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Rae1 drives NKG2D binding-dependent tumor development in mice by activating mTOR and STAT3 pathways in tumor cells

Peiyan Zhao[1] | Lei Yang[1] | Xin Li[1] | Wenting Lu[1] | Fangjie Lu[1] | Shengnan Wang[1] | Ying Wang[1] | Li Hua[2] | Cuiyun Cui[2] | Boqi Dong[2] | Yongli Yu[2] | Liying Wang[1]

[1]Department of Molecular Biology, College of Basic Medical Sciences and Institute of Pediatrics, The First Hospital of Jilin University, Jilin University, Changchun, China

[2]Department of Immunology, College of Basic Medical Sciences, Jilin University, Changchun, China

Correspondence
Liying Wang or Yongli Yu, Department of Molecular Biology, College of Basic Medical Sciences and Institute of Pediatrics, The First Hospital of Jilin University or Department of Immunology, College of Basic Medical Sciences, Jilin University, Changchun, China.
Email: wangliy@jlu.edu.cn or ylyu@jlu.edu.cn

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Abstract

Natural killer group 2 member D (NKG2D) ligands (NKG2DLs) on tumor cells engage NKG2D and mediate killing by NKG2D$^+$ immune cells. However, tumor cells with high levels of NKG2DLs are still malignant and proliferate rapidly. We investigated the reason for NKG2DL-expressing cell progression. Tumor cells in mice were assessed for their NKG2DL expression, ability to attract immune cells, tumorigenicity, mTOR, and signal transducer and activator of transcription 3 (STAT3) signaling activation. Antibody blockade was used to determine the effect of NKG2DL-NKG2D interaction on signaling activation in vitro. Retinoic acid early inducible gene 1 (Rae1) was related to the expression of other NKG2DLs, the promotion of tumorigenicity, *Mmp2* expression, mTOR and STAT3 phosphorylation in GL261 cells, and the recruitment of NKG2D$^+$ cells in mice. Rae1 also induced NKG2DL expression, mTOR, and STAT3 phosphorylation in GL261 cells and LLC cells, but not in B16 and Pan02 cells, which did not express NKG2DLs, when cocultured with PBMCs; the induced phosphorylation was eliminated by Rae1-NKG2D blockade. Inhibition of mTOR and/or STAT3 decreased PBMC-induced migration and proliferation of GL261 cells in vitro. Rae1, a NKG2DL on tumor cells, plays a driving role in the expression of other NKG2DLs and in tumor development in mice by activating mTOR and STAT3 pathways, relying on its interaction with NKG2D on immune cells.

KEYWORDS

mTOR, natural killer group 2 member D ligands, retinoic acid early inducible gene 1, signal transducer and activator of transcription 3, tumor development

1 | INTRODUCTION

The activating receptor NKG2D is present on NK cells and on a subset of T cells, and its ligands are recognized as a potent immune axis for controlling tumor growth and microbial infections.[1] NKG2DLs are cell surface proteins structurally related to MHC. MHC-I chain-related proteins A and B (MICA/B) and a family of unique long 16-binding proteins (ULBPs) are the NKG2DLs in

Abbreviations: H60, murine minor histocompatibility 60 protein; i.c., intracranially; IFN-γ, interferon-gamma; LN, lymph node; MICA/B, MHC-I chain-related protein A and B; MULT1, murine UL16-binding protein-like transcript 1; NK, natural killer; NKG2D, natural killer group 2 member D; NKG2DL, NKG2D ligand; Rae1, retinoic acid early inducible gene 1; s.c., subcutaneously;STAT3, signal transducer and activator of transcription 3; ULBP, unique long 16-binding protein.

humans. Murine minor histocompatibility 60 protein (H60), Rae1 and murine UL16-binding protein-like transcript 1 (MULT1) are the NKG2DLs in mice.[2] In general, NKG2DLs are not expressed on the surface of healthy cells but can be induced on tumor cells. Therefore, the NKG2D-NKG2DL axis serves as a mechanism for immune surveillance to detect and eliminate tumor cells. NKG2DL-expressing cells are susceptible to killing by NKG2D-expressing immune cells.[3-5] Together, high levels of expression of NKG2DLs on tumor cells or large numbers of recruited NKG2D$^+$ immune cells are conducive to tumor rejection. But this is not always the case.

