 reported that Treg cells can abrogate the T-cell activation capacity of antigen-presenting
312 cells (APCs) such as macrophages through down-regulating the CD86
313 expression.[22][23] Then, we examined the existence of Treg cells within the 4T1 tumors
314 and found that the number of them was drastically decreased in KO tumors compared to
315 Ctrl tumors (Fig. 1e and Fig. S3a, b).

316 We then explored whether the difference in Treg cells' infiltration was direct or
317 indirect effects by IL-34. Interestingly, we found that most Treg cells infiltrating into the
318 4T1 tumors expressed IL-34 receptor, colony-stimulating factor 1 receptor (CSF-1R) (Fig.
319 S3c), while its expression was barely observed in other tumor-infiltrating T cells (Fig.
320 S3d). To test whether IL-34 directly affects tumor-infiltrating Treg cells through binding
321 to CSF-1R, recombinant IL-34 was added to *in vitro* culture system that induces Treg
322 cells from naive T cells. However, notable change was not observed (data not shown),
323 suggesting that IL-34 does not directly affect the number of infiltrated Treg cells, although
324 they apparently express receptors for IL-34. We then set out to examine the possibility of
325 indirect pathways with which IL-34 changed the Treg cells infiltration. Treg cells are
326 known to be induced by intra-tumoral immunosuppressive myeloid cells such as TAMs

327 or MDSCs,[24][25] thus we focused on MDSCs because the infiltration of TAMs was not
328 different between Ctrl and KO tumors. In consequence, there was a remarkable alteration
329 in the MDSCs fraction infiltrated into each tumor (Fig. 1f). Two major subsets have been
330 identified to compose MDSCs; one is monocytic MDSCs (M-MDSCs), which are similar
331 to monocytes and are known to differentiate into tumor-associated macrophages (TAMs),
332 and another one is polymorphonuclear MDSCs (PMN-MDSCs), which share phenotypic
333 features with neutrophils.[26] In KO tumors, the population of CD11b⁺Ly6C⁺Ly6G⁻ cells,
334 representing M-MDSCs in mice, was decreased sharply compared with Ctrl tumors (Fig.
335 1f). Contrary, the population of CD11b⁺Ly6C^{lo}Ly6G⁺ cells, representing PMN-MDSCs
336 in mice, was increased in KO tumors compared with Ctrl tumors (Fig. 1f). Because M-
337 MDSCs are known to have a strong immunosuppressive ability and highly express CSF-
338 1R,[27] these results suggest that IL-34 may promote *in vivo* tumor growth by affecting
339 intra-tumoral M-MDSCs. Additionally, immunosuppressive effects via M-MDSCs are
340 further supported by the results that M-MDSCs infiltrating in Ctrl tumors showed not
341 only larger frequency but also a higher expression of *Ccl22*, which is critical for the
342 migration of Treg cells, than M-MDSCs infiltrating in KO tumors (Fig. S3e).

343 The relationship between intra-tumoral IL-34 expression and MDSCs has not
344 been well described. Thus, we examined the direct effect of IL-34 on the induction of

345 MDSCs by *in vitro* experiments. MDSCs were induced from bone marrow with
346 granulocyte-macrophage colony-stimulating factor (GM-CSF) as previously
347 described.[28] When recombinant IL-34 was added to the culture, a drastic increase in
348 the induction of M-MDSCs was observed. On the other hand, PMN-MDSCs induction
349 was slightly decreased (Fig. 2a). Of note, in human breast cancer patient-derived
350 organoids, IL-34 inhibition slightly decreased M-MDSCs and increased PMN-MDSCs
351 (Fig. S4a). To further consolidate the view that IL-34 affects MDSCs, we administered
352 anti-IL-34 antibodies to MMTV-PyMT mice that spontaneously developed breast cancer.
353 Thereby, an apparent decrease in M-MDSCs and an increase in PMN-MDSCs were
354 observed in the TME of the IL-34 inhibitory group (Fig. S4b). In addition, accompanied
355 by IL-34 inhibition-derived decrement of M-MDSCs, Treg cells also decreased (Fig. S4c).
356 These data strongly suggest that IL-34 alters the balance of M-MDSCs and PMN-MDSCs.
357 Collectively, these results support the notion that an increase of M-MDSCs (and a
358 decrease of PMN-MDSCs) in Ctrl tumors is caused by tumor-derived IL-34, and the IL-
359 34-MDSCs axis may enhance the tumor growth.

360

361 **IL-34-MDSCs axis promotes tumor growth by creating an immunosuppressive**
362 **TME**

363 Although M-MDSCs have been reported to have a strong immunosuppressive ability,[27]
364 the role of M-MDSCs in TNBC tumor growth has still been unidentified. To test the
365 hypothesis that IL-34-induced M-MDSCs promote tumor growth, methylpiperidino
366 pyrazole (MPP), an estrogen receptor inhibitor that can selectively deplete M-
367 MDSCs,[29] was administered to the mice bearing Ctrl tumor. Administration of MPP
368 resulted in significantly decreased growth of Ctrl tumors (Fig. 2b and Fig. S5a), along
369 with a selective reduction of M-MDSCs in TME (Fig. 2c). The population of PMN-
370 MDSCs, T cells, and macrophages did not change by the MPP administration (Fig. 2c
371 and Fig. S5b), while, of note, a decrease of Treg cells was observed in the group with
372 MPP treatment (Fig. S5c). These observations support the hypothesis that tumor-derived
373 IL-34 induces M-MDSCs, which then recruits Treg cells to TME to support tumor growth
374 by suppressing antitumor immune responses.

375

376 **IL-34 lowers the therapeutic effect of chemotherapy via its effects on MDSCs**

377 Given an immunosuppressive function of IL-34 in TME shown in our previous
378 studies,[18]-[20] in addition to evidence regarding the relationship between
379 chemoresistance and MDSCs,[17] we then explored the antitumor effect of paclitaxel
380 (PTX), which is one of the standard treatments of TNBC, on *in vivo* tumor growth in the

381 presence or absence of IL-34. Interestingly, PTX exerted its therapeutic effect in KO but
382 not in Ctrl tumor-bearing mice (Fig. 3a-d). Since there was no significant change in the
383 immune cell component of TME with or without PTX treatment (Fig. S6a, b), a scenario
384 was suggested in which tumor-derived IL-34 limits the PTX's effect by regulating
385 MDSCs balance, namely, inducing M-MDSCs and suppressing PMN-MDSCs.

386 To verify the hypothesis that acquirement of chemoresistance is due to the IL-
387 34-MDSCs axis, combination therapy of PTX and MPP was performed for Ctrl tumors.
388 Consistent with the results shown in Fig. 2b, monotherapy of MPP delayed tumor growth,
389 and administration of PTX combined with MPP showed a synergistic antitumor effect
390 (Fig. 3e and Fig. S6c). These results suggest that the antitumor effect by the combination
391 therapy with PTX and MPP was mediated by an alteration of MDSCs' balance in the
392 TME that occurred by the administration of MPP. In fact, TME analysis exhibited a
393 decrease in M-MDSCs and a contrasting increase in PMN-MDSCs in the MPP-treated
394 and MPP/PTX combination therapy groups compared to the PTX monotherapy group
395 (Fig. 3f and Fig. S6d). Taken together, the above observations are indicative of a negative
396 involvement of the IL-34-MDSCs axis in the therapeutic effect of chemotherapy.

397

398 **IL-34-MDSCs axis dramatically suppresses angiogenesis and forms a**

399 **chemoresistant tumor**

400 We next asked how the IL-34-MDSCs axis limits the therapeutic effect of chemotherapy.
401 To address this issue, we evaluated the vascular invasion into the tumors, which has been
402 reported as a representative mechanism for chemoresistance caused by
403 MDSCs.[30][31][32] Of note, blood vessels did not invade the central part of the Ctrl
404 tumors and were observed only in the outer layer. Conversely, massive invasion of blood
405 vessels into the central part was observed in KO tumors (Fig. 4a). In addition to the clear
406 difference of blood vessel distribution between the two tumors represented by the
407 histological images, quantification of vascular endothelial cells revealed their dramatic
408 increase in KO tumors compared to Ctrl tumors (Fig. 4a).

409 We then investigated the angiogenic ability of MDSCs in the tumors, mainly
410 focusing on PMN-MDSCs that increased in KO tumors. Interestingly, PMN-MDSCs
411 isolated from Ctrl tumors showed a marked reduction of *Vegfa* and *Vegfb* expression
412 levels compared to those from KO tumors (Fig. 4b), and similar trends were observed for
413 M-MDSCs (Fig. S7a). The levels of these factors were not different between Ctrl and KO
414 tumor cells (Fig. S7b). These results suggest that the IL-34-MDSCs axis suppresses
415 angiogenesis and then causes chemoresistance through inhibiting chemotherapeutic
416 agents from reaching the entire tumor. In fact, many MDSCs (Gr-1⁺ cells) that can induce

417 angiogenesis invaded the central part of KO tumors, whereas, in Ctrl tumors, their
418 invasion was observed only in the outer layer (Fig. 4c). Collectively, the above
419 observations suggest that tumor-derived IL-34 acts on MDSCs to create an
420 immunosuppressive and chemoresistant TME.

421

422 **IL-34 blockade restores chemoresistance *in vivo***

423 To gain further understanding of IL-34's effect on MDSCs in the TME, we next examined
424 whether IL-34 inhibiting reagents affected chemoresistance. Administration of an anti-
425 IL-34 antibody led to a significant increase in the density of blood vessels within the
426 tumors, accompanied by an increasing trend of PMN-MDSCs (Fig. 5a and Fig. S8a). This
427 result suggests that the IL-34 blockade may restore the penetration of chemotherapeutic
428 agents. In addition, IL-34 inhibition tended to decrease the frequency of M-MDSCs and
429 Treg cells in the TME (Fig. S8b), suggesting that the immunosuppressive circumstance
430 in the TME of Ctrl tumors was also improved by the IL-34 blockade.

431 We finally treated Ctrl tumors with a combination of IL-34 blockade and PTX.
432 Although Ctrl tumors has not sensitive to PTX (Fig. 3a, b), this combinatory treatment
433 induced significant growth retardation (Fig. 5b). These results demonstrate that the
434 efficacy of PTX could be restored by targeting the IL-34-MDSCs axis. Notably, the above

435 results further illustrate that tumor-derived IL-34 indeed contributes to chemoresistance
436 *in vivo*. Moreover, the combination therapy of PTX and IL-34 inhibition was
437 accompanied by a decrease in M-MDSCs (Fig. 5c), suggesting that this therapy could
438 induce weaning from immunosuppressive TME. In fact, an increase in T cells and TAMs
439 was observed in the combination therapy group compared to the PTX monotherapy group
440 (Fig. S8c). Considering that chemotherapy is originally used to induce cell death in
441 tumors and augment immune responses to cancer, the above findings may have an
442 implication for developing future chemotherapy because most tumors show certain levels
443 of resistance against chemotherapy.

444

445 **Involvement of IL-34-MDSCs axis in human TNBC**

446 To assess whether the findings obtained in our mouse experiments can be extrapolated to
447 human TNBC's TME, we analyzed human data from The Cancer Genome Atlas (TCGA).
448 This analysis revealed that there was a positive correlation between IL-34 expression in
449 human TNBC tumors and intra-tumoral M-MDSCs (Fig. 6a). Furthermore, we found that
450 intra-tumoral M-MDSCs positively correlated with intra-tumoral Treg cells (Fig. 6b),
451 suggesting that immunosuppressive TME through the IL-34-MDSCs axis is also
452 established in human TNBC. In fact, consistent with our mouse experiments (Fig. 1f and

453 Fig. S2b), high IL-34 expression and high M-MDSCs infiltration in human TNBC tumors
454 showed worse patient survival (Fig. 6c). Hence, when we then analyzed the relationship
455 between intra-tumoral IL-34 expression and PMN-MDSCs by using SLC27A2, a marker
456 of human PMN-MDSCs,[33] expression levels of IL-34 and SLC27A2 were negatively
457 correlated (Fig. 6d). In addition, there was a positive correlation between intra-tumoral
458 PMN-MDSCs and vascular endothelial cells (Fig. 6e), which supports our results
459 obtained in the mouse experiments (Fig. 4, 5).

460 Finally, we confirmed if the results from gene expression analyses of the TCGA
461 dataset are consistent with protein expression in TNBC tissues, using the IHC
462 examination for each candidate molecule (Fig. S9a). Of note, within the TNBC tissues,
463 IL-34 expression positively correlated with M-MDSC infiltration, and moreover, M-
464 MDSCs and Treg cells infiltration also related positively. On the other hand, despite no
465 relation between IL-34 expression and PMN-MDSCs infiltration in the TNBC cohort
466 used in the present study, the infiltration of PMN-MDSCs showed a strong and positive
467 association with vascular endothelial cells' invasion in the TNBC tumors (Fig. S9b).

468 Collectively, the mouse data in this study overall represent the pathological
469 situation of human TNBC's TME, suggesting that IL-34-targeted therapy may derepress
470 immunosuppression and increase the effectiveness of chemotherapy in TNBC. However,

471 there are some exceptions due to the complex composition of TME in TNBC patients, as
472 the relationship between IL-34 and PMN-MDSCs indicated at last, and further studies in
473 large cohorts are needed.

474

475 **DISCUSSION**

476 We turn our eyes to previous research regarding IL-34 and cancer; in esophageal cancer,
477 it has been reported that IL-34 expression in chemotherapy-nonresponsive patients was
478 significantly higher than that in chemotherapy-responsive patients.[34] IL-34 expression
479 converts the TME toward a TAM-rich immunosuppressive one resulting in a
480 chemoresistance and worse prognosis in IL-34-high expressing patients.[34] In also lung
481 cancer, we previously demonstrated that chemoresistant tumor-derived IL-34 enhanced
482 local immunosuppressive degree by inducing TAMs from monocytes via paracrine
483 pathways and promoted chemoresistant cancer cells' survival via autocrine pathways.[19]
484 In addition, we indicated that both existences of high IL-34 expression and high TAM
485 infiltration in TME were associated with worse progression-free survival in an ovarian
486 cancer study.[18] Similar results have been documented in also colorectal cancer.[35]
487 Thus, IL-34 induces immunosuppressive TAMs within the TME, leading to therapeutic
488 resistance and poor outcomes in various cancers.

489 While studies regarding IL-34 and cancer have hitherto exhibited the TAMs-
490 mediated dynamics by IL-34 in TME, in this study, we have demonstrated for the first
491 time that TNBC tumor-derived IL-34 functions as an MDSCs balance regulator; namely,
492 it induces differentiation of M-MDSCs from the bone marrow and in turn suppresses

493 differentiation of PMN-MDSCs. As IL-34 has been so far reported as a TAMs-mediated
494 immunosuppressive factor rather than an MDSC inducer, our findings provide new
495 insights into how IL-34 contributes to the regulation of immune response within TME.

496 The induction of MDSCs is an important immune-evading mechanism elicited
497 by tumors. Intra-tumoral M-MDSCs, in particular, are more potent immunosuppressors
498 than PMN-MDSCs,[27] and indeed, a previous human study showed that the number of
499 M-MDSCs but not of PMN-MDSCs correlated directly with antitumor T cell
500 suppression.[36] In addition to their intrinsic immunosuppressive ability, M-MDSCs
501 indirectly attenuate immune response by recruiting Treg cells via CCL22 production in
502 TME.[37] Our results indicated that IL-34 strongly triggers this pathway, orchestrates
503 MDSCs (increases M-MDSC) to create the immunosuppressive TME, and consequently
504 promotes tumor growth. In contrast, the resulting decrease of PMN-MDSCs could
505 negatively regulate angiogenesis in TME, making it difficult for chemotherapeutic agents
506 to reach the entire tumor. Therefore, IL-34, which regulates the balance of these two types
507 of MDSCs, is a crucial factor in controlling TME.

508 The relationship between angiogenesis and tumor malignancy has been
509 discussed in many studies.[30][31][32] In general, angiogenesis is an event that promotes
510 tumor growth;[30] however, it has advantages in terms of drug delivery that allows

511 antitumor drugs to penetrate tumors.[38] In fact, our results have consistently indicated
512 dramatic positive effects of chemotherapy for blood vessel-rich tumors. Moreover, tumor-
513 infiltrated immune cell analyses revealed much infiltration of PMN-MDSCs in
514 chemotherapy-susceptible tumors, which contained much vascular structure.

515 One of the most common and well-studied mechanisms inducing angiogenesis
516 is the production of VEGF by MDSCs.[30][31] VEGF is a growth factor secreted by
517 various cells and plays an important role in the general process of angiogenesis.[39]
518 Considering our results based on these backgrounds, it is worth noting that many PMN-
519 MDSCs infiltrated into IL-34-deficient tumors in which many blood vessels were
520 observed. In fact, a previous study has shown that co-injection of tumor-derived PMN-
521 MDSCs and tumor cells exhibit significantly higher intra-tumoral vascular density than
522 mice injected with tumor cells alone.[32] In short, PMN-MDSCs have a strong ability to
523 contribute to tumor angiogenesis, which increment may lead to a dramatic increase of
524 vasculature within IL-34-deficient tumors.

525 In addition to the quantitative changes in PMN-MDSCs, the MDSCs isolated
526 from IL-34-deficient tumors displayed remarkably increased *Vegfa* and *Vegfb* expression
527 levels compared to those isolated from IL-34-producing tumors. These observations
528 suggest that tumor-derived IL-34 may contribute to the suppression of VEGF expression

529 through regulating downstream signaling of CSF-1R. Similarly, we have previously
530 shown that IL-34-deficient tumors are IL-6-rich,[4] which may stimulate MDSCs via
531 activation of the STAT3-mediated pathway.[40] This activation may induce more VEGF
532 production by MDSCs, probably establishing a positive feedback loop that sustains their
533 angiogenic activity.[41] These observations overall support the notion that PMN-MDSCs
534 infiltrating into IL-34-deficient tumors are a promoting factor for angiogenesis. In
535 addition, administration of anti-IL-34 antibody to IL-34-producing tumors increased the
536 frequency of intra-tumoral PMN-MDSCs and resulted in increased neo-angiogenesis.
537 Collectively, the IL-34-MDSCs axis in tumors seems to play a major role in angiogenic
538 suppression and contribute to chemoresistance.

539 TNBC is a subtype of breast cancer with the worst susceptibility to anti-tumor treatment,
540 including chemotherapy. It has been demonstrated that TNBC shows various levels of
541 chemoresistance via different mechanisms.[6] We have indicated that IL-34 is
542 characteristically expressed in TNBC and acts as an essential factor for chemoresistance.
543 In this study, we have shown for the first time a novel mechanism regulating intra-tumoral
544 MDSCs by IL-34, which then affected Treg infiltration and angiogenesis. Our study may
545 provide insights on how to manipulate the immune system in TNBC to enhance the
546 effectiveness of chemotherapeutic agents. Indeed, our data demonstrating that the

547 combination therapy of IL-34 blockade and chemotherapeutic agents was very effective

548 could be clinically significant.

549

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656

657

658 **FIGURE LEGENDS**

659 **Fig. 1 IL-34 promotes in vivo tumor growth by modulating TME in TNBC.**

660 (a) Subcutaneous tumor growth in BALB/c mice inoculated with control (Ctrl) and IL-34
661 knockout (KO) 4T1 cells (1×10^5 cells, n=6). Similar results were obtained in several
662 independent experiments. (b) Bar graphs representing tumor weight of harvested Ctrl and
663 IL-34 KO 4T1 tumors at 14 days post tumor inoculation (n=6). (c) Bar graphs
664 representing the frequency of TAM ($CD11b^+F4/80^+$ cells) and T cell ($CD3^+$ cells) within
665 $CD45^+$ cells infiltrated in the tumors described in Fig. 1a (n=6). (d) Representative
666 histograms indicated for Ctrl (Blue), KO (red), and isotype (gray) (left) and mean
667 fluorescence intensity (MFI) (right) of CD86 expression on $CD86^+$ TAM
668 ($CD45^+CD11b^+F4/80^+CD86^+$ cells) infiltrated in the tumors described in Fig. 1a (n=6).
669 (e) Representative IHC staining of CD4 (brown) and FOXP3 (black) in Ctrl and IL-34
670 KO 4T1 tumors at the time of day 14 from tumor inoculation (left). Scale bars=50 μ m.
671 Quantification of $CD4^+FOXP3^+$ cells in Ctrl and KO tumors from a field of view (n=3,
672 right). (f) Representative plots of M-MDSC ($CD11b^+Ly6C^+Ly6G^-$ cells) and PMN-
673 MDSC ($CD11b^+Ly6C^loLy6G^+$ cells) (left). Bar graphs representing the frequency of M-
674 MDSC and PMN-MDSC within $CD45^+$ cells infiltrated in the tumors described in Fig.
675 1a (n=6, right). Values were analyzed by a two-sided Student's *t*-test (a-f) and are shown

676 as mean \pm SEM. Asterisk indicates a significant difference; * p <0.05, ** p <0.01,
677 *** p <0.001, compared with the control group.

678

679 **Fig. 2 IL-34 creates an immunosuppressive TME via induction of M-MDSCs in**
680 **TNBC.**

681 (a) In vitro induction assay of MDSCs from bone marrow in the presence or absence of
682 IL-34. Bar graphs representing the frequency of M-MDSC (CD11b⁺Ly6C⁺Ly6G⁻ cells)
683 and PMN-MDSC (CD11b⁺Ly6C^{lo}Ly6G⁺ cells) within CD45⁺ cells differentiated from
684 bone marrow (n=3). (b) Subcutaneous tumor growth in BALB/c mice inoculated with
685 Ctrl and IL-34 KO 4T1 cells (1 x 10⁵ cells, n=4-8). Methylpiperidino pyrazole (MPP)
686 (1.0 mg/kg) or control DMSO was administered intraperitoneally daily for 5-14 days. (c)
687 Representative plots of M-MDSC (CD11b⁺Ly6C⁺Ly6G⁻ cells) and PMN-MDSC
688 (CD11b⁺Ly6C^{lo}Ly6G⁺ cells) (top). Bar graphs representing the frequency of M-MDSC
689 and PMN-MDSC within CD45⁺ cells infiltrated in the tumors described in Fig. 2b (n=4-
690 8, bottom). Values were analyzed by a two-sided Student's *t*-test (a) and one-way ANOVA
691 with Tukey's test (b-c) and are shown as mean \pm SEM. Asterisk indicates a significant
692 difference; * p <0.05, *** p <0.001, compared with the control group.