Highly NKG2DL-expressing tumors still develop rapidly in the presence of sufficient NKG2D$^+$ immune cells. The levels of NKG2DLs found in breast cancer, lung cancer, and ovarian cancer[6,7] are inversely proportional to patient survival. Studies on mice with genetically modified prostate cancer determined that slow-growing tumors with high levels of NKG2DLs cannot be eliminated by NKG2D$^+$ immune cells, but grow faster when large numbers of NKG2D$^+$ immune cells have infiltrated.[3] Fibrosarcomas with high-level NKG2DLs tend to develop earlier in NKG2D-sufficient mice than in NKG2D-deficient mice.[3] In a hepatocellular carcinoma model, Rae1 high-expressing tumor cells developed a large tumor in NKG2D-sufficient mice, which had reduced survival compared with NKG2D-dificient mice,[8] suggesting an accelerating role for NKG2D in the development of NKG2DL-expressing tumors. In humans, glioma cells were shown to express various NKG2DLs,[9-11] but glioma still represents one of the deadliest types of brain tumor, manifested by a high rate of recurrence and poor prognosis.[12-14] To date, it has not been clear how NKG2DL-expressing tumor cells can escape killing by NKG2D$^+$ immune cells.

Generally, NKG2D$^+$ immune cells, such as NK cells, display their killing activities through the release of numerous deadly proteins or cytokines such as interferon-gamma (IFN-γ) after activation by NKG2DLs on their target cells, such as tumor cells.[15-17] However, IFN-γ has been demonstrated to promote the progression of lung cancers[18] and melanomas[19] via the JAK-STAT and PI3K-AKT-mTOR signaling pathways. Whether NKG2DLs on tumor cells are not sufficient to induce the cytotoxic effects of NKG2D$^+$ immune cells, and thereby promote tumor development, is still unclear. The sustained engagement of NKG2D and NKG2DLs could reduce NK cell responsiveness[20] and increase the tumor burden of skin cancers[21] and the incidence of B cell lymphomas.[4] However, the effect on tumor cells of the NKG2DL-NKG2D interaction has been studied to a lesser extent.

In this study, we selected GL261 glioma cells and LLC cells that had high expression levels of Rae1, Rae1 knockout GL261 cells, and B16 and Pan02 cells without NKG2DL expression, as subjects to determine if NKG2DLs on tumor cells were able to promote tumor development along with immune cell recruitment and to investigate how this process occurs. This study will provide new insight into the tumor-promoting effects of the NKG2DL-NKG2D axis, which has been considered as a target for immunotherapy.

2 | MATERIALS AND METHODS

2.1 | Construction of the plasmids

The CRISPR/Cas9 system was used to knock out the *Rae1* gene. Plasmids for the *Rae1* gene knockout and GFP gene expression were made by the YSY Biotech Company Ltd and named Cas9-Rae1 KO. The GFP coding sequence was cloned into the pcDNA3 plasmid, verified by DNA sequencing, and named pcDNA3-GFP.

2.2 | Cells and cell lines

C57BL/6 mouse-derived GL261 glioma cells (American Type Culture Collection) were transfected with Cas9-Rae1 KO and pcDNA3-GFP plasmids using Lipofectamine 3000 (Invitrogen) and selected with G418 antibiotic (Millipore). C57BL/6 mouse-derived GL261 cells, LLC Lewis lung carcinoma cells, B16 melanoma cells and Pan02 pancreatic cancer cells (American Type Culture Collection) were maintained at 37°C in RPMI 1640 medium supplemented with 10% (v/v) FBS and antibiotics (100 IU/ml of penicillin and streptomycin) in a 5% CO_2 in air humidified incubator.

2.3 | Mice

Six- to eight-wk-old female C57BL/6 mice were purchased from the Experimental Animal Center, Medical College of Norman Bethune, Jilin University (Changchun, China). Experimental manipulation of mice was undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Science & Technology of Jilin Province.

2.4 | Establishment of mouse tumor models

For the intracranial glioma mouse model, mice were anesthetized and injected with 1×10^5 GL261 cells at 2 mm to the right of the bregma and 3 mm deep using a stereotaxic instrument (Kopf Instruments). After surgery, mice showing pathologic signs caused by the surgery was excluded from the experiment. For the subcutaneous mouse model, tumor cells were injected subcutaneously into the right back near the hind leg of the mice. Tumor volume = length × width^2 × 0.5.

2.5 | Cytotoxicity assay

Here, 4×10^3 GL261 cells (T) per well were seeded into 96-well plates and cocultured with murine splenocytes (E) at the E/T ratio of 200:1. After 4 h, lysate of GL261 cells was detected using an LDH Cytotoxicity Assay Kit. Absorbance at 490 nm was measured using a microplate reader (Synergy H1M).

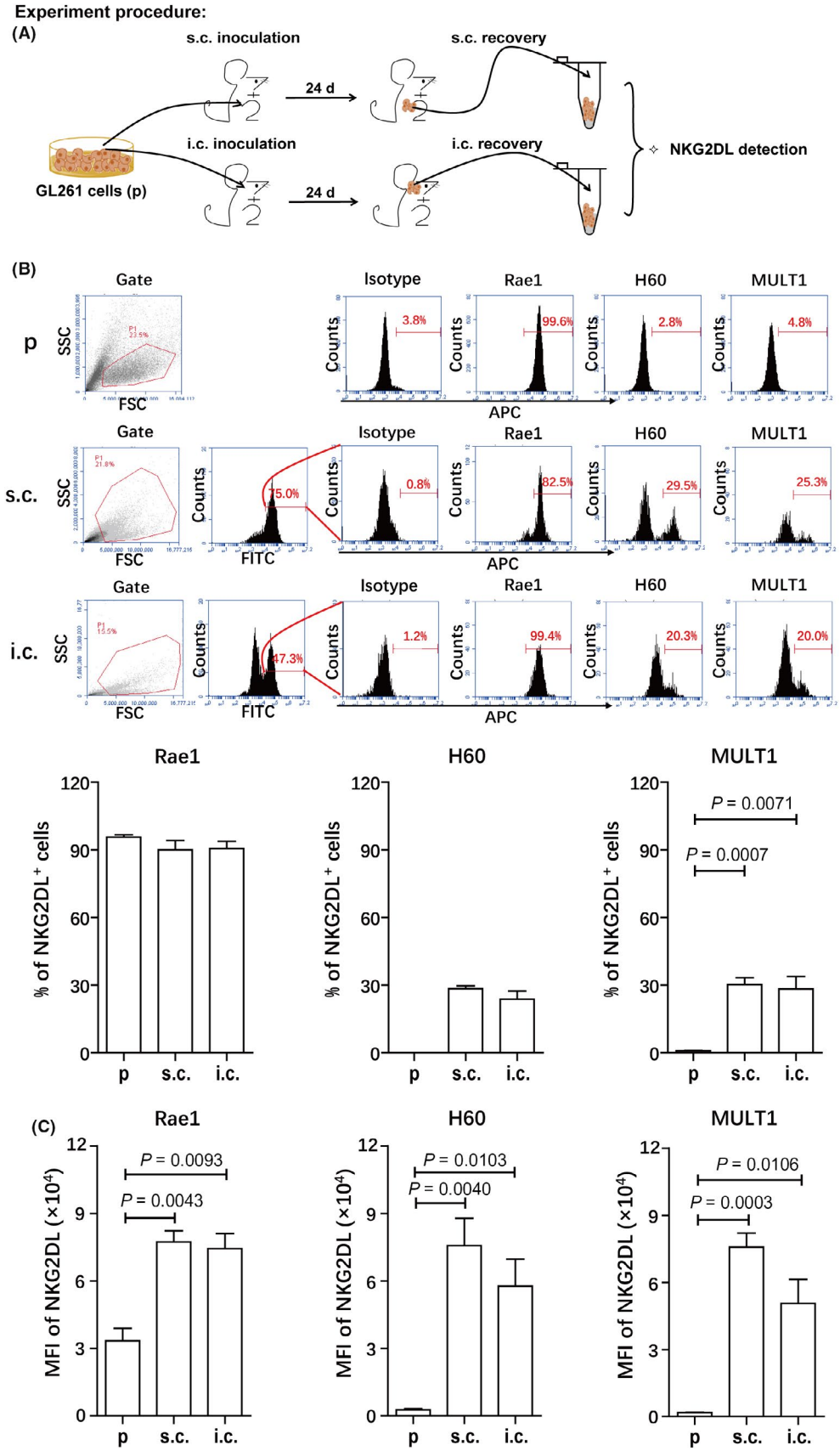

FIGURE 1 Effect of in vivo inoculation on NKG2DL expression in GL261 cells. As shown in (A) parental GL261 cells (p) were inoculated intracranially (i.c.) or subcutaneously (s.c.) into mice. After 24 d, GL261 cells were recovered and the percentage of NKG2DL (Rae1, H60 and MULT1)-expressing GL261 cells (B) and the NKG2DL levels (C) were tested by flow cytometry. Data are represented as mean ± SD (n = 3)