693

694 **Fig. 3 IL-34-MDSCs axis negates the antitumor effects of Paclitaxel (PTX) in TNBC.**

695 (a) Subcutaneous tumor growth in BALB/c mice inoculated with Ctrl 4T1 cells (1×10^5
696 cells, n=6). PTX (1.3 mg/kg) or control saline was administered intraperitoneally daily
697 for 5-14 days. (b) Bar graphs representing tumor weight of harvested Ctrl 4T1 tumors at
698 14 days post tumor inoculation (n=6). (c) Subcutaneous tumor growth in BALB/c mice
699 inoculated with IL-34 KO 4T1 cells (1×10^5 cells, n=6). PTX (1.3 mg/kg) or control
700 Saline was administered intraperitoneally daily for 5-14 days. (d) Bar graphs representing
701 tumor weight of harvested IL-34 KO 4T1 tumors at 14 days post tumor inoculation (n=6).
702 (e) Subcutaneous tumor growth in BALB/c mice inoculated with Ctrl 4T1 cells (1×10^5
703 cells, n=12). PTX (1.3 mg/kg), control Saline, MPP (1.0 mg/kg), control DMSO, or their
704 combination was administered intraperitoneally daily for 5-14 days. (f) Bar graphs
705 representing the frequency of M-MDSC ($CD11b^+Ly6C^+Ly6G^-$ cells) and PMN-MDSC
706 ($CD11b^+Ly6C^loLy6G^+$ cells) within $CD45^+$ cells infiltrated in the tumors described in Fig.
707 3e (n=12). Values were analyzed by a two-sided Student's *t*-test (a-d) and one-way
708 ANOVA with Tukey's test (e-f) and are shown as mean \pm SEM. Asterisk indicates a
709 significant difference; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the control group.

710

711

712 **Fig. 4 IL-34-MDSCs axis has the potential to suppress angiogenesis.**

713 (a) Ctrl and IL-34 KO 4T1 cells (1×10^5 cells) were subcutaneously inoculated into
714 BALB/c mice. Representative IHC staining of CD31 (brown) in Ctrl and IL-34 KO 4T1
715 tumors at the time of day 14 from tumor inoculation (left). Scale bars=200 μ m.
716 Quantification of CD31⁺ cells in Ctrl and IL-34 KO 4T1 tumors from a field of view (n=3,
717 right). (b) PMN-MDSCs were sorted from cell suspensions of tumor-infiltrated immune
718 cells at the time of day 14 from tumor inoculation. Bar graphs representing fold induction
719 of *Vegfa* and *Vegfb* expression of PMN-MDSCs in Ctrl and IL-34 KO 4T1 tumors (n=3).
720 Gene expression levels were determined by RT-PCR, as normalized to *Gapdh*. (c)
721 Representative IHC staining of Gr-1 (brown) in Ctrl and IL-34 KO 4T1 tumors at the time
722 of day 14 from tumor inoculation. Scale bars=100 μ m. Values were analyzed by a two-
723 sided Student's *t*-test (a-b) and are shown as mean \pm SEM. Asterisk indicates a significant
724 difference; ***p*<0.01, ****p*<0.001, compared with the control group.

725

726 **Fig. 5 Effects of IL-34 blockade on chemoresistance including in vivo tumor growth**
727 **and TME.**

728 (a) Ctrl 4T1 cells (1×10^5 cells) were subcutaneously inoculated into BALB/c mice. Anti-
729 IL-34 antibody (200 μ g) or control IgG was administered intraperitoneally daily for 5-14

730 days. Representative IHC staining of CD31 (brown) in Ctrl 4T1 tumors treated
731 with/without α IL-34 at the time of day 14 from tumor inoculation (left). Scale bars=200
732 μ m. Quantification of CD31⁺ cells in Ctrl 4T1 tumors treated with/without α IL-34 from
733 a field of view (n=3, right). (b) Subcutaneous tumor growth in BALB/c mice inoculated
734 with Ctrl 4T1 cells (1×10^5 cells, n=12). PTX (1.3 mg/kg), anti-IL-34 antibody (200 μ g),
735 control saline, control IgG, or their combination was administered intraperitoneally daily
736 for 5-14 days. (c) Bar graphs representing the frequency of M-MDSC
737 (CD11b⁺Ly6C⁺Ly6G⁻ cells) and PMN-MDSC (CD11b⁺Ly6C^{lo}Ly6G⁺ cells) within
738 CD45⁺ cells infiltrated in the tumors described in Fig. 5b (n=12). Values were analyzed
739 by a two-sided Student's *t*-test (a) and one-way ANOVA with Tukey's test (b-c) and are
740 shown as mean \pm SEM. Asterisk indicates a significant difference; **p*<0.05, ***p*<0.01,
741 compared with the control group.

742

743 **Fig. 6 Potential involvement of IL-34-MDSCs axis in human TNBC.**

744 (a) Pearson correlation coefficient between relative IL-34 expression in tumors and intra-
745 tumoral M-MDSCs infiltration is shown. (b) Pearson correlation coefficient between
746 intra-tumoral Treg cells infiltration and intra-tumoral M-MDSCs infiltration is shown. (c)
747 Kaplan-Meier plot of overall survival of TNBC patients with high IL-34 expression/high

748 M-MDSCs infiltration and low IL-34 expression/low M-MDSCs infiltration stratified by
749 PAM50 subtype. Values were analyzed by a Log-rank test. (d) Spearman correlation
750 coefficient between relative IL-34 expression in tumors and intra-tumoral PMN-MDSCs
751 infiltration is shown. (e) Pearson correlation coefficient between intra-tumoral vascular
752 endothelial cells infiltration and intra-tumoral PMN-MDSCs infiltration is shown.

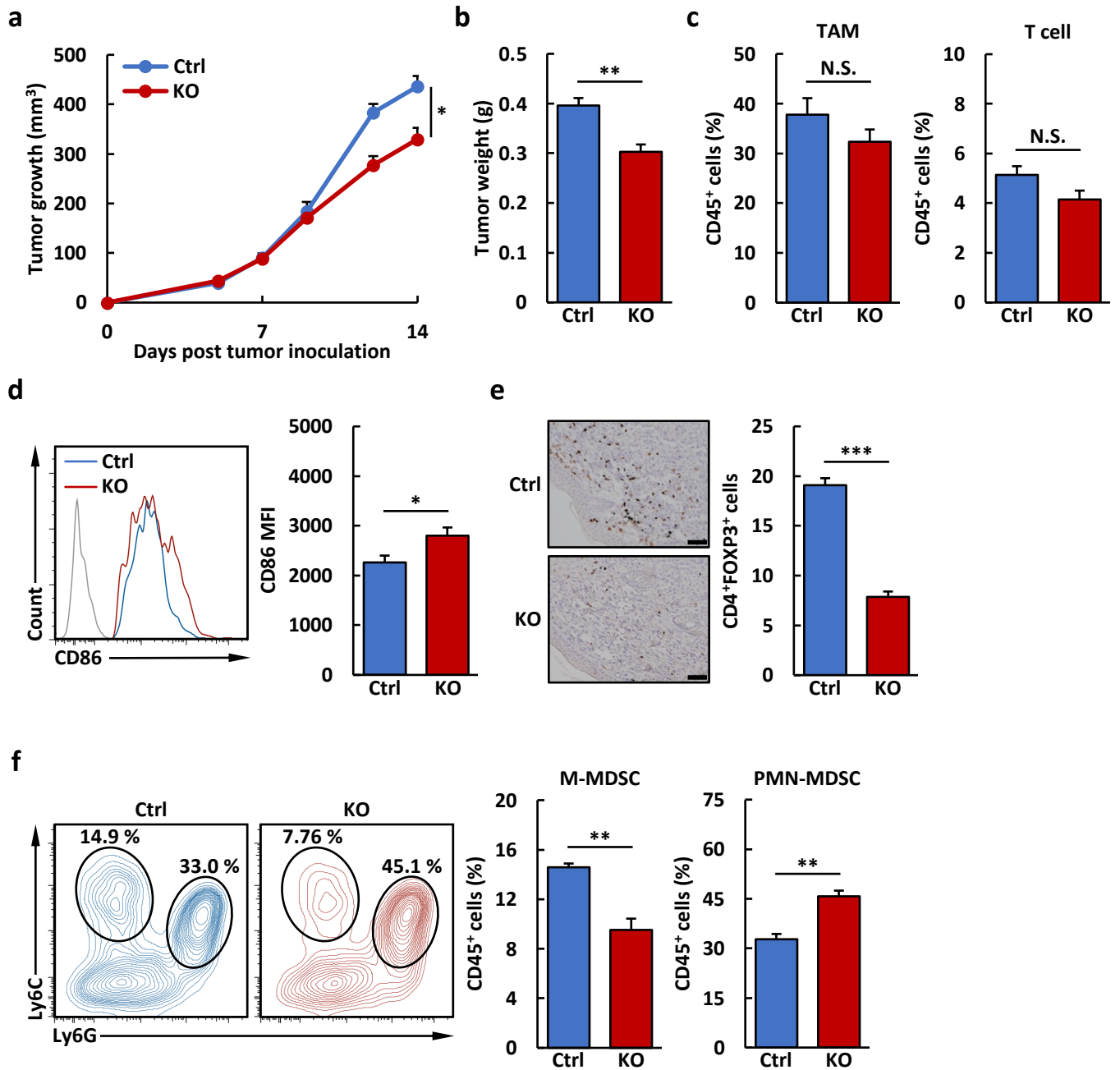


Fig. 1 IL-34 promotes in vivo tumor growth by modulating TME in TNBC.

(a) Subcutaneous tumor growth in BALB/c mice inoculated with control (Ctrl) and IL-34 knockout (KO) 4T1 cells (1×10^5 cells, $n=6$). Similar results were obtained in several independent experiments. (b) Bar graphs representing tumor weight of harvested Ctrl and IL-34 KO 4T1 tumors at 14 days post tumor inoculation ($n=6$). (c) Bar graphs representing the frequency of TAM ($CD11b^+F4/80^+$ cells) and T cell ($CD3^+$ cells) within $CD45^+$ cells infiltrated in the tumors described in Fig. 1a ($n=6$). (d) Representative histograms indicated for Ctrl (Blue), KO (red), and isotype (gray) (left) and mean fluorescence intensity (MFI) (right) of CD86 expression on $CD86^+$ TAM ($CD45^+CD11b^+F4/80^+CD86^+$ cells) infiltrated in the tumors described in Fig. 1a ($n=6$). (e) Representative IHC staining of CD4 (brown) and FOXP3 (black) in Ctrl and IL-34 KO 4T1 tumors at the time of day 14 from tumor inoculation (left). Scale bars=50 μ m. Quantification of $CD4^+FOXP3^+$ cells in Ctrl and KO tumors from a field of view ($n=3$, right). (f) Representative plots of M-MDSC ($CD11b^+Ly6C^+Ly6G^-$ cells) and PMN-MDSC ($CD11b^+Ly6C^loLy6G^+$ cells) (left). Bar graphs representing the frequency of M-MDSC and PMN-MDSC within $CD45^+$ cells infiltrated in the tumors described in Fig. 1a ($n=6$, right). Values were analyzed by a two-sided Student's *t*-test (a-f) and are shown as mean \pm SEM. Asterisk indicates a significant difference; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared with the control group.

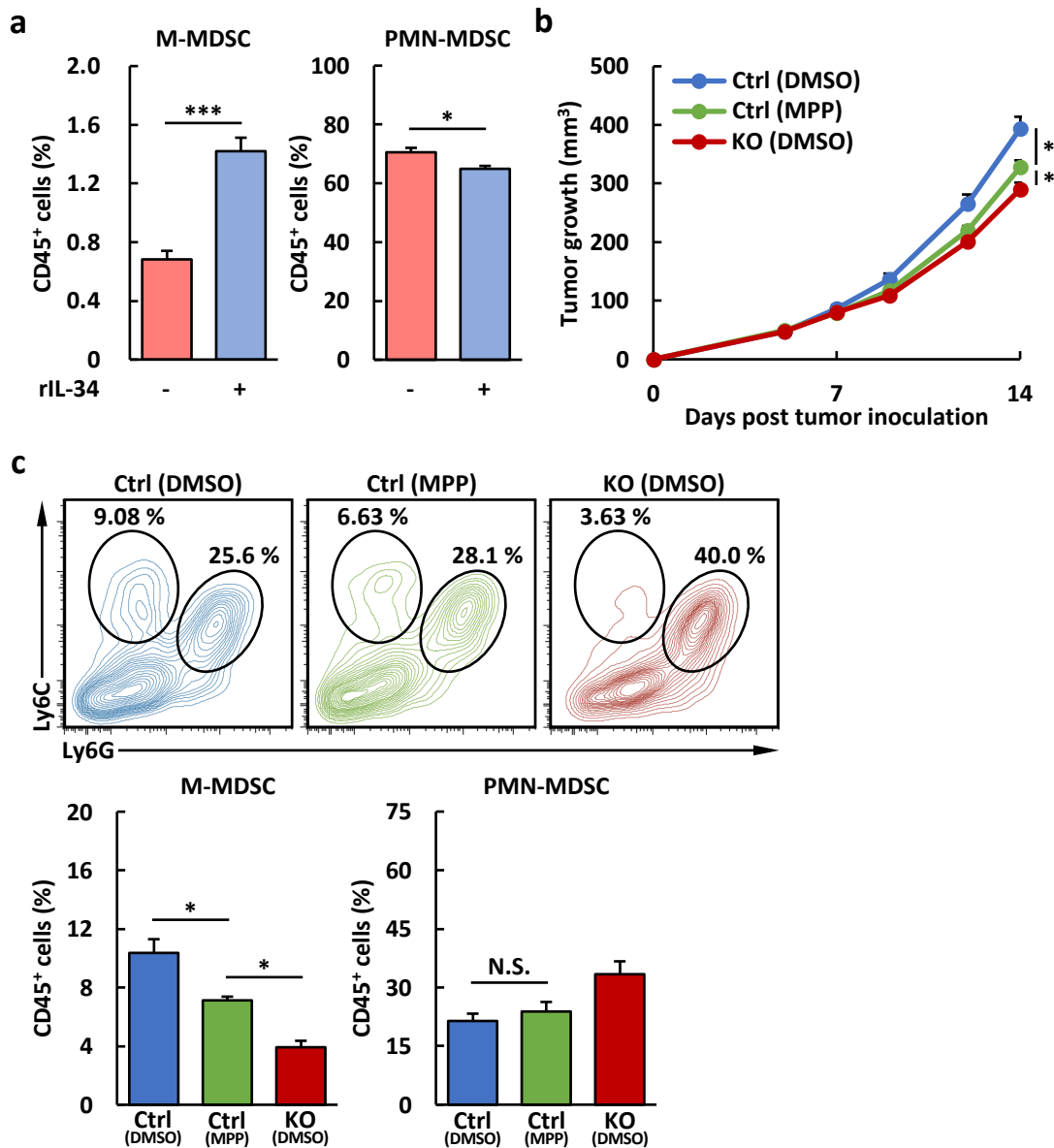


Fig. 2 IL-34 creates an immunosuppressive TME via induction of M-MDSCs in TNBC.

(a) In vitro induction assay of MDSCs from bone marrow in the presence or absence of IL-34. Bar graphs representing the frequency of M-MDSC (CD11b⁺Ly6C⁺Ly6G⁻ cells) and PMN-MDSC (CD11b⁺Ly6C^{lo}Ly6G⁺ cells) within CD45⁺ cells differentiated from bone marrow (n=3). (b) Subcutaneous tumor growth in BALB/c mice inoculated with Ctrl and IL-34 KO 4T1 cells (1 × 10⁵ cells, n=4-8). Methylpiperidino pyrazole (MPP) (1.0 mg/kg) or control DMSO was administered intraperitoneally daily for 5-14 days. (c) Representative plots of M-MDSC (CD11b⁺Ly6C⁺Ly6G⁻ cells) and PMN-MDSC (CD11b⁺Ly6C^{lo}Ly6G⁺ cells) (top). Bar graphs representing the frequency of M-MDSC and PMN-MDSC within CD45⁺ cells infiltrated in the tumors described in Fig. 2b (n=4-8, bottom). Values were analyzed by a two-sided Student's *t*-test (a) and one-way ANOVA with Tukey's test (b-c) and are shown as mean ± SEM. Asterisk indicates a significant difference; **p*<0.05, ****p*<0.001, compared with the control group.

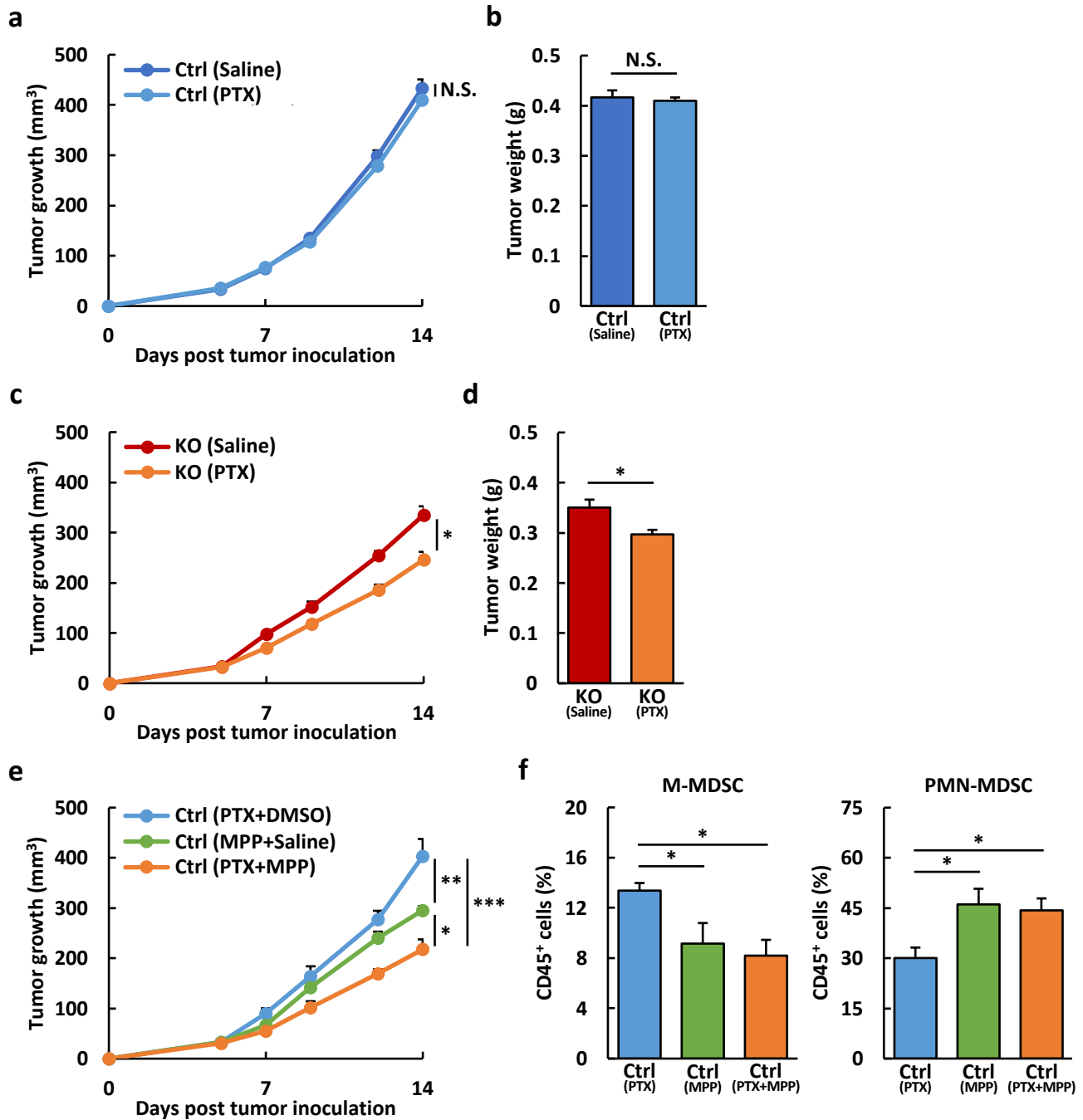


Fig. 3 IL-34-MDSCs axis negates the antitumor effects of Paclitaxel (PTX) in TNBC.

(a) Subcutaneous tumor growth in BALB/c mice inoculated with Ctrl 4T1 cells (1×10^5 cells, $n=6$). PTX (1.3 mg/kg) or control saline was administered intraperitoneally daily for 5-14 days. (b) Bar graphs representing tumor weight of harvested Ctrl 4T1 tumors at 14 days post tumor inoculation ($n=6$). (c) Subcutaneous tumor growth in BALB/c mice inoculated with IL-34 KO 4T1 cells (1×10^5 cells, $n=6$). PTX (1.3 mg/kg) or control Saline was administered intraperitoneally daily for 5-14 days. (d) Bar graphs representing tumor weight of harvested IL-34 KO 4T1 tumors at 14 days post tumor inoculation ($n=6$). (e) Subcutaneous tumor growth in BALB/c mice inoculated with Ctrl 4T1 cells (1×10^5 cells, $n=12$). PTX (1.3 mg/kg), control Saline, MPP (1.0 mg/kg), control DMSO, or their combination was administered intraperitoneally daily for 5-14 days. (f) Bar graphs representing the frequency of M-MDSC (CD11b⁺Ly6C⁺Ly6G⁻ cells) and PMN-MDSC (CD11b⁺Ly6C^{lo}Ly6G⁺ cells) within CD45⁺ cells infiltrated in the tumors described in Fig. 3e ($n=12$). Values were analyzed by a two-sided Student's *t*-test (a-d) and one-way ANOVA with Tukey's test (e-f) and are shown as mean \pm SEM. Asterisk indicates a significant difference; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the control group.

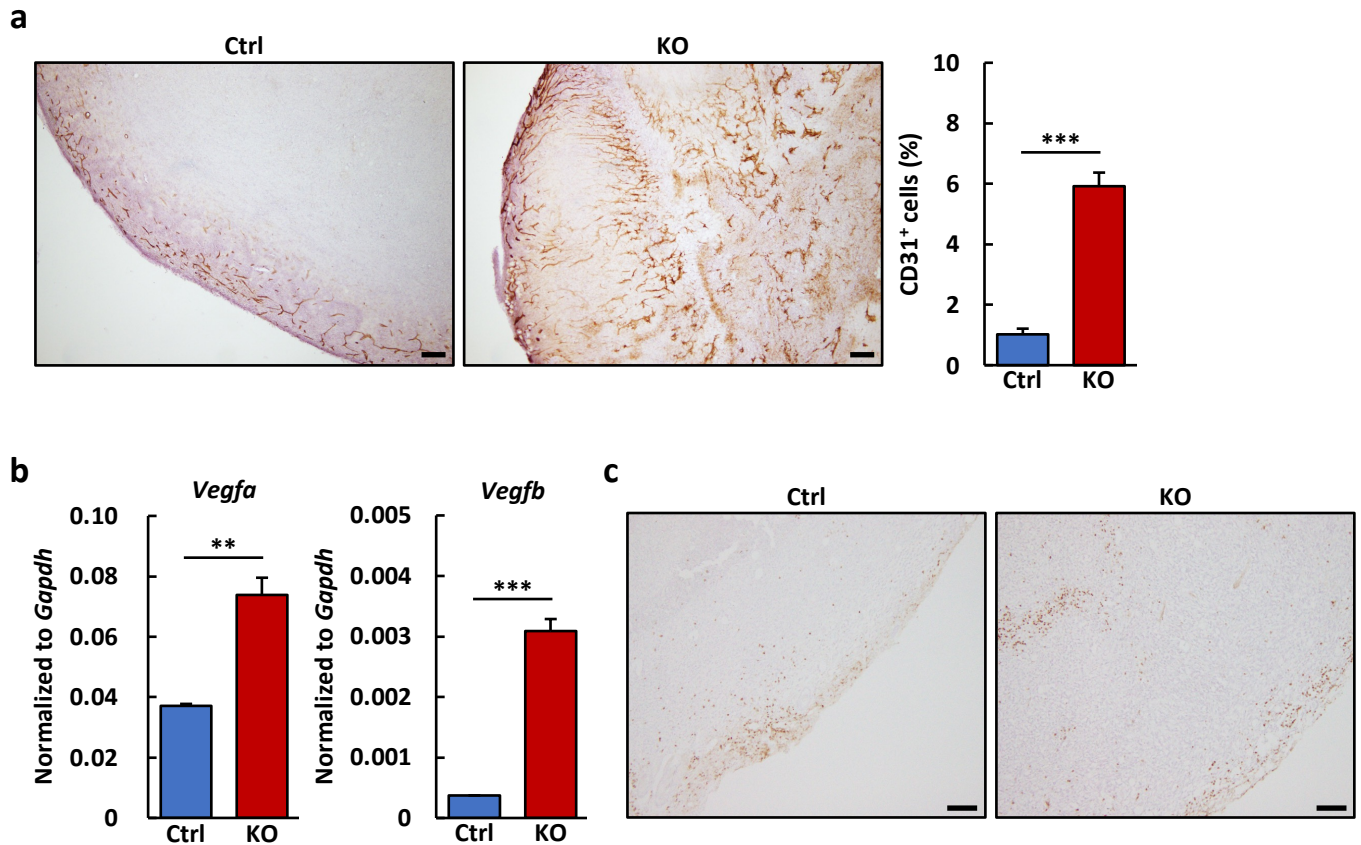


Fig. 4 IL-34-MDSCs axis has the potential to suppress angiogenesis.