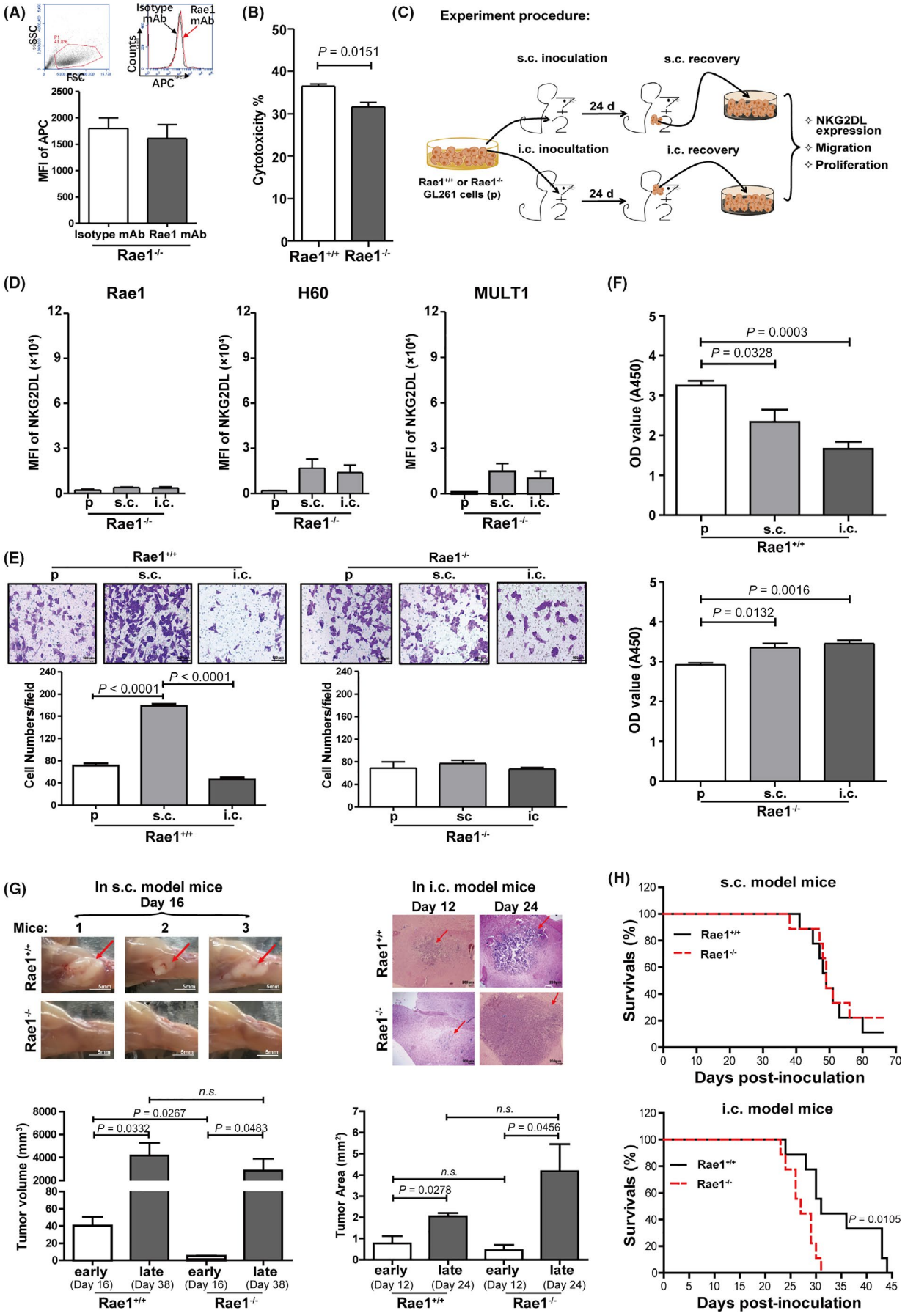
(A)
SSC
FSC
Isotype mAb
Rae1 mAb
Counts
APC
MFI of APC
Isotype mAb Rae1 mAb
Rae1-/-
(B)
P = 0.0151
Cytotoxicity %
Rae1+/+ Rae1-/-
(C)
Experiment procedure:
s.c. inoculation
s.c. recovery
24 d
i.c. inocultation
i.c. recovery
Rae1+/+ or Rae1-/-
GL261 cells (p)
NKG2DL expression
Migration
Proliferation
(D)
Rae1
H60
MULT1
MFI of NKG2DL (×10⁴)
p s.c. i.c.
Rae1-/-
(E)
Rae1+/+
Rae1-/-
p s.c. i.c.
Cell Numbers/field
P < 0.0001
P < 0.0001
p sc ic
(F)
P = 0.0003
P = 0.0328
OD value (A450)
Rae1+/+
P = 0.0016
P = 0.0132
Rae1-/-
(G)
In s.c. model mice
Day 16
Mice: 1 2 3
Rae1+/+
Rae1-/-
In i.c. model mice
Day 12
Day 24
n.s.
P = 0.0267
P = 0.0332
P = 0.0483
Tumor volume (mm³)
early (Day 16) late (Day 38) early (Day 16) late (Day 38)
n.s.
P = 0.0456
n.s.
P = 0.0278
Tumor Area (mm²)
early (Day 12) late (Day 24) early (Day 12) late (Day 24)
(H)
s.c. model mice
Survivals (%)
Days post-inoculation
i.c. model mice
P = 0.0105