(a) Ctrl and IL-34 KO 4T1 cells (1×10^5 cells) were subcutaneously inoculated into BALB/c mice. Representative IHC staining of CD31 (brown) in Ctrl and IL-34 KO 4T1 tumors at the time of day 14 from tumor inoculation (left). Scale bars=200 μ m. Quantification of CD31⁺ cells in Ctrl and IL-34 KO 4T1 tumors from a field of view (n=3, right). (b) PMN-MDSCs were sorted from cell suspensions of tumor-infiltrated immune cells at the time of day 14 from tumor inoculation. Bar graphs representing fold induction of *Vegfa* and *Vegfb* expression of PMN-MDSCs in Ctrl and IL-34 KO 4T1 tumors (n=3). Gene expression levels were determined by RT-PCR, as normalized to *Gapdh*. (c) Representative IHC staining of Gr-1 (brown) in Ctrl and IL-34 KO 4T1 tumors at the time of day 14 from tumor inoculation. Scale bars=100 μ m. Values were analyzed by a two-sided Student's *t*-test (a-b) and are shown as mean \pm SEM. Asterisk indicates a significant difference; ***p*<0.01, ****p*<0.001, compared with the control group.

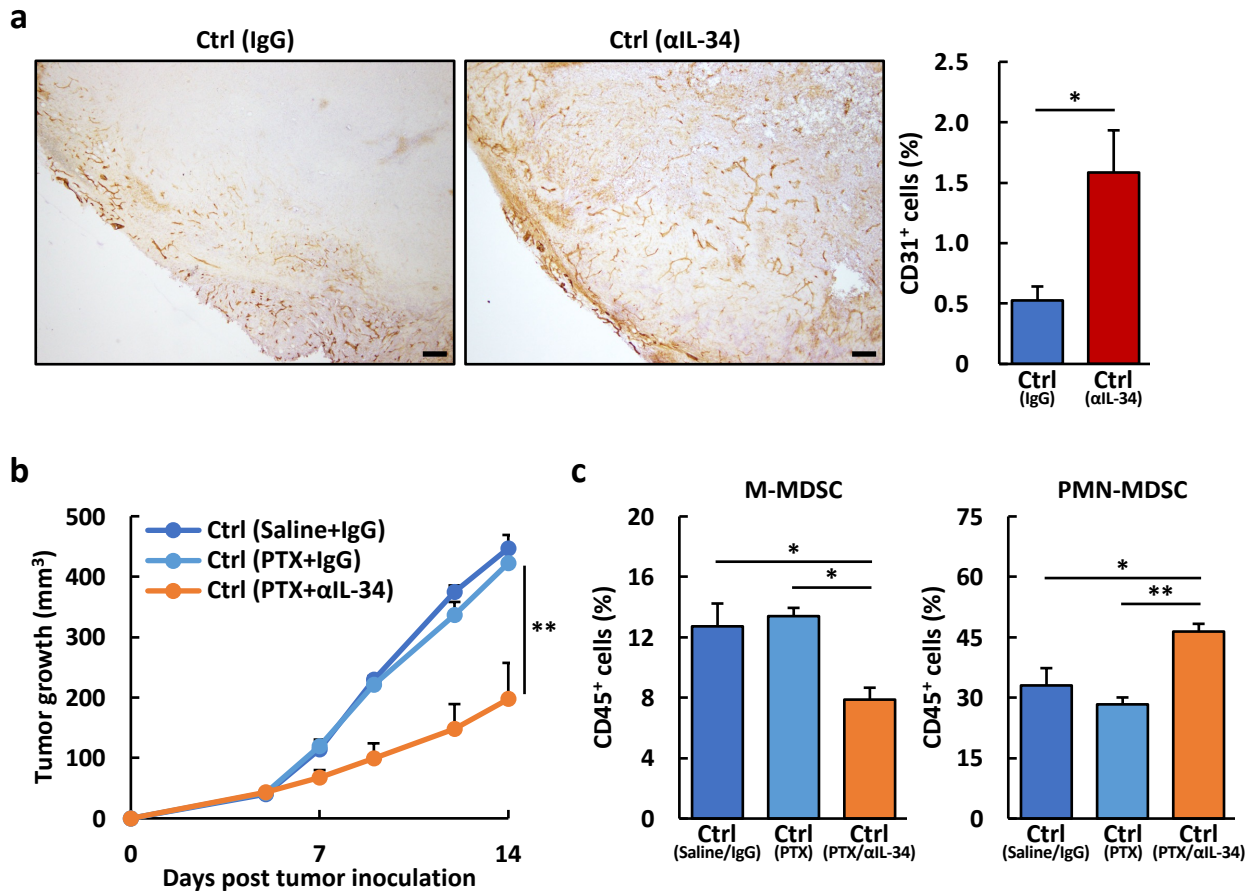


Fig. 5 Effects of IL-34 blockade on chemoresistance including in vivo tumor growth and TME.

(a) Ctrl 4T1 cells (1×10^5 cells) were subcutaneously inoculated into BALB/c mice. Anti-IL-34 antibody (200 μ g) or control IgG was administered intraperitoneally daily for 5-14 days. Representative IHC staining of CD31 (brown) in Ctrl 4T1 tumors treated with/without α IL-34 at the time of day 14 from tumor inoculation (left). Scale bars=200 μ m. Quantification of CD31⁺ cells in Ctrl 4T1 tumors treated with/without α IL-34 from a field of view (n=3, right). (b) Subcutaneous tumor growth in BALB/c mice inoculated with Ctrl 4T1 cells (1×10^5 cells, n=12). PTX (1.3 mg/kg), anti-IL-34 antibody (200 μ g), control saline, control IgG, or their combination was administered intraperitoneally daily for 5-14 days. (c) Bar graphs representing the frequency of M-MDSC (CD11b⁺Ly6C⁺Ly6G⁻ cells) and PMN-MDSC (CD11b⁺Ly6C^{lo}Ly6G⁺ cells) within CD45⁺ cells infiltrated in the tumors described in Fig. 5b (n=12). Values were analyzed by a two-sided Student's *t*-test (a) and one-way ANOVA with Tukey's test (b-c) and are shown as mean \pm SEM. Asterisk indicates a significant difference; **p*<0.05, ***p*<0.01, compared with the control group.

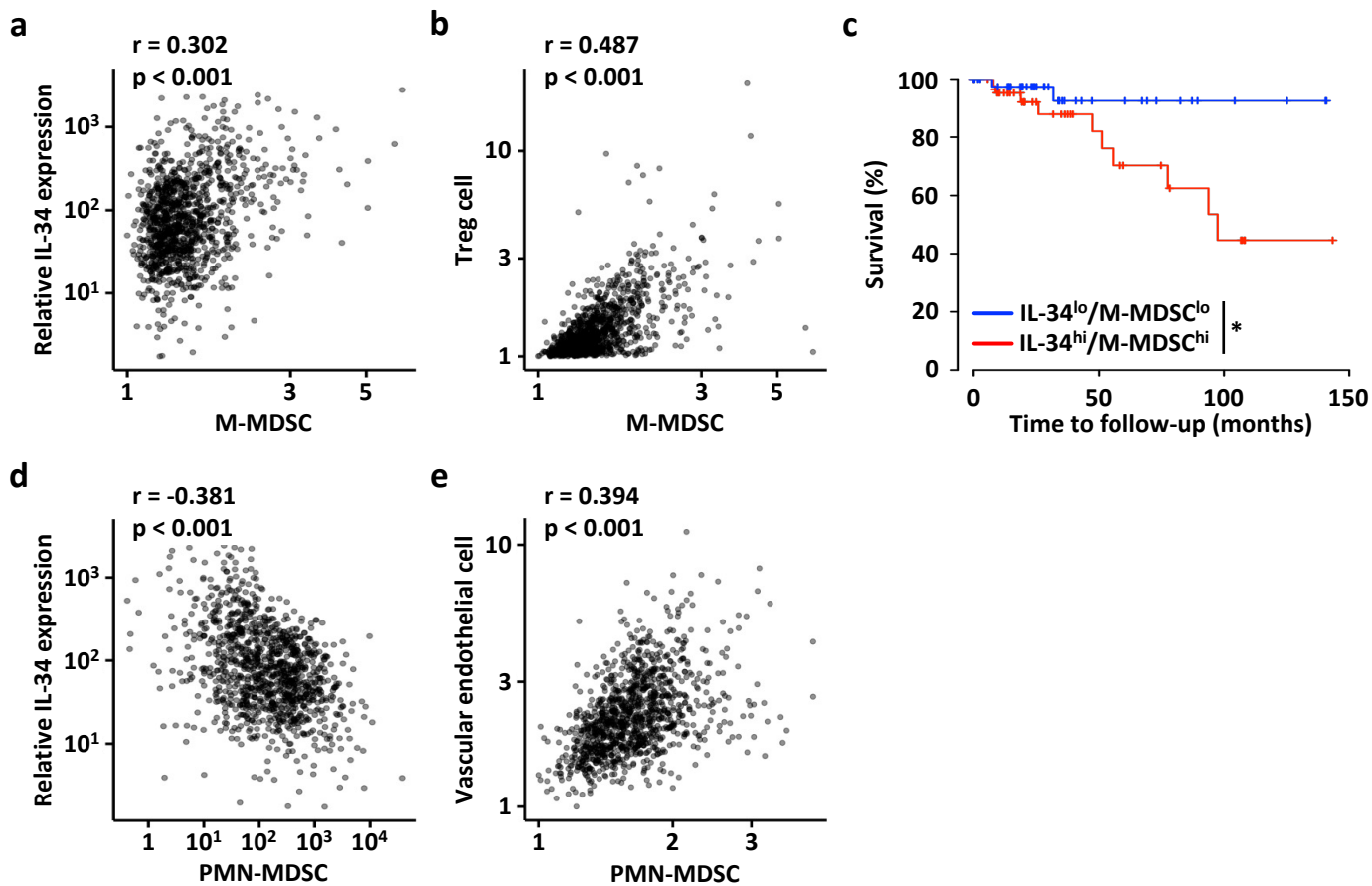


Fig. 6 Potential involvement of IL-34-MDSCs axis in human TNBC.

(a) Pearson correlation coefficient between relative IL-34 expression in tumors and intra-tumoral M-MDSCs infiltration is shown. (b) Pearson correlation coefficient between intra-tumoral Treg cells infiltration and intra-tumoral M-MDSCs infiltration is shown. (c) Kaplan-Meier plot of overall survival of TNBC patients with high IL-34 expression/high M-MDSCs infiltration and low IL-34 expression/low M-MDSCs infiltration stratified by PAM50 subtype. Values were analyzed by a Log-rank test. (d) Spearman correlation coefficient between relative IL-34 expression in tumors and intra-tumoral PMN-MDSCs infiltration is shown. (e) Pearson correlation coefficient between intra-tumoral vascular endothelial cells infiltration and intra-tumoral PMN-MDSCs infiltration is shown.

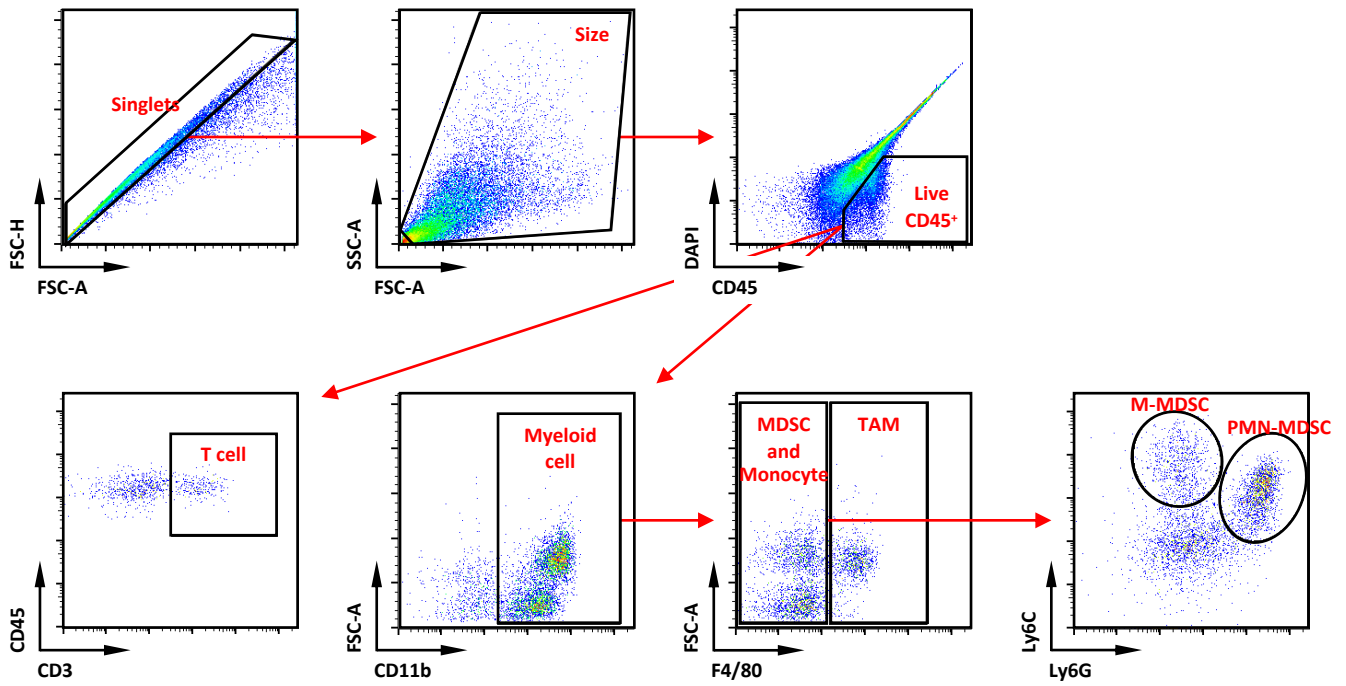


Fig. S1 Flow cytometry gating strategy that used to define each immune cell in the 4T1 tumors.

Singlets were selected from the FSC-A versus FSC-H dot plot, remained-erythrocytes and debris were removed based on FSC-A versus SSC-A, and dead cells were excluded with 4', 6-diamidino-2-phenylindole (DAPI), simultaneously, immune cells were gated on CD45⁺ cells, then a target population was expanded. The flow cytometry gating strategy used to define T cell (CD3⁺ cells), TAM (CD11b⁺F4/80⁺ cells), M-MDSC (CD11b⁺F4/80⁻Ly6C⁺Ly6G⁻ cells), and PMN-MDSC (CD11b⁺F4/80⁻Ly6C^{lo}Ly6G⁺ cells) within CD45⁺ cells infiltrated in the 4T1 tumors.

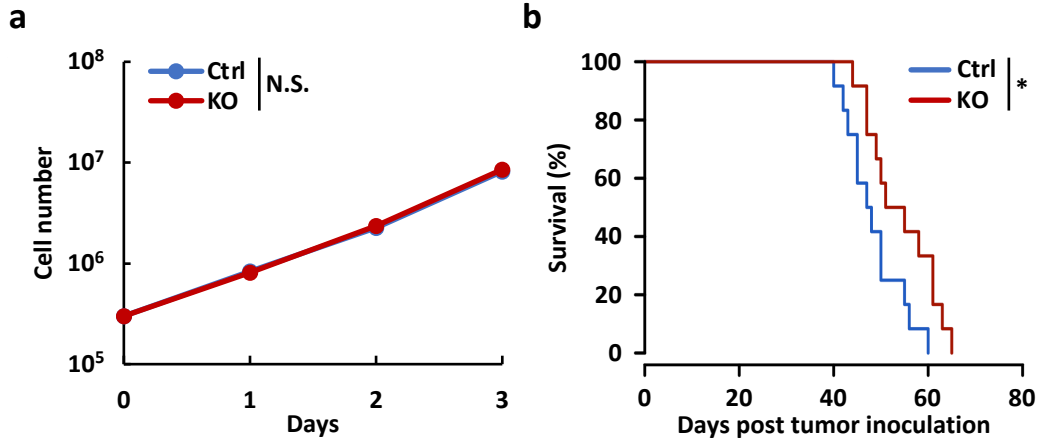


Fig. S2 Effects of IL-34 on in vitro cell proliferation and in vivo survival.

(a) Numbers of Ctrl and IL-34 KO 4T1 cells in cell culture were monitored for 3 days (n=3). (b) Kaplan–Meier plots of survival in mice inoculated with Ctrl and IL-34 KO 4T1 cells (1×10^5 cells, n=12). Values were analyzed by a two-sided Student's *t*-test (a) and Log-rank test (b) and are shown as mean \pm SEM. Asterisk indicates a significant difference; * $p < 0.05$, compared with the control group.

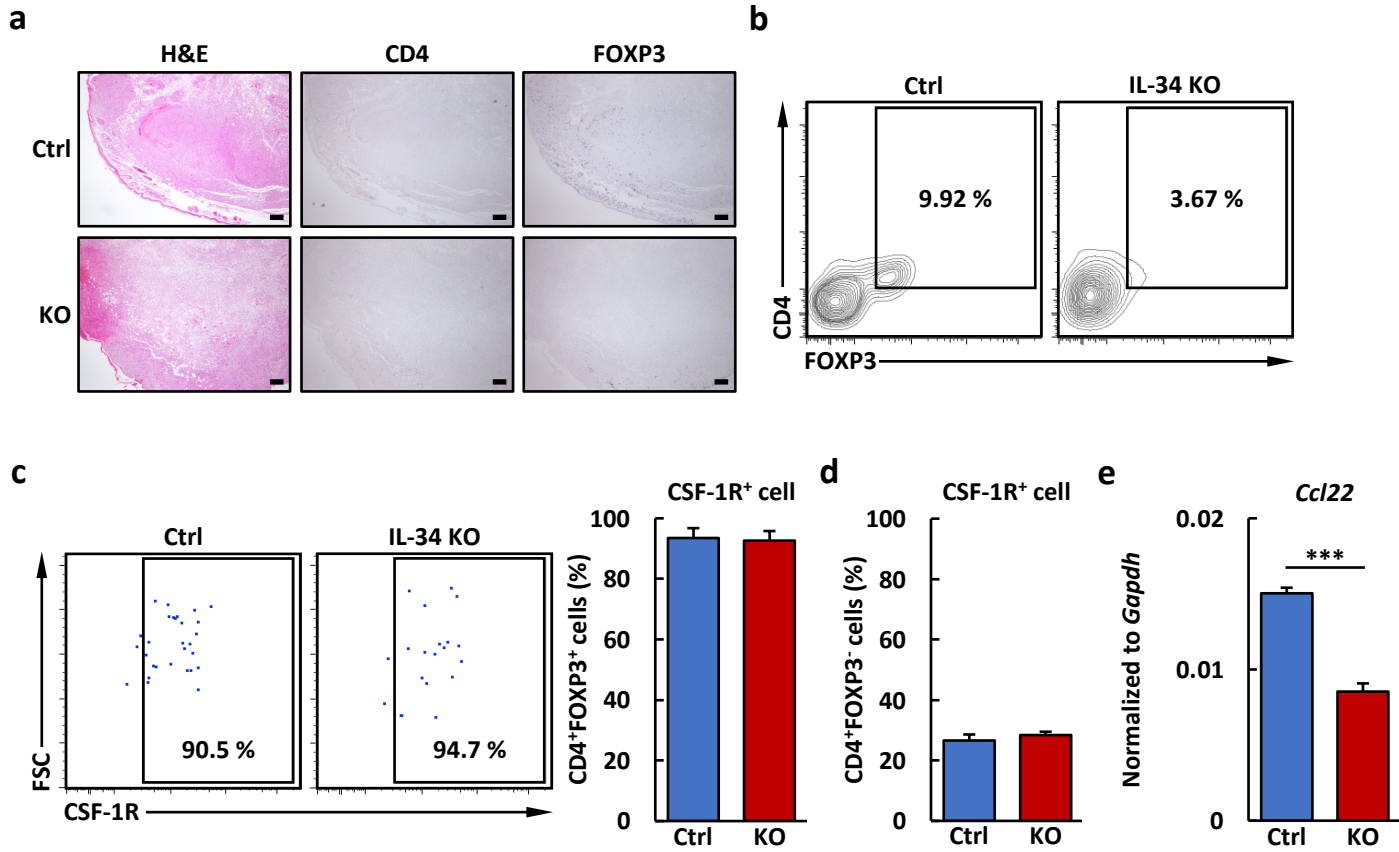


Fig. S3 Potential involvement of IL-34 in intra-tumoral Treg cells induction.

(a) Ctrl and IL-34 KO 4T1 cells (1×10^5 cells) were subcutaneously inoculated into BALB/c mice. Representative staining of H&E, CD4, and FOXP3 in injected Ctrl and IL-34 KO 4T1 tumors at the time of day 14. Scale bars=200 μ m. (b) Representative plots of Treg cell (CD4⁺FOXP3⁺ cells) within CD3⁺ cells infiltrated in the tumors described in Fig. S1a. (c) Representative plots (left). Bar graphs representing the frequency of CSF-1R⁺ Treg cell (CD4⁺FOXP3⁺CSF-1R⁺ cells) within CD4⁺FOXP3⁺ infiltrated in the tumors described in Fig. S1a (n=3, right). (d) Bar graphs representing the frequency of CSF-1R⁺ T cell except for Treg cell (CD4⁺FOXP3⁻CSF-1R⁺ cells) within CD4⁺FOXP3⁺ infiltrated in the tumors described in Fig. S1a (n=3). (e) M-MDSCs were sorted from cell suspensions of tumor-infiltrated immune cells at the time of day 14 from tumor inoculation. Bar graphs representing fold induction of *Ccl22* expression of M-MDSCs in Ctrl and IL-34 KO 4T1 tumors (n=3). Gene expression levels were determined by RT-PCR, as normalized to *Gapdh*. Values were analyzed by a two-sided Student's *t*-test (c-e) and are shown as mean \pm SEM. Asterisk indicates a significant difference; ***p<0.001, compared with the control group.

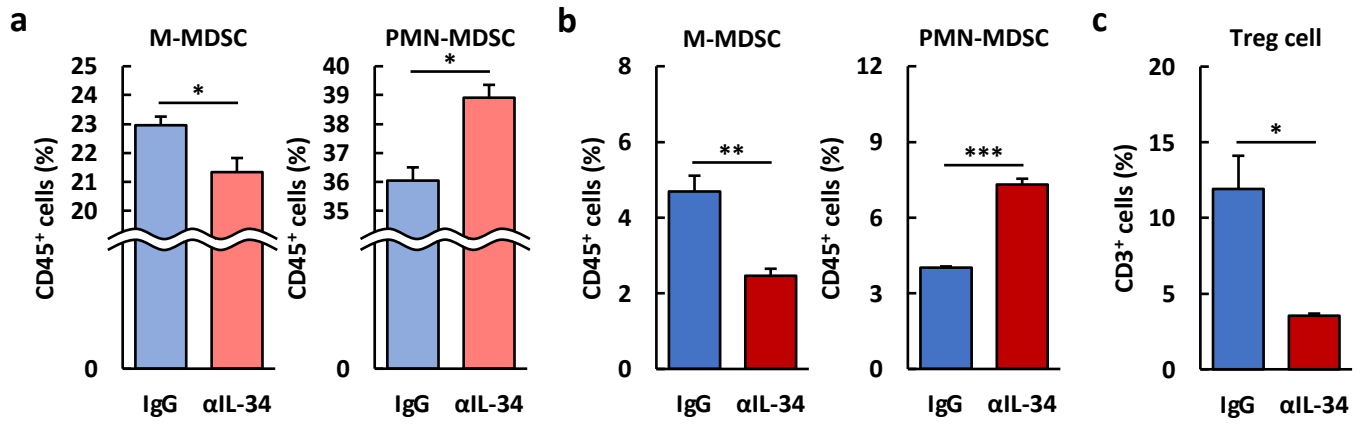


Fig. S4 Effects of IL-34 on TME within breast cancer patient-derived organoids and murine spontaneous TNBC tumors. (a) Anti-IL-34 antibodies (α IL-34) or control IgG were added to breast cancer patient-derived organoids containing immune cells, and MDSCs phenotype within the organoids was observed. Bar graphs representing the frequency of M-MDSC (CD11b⁺CD33⁺CD14⁺CD15⁻ cells) and PMN-MDSC (CD11b⁺CD33⁺CD15⁺CD14⁻ cells) within CD45⁺ cells (n=3). (b) Bar graphs representing the frequency of M-MDSC (CD11b⁺Ly6C⁺Ly6G⁻ cells) and PMN-MDSC (CD11b⁺Ly6C^{lo}Ly6G⁺ cells) within CD45⁺ cells infiltrated in the spontaneous tumors of MMTV-PyMT mice (n=3). (c) Bar graphs representing the frequency of Treg cell (CD3⁺CD4⁺FOXP3⁺ cells) within CD45⁺ cells infiltrated in the spontaneous tumors of MMTV-PyMT mice (n=3). Values were analyzed by a two-sided Student's *t*-test and are shown as mean \pm SEM. Asterisk indicates a significant difference; **p*<0.05, ***p*<0.01, ****p*<0.001, compared with the control group.

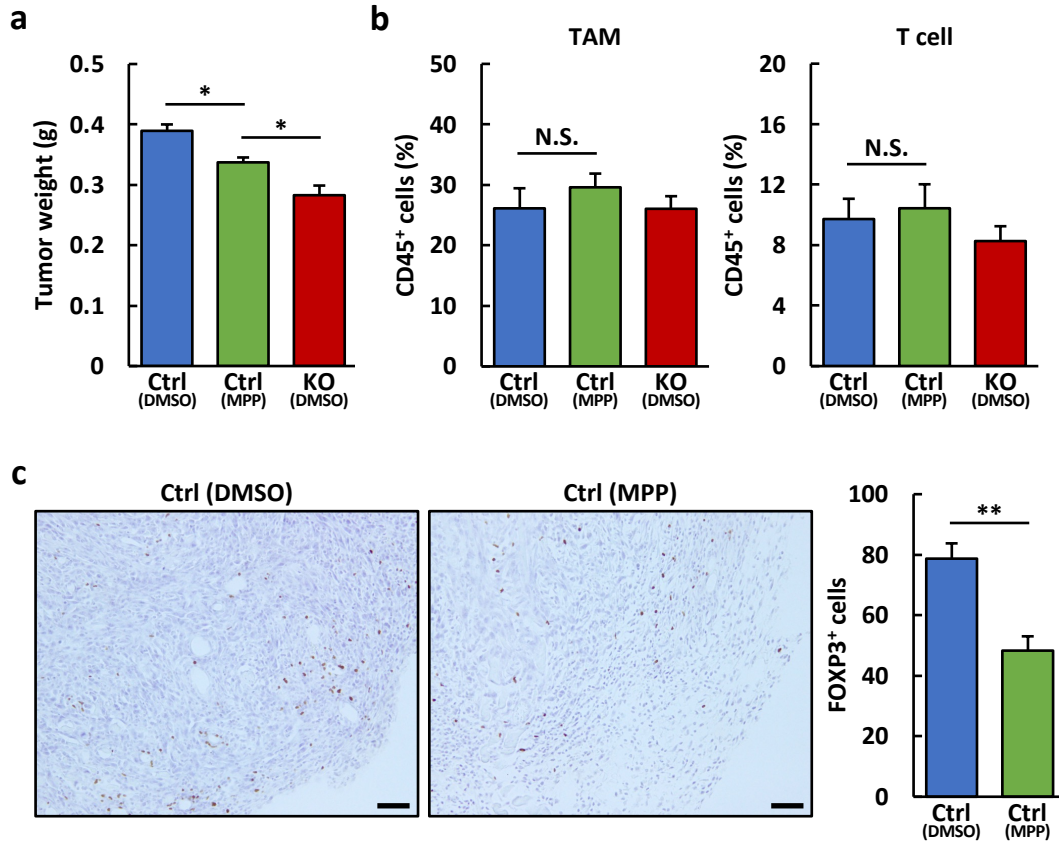


Fig. S5 Effects of MPP administration aimed at M-MDSCs depletion on tumor weight, tumor-infiltrating macrophages, and T cells, including Treg cells.

(a) Ctrl and IL-34 KO 4T1 cells (1×10^5 cells) were subcutaneously inoculated into BALB/c mice. MPP (1.0 mg/kg) or control DMSO was administered intraperitoneally daily for 5-14 days. Bar graphs representing tumor weight of harvested Ctrl and IL-34 KO 4T1 tumors at 14 days post tumor inoculation ($n=4-8$). (b) Bar graphs representing the frequency of TAM (CD11b⁺F4/80⁺ cells) and T cell (CD3⁺ cells) within CD45⁺ cells infiltrated in the tumors described in Fig. S4a ($n=4-8$). (c) Representative IHC staining of FOXP3 (red) in Ctrl tumors treated with/without MPP at the time of day 14 from tumor inoculation (left). Scale bars=50 μ m. Quantification of FOXP3⁺ cells in Ctrl tumors treated with/without MPP from a field of view ($n=3$, right). Values were analyzed by a one-way ANOVA with Tukey's test (a-b) and two-sided Student's *t*-test (c) and are shown as mean \pm SEM. Asterisk indicates a significant difference; * $p < 0.05$, ** $p < 0.01$, compared with the control group.

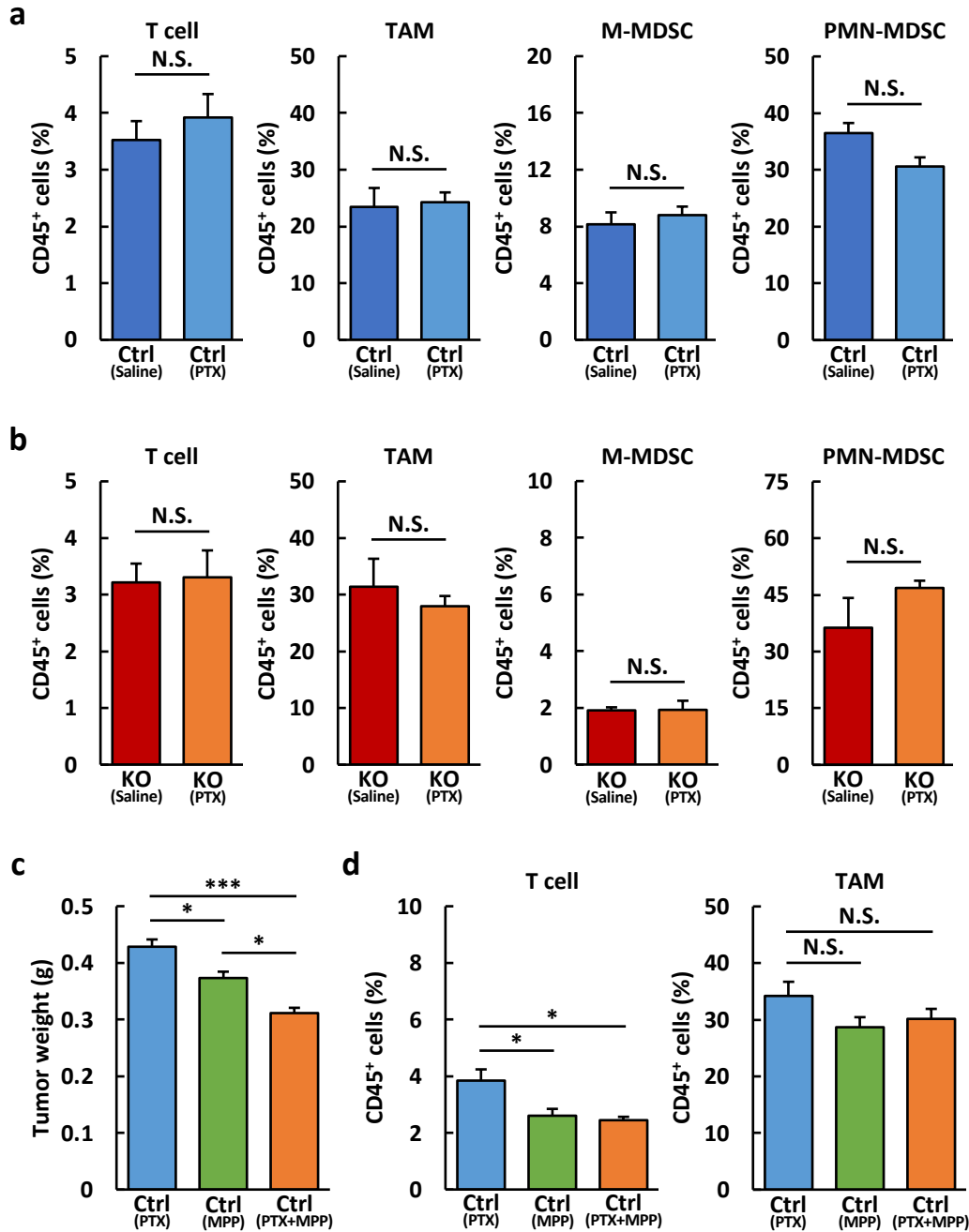


Fig. S6 Effects of PTX and/or MPP treatment on tumor-infiltrating immune cells and tumor weight in Ctrl and IL-34 KO 4T1 tumors.

(a-b) Ctrl and IL-34 KO 4T1 cells (1×10^5 cells) were subcutaneously inoculated into BALB/c mice. PTX (1.3 mg/kg) or control saline was administered intraperitoneally daily for 5-14 days. Bar graphs representing the frequency of T cell (CD3⁺ cells) and TAM (CD11b⁺F4/80⁺ cells) and M-MDSC (CD11b⁺Ly6C⁺Ly6G⁻ cells) and PMN-MDSC (CD11b⁺Ly6C^{lo}Ly6G⁺ cells) within CD45⁺ cells infiltrated in the Ctrl and IL-34 KO 4T1 tumors (n=6). (c) Ctrl 4T1 cells (1×10^5 cells) were subcutaneously inoculated into BALB/c mice. PTX (1.3 mg/kg), control Saline, MPP (1.0 mg/kg), control DMSO, or their combination was administered intraperitoneally daily for 5-14 days. Bar graphs representing tumor weight of harvested Ctrl 4T1 tumors at 14 days post tumor inoculation (n=12). (d) Bar graphs representing the frequency of T cell (CD3⁺ cells) and TAM (CD11b⁺F4/80⁺ cells) within CD45⁺ cells infiltrated in the Ctrl 4T1 tumors (n=12). Values were analyzed by a two-sided Student's *t*-test (a-b) and one-way ANOVA with Tukey's test (c-d) and are shown as mean \pm SEM. Asterisk indicates a significant difference; **p*<0.05, ****p*<0.001, compared with the control group.

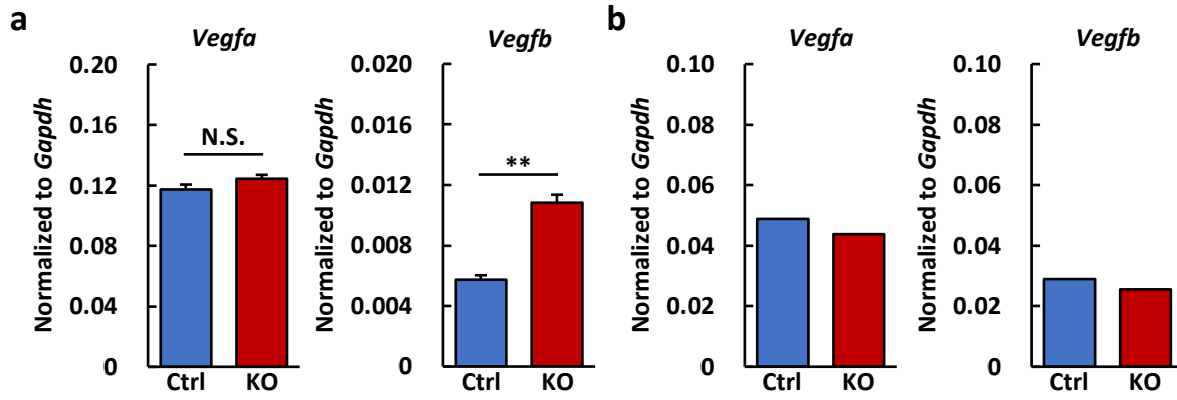


Fig. S7 The angiogenic ability of M-MDSCs and cancer cells in the presence or absence of IL-34.

(a) M-MDSCs were sorted from cell suspensions of tumor-infiltrated immune cells at the time of day 14 from tumor inoculation. Bar graphs representing fold induction of *Vegfa* and *Vegfb* expression of M-MDSCs in Ctrl and IL-34 KO 4T1 tumors (n=3). Gene expression levels were determined by RT-PCR, as normalized to *Gapdh*. (b) Bar graphs representing fold induction of *Vegfa* and *Vegfb* expression of Ctrl and IL-34 KO 4T1 cells. Gene expression levels were determined by RT-PCR, as normalized to *Gapdh*. Values were analyzed by a two-sided Student's *t*-test (a-b) and are shown as mean \pm SEM. Asterisk indicates a significant difference; **p<0.01, compared with the control group.

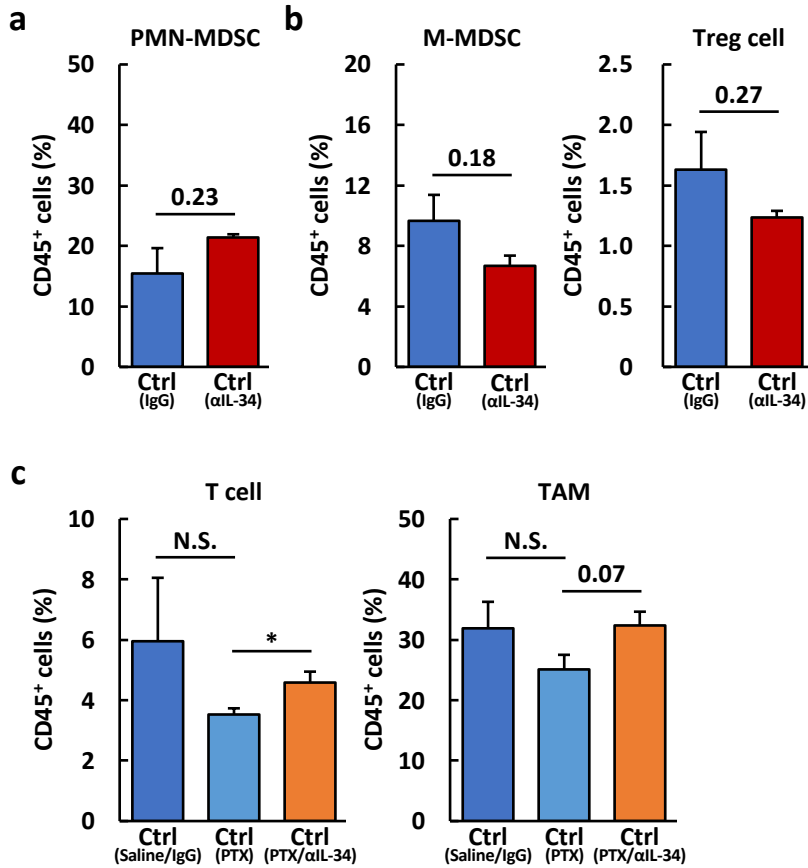


Fig. S8 Effects of IL-34 blockade and/or PTX treatment on tumor-infiltrating immune cells in Ctrl 4T1 tumors.

(a-b) Ctrl 4T1 cells (1×10^5 cells) were subcutaneously inoculated into BALB/c mice. Anti-IL-34 antibody (200 μ g) or control IgG was administered intraperitoneally daily for 5-14 days. Bar graphs representing the frequency of PMN-MDSC (CD11b⁺Ly6C^{lo}Ly6G⁺ cells) and M-MDSC (CD11b⁺Ly6C⁺Ly6G⁻ cells) and Treg cell (CD3⁺CD4⁺FOXP3⁺ cells) within CD45⁺ cells infiltrated in Ctrl 4T1 tumors treated with/without α IL-34 (n=6). (c) Bar graphs representing the frequency of T cell (CD3⁺ cells) and TAM (CD11b⁺F4/80⁺ cells) within CD45⁺ cells infiltrated in the tumors described in Fig. 5b (n=6-12). Values were analyzed by a two-sided Student's *t*-test (a-b) and one-way ANOVA with Tukey's test (c) and are shown as mean \pm SEM. Asterisk indicates a significant difference; *p<0.05, compared with the control group.

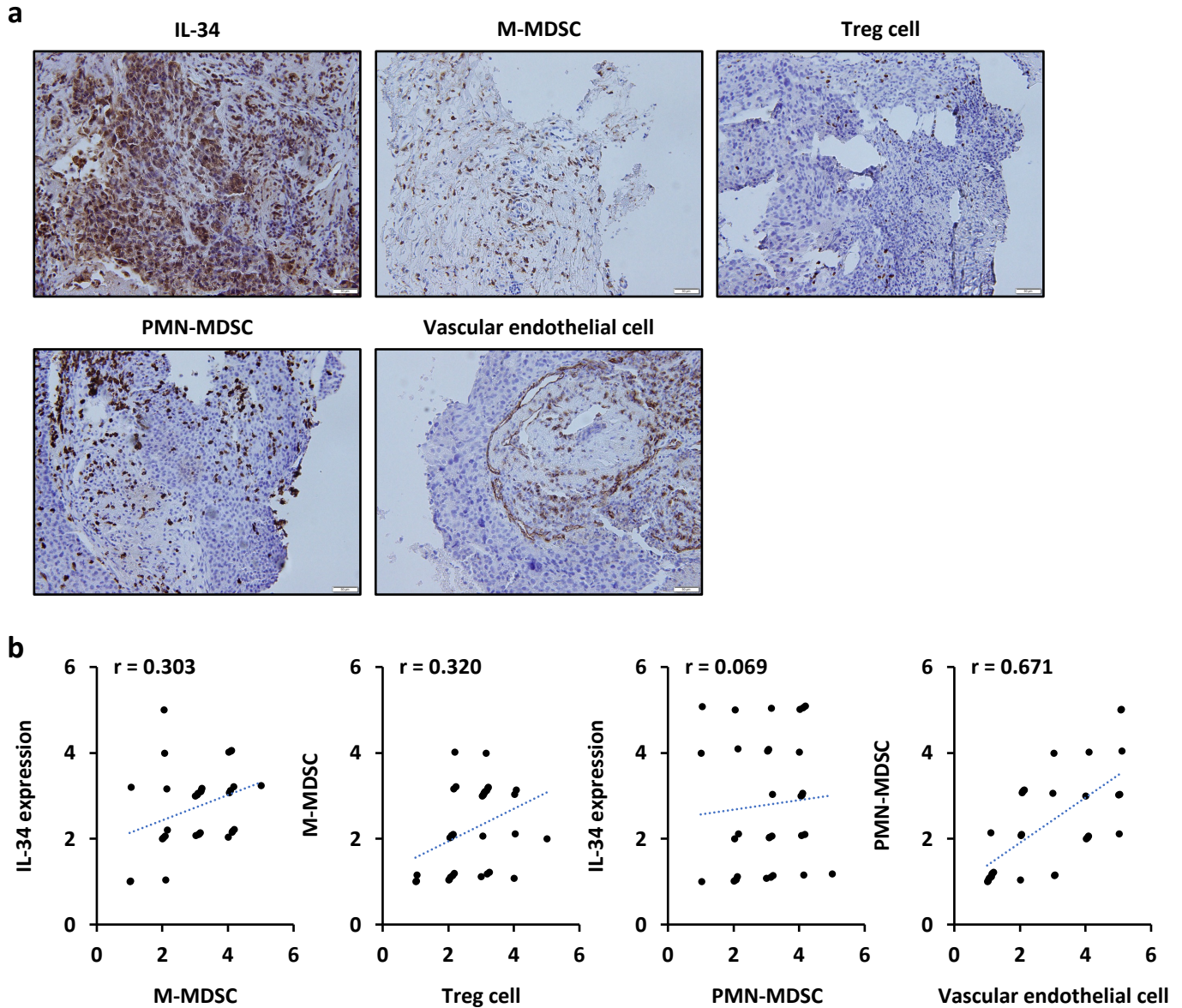


Fig. S9 Potential involvement of IL-34-MDSCs axis in real-world human TNBC.

(a) Representative staining of IL-34, CD14 (used as M-MDSC marker), FOXP3 (used as Treg cell marker), CD15 (used as PMN-MDSC marker), and CD31 (used as vascular endothelial cell marker) in formalin-fixed paraffin-embedded TNBC tissues. Scale bars=50 μ m. (b) Pearson correlation coefficient between IL-34 expression in tumors and intra-tumoral M-MDSCs infiltration, intra-tumoral M-MDSCs infiltration and intra-tumoral Treg cells infiltration, IL-34 expression in tumors and intra-tumoral PMN-MDSCs infiltration, intra-tumoral PMN-MDSCs infiltration and intra-tumoral vascular endothelial cells infiltration are shown.