FIGURE 2 Effects of Rae1 on NKG2DL expression, susceptibility to NK cell killing, and tumorigenicity of GL261 cells. $Rae1^{-/-}$ GL261 cells were identified by detecting their Rae1 expression (A) and susceptibility to splenocyte killing (B). As shown in (C), parental $Rae1^{+/+}$ and $Rae1^{-/-}$ GL261 cells (p) were inoculated subcutaneously (s.c.) or intracranially (i.c.) into mice and recovered to evaluate their NKG2DL expression (D), migration capacity (E), and proliferation capacity (F). Tumor growth was evaluated by the tumor volume (left panel in G) of s.c. inoculated mice, tumor area (right panel in G) of i.c. inoculated mice, and their survival rate (9 mice/group) (H). Red arrows indicate the tumor site; *n.s.* denotes non-significant. Data are represented as mean ± SD (n = 3)

2.6 | Proliferation assay

Here, 2×10^3 cells per well were seeded into 96-well plates and cultured for 4 d. Live cell numbers were determined using a cell counting kit CCK-8 (TransGen Biotech). Absorbance at 450 nm was measured using a microplate reader (Synergy H1M).

2.7 | Transwell assay

Here, 5×10^4 cells per well were seeded into the upper chamber of 24-well BD BioCoat Matrigel coated plates (Corning Life Sciences). After 24 h, migrated cells were fixed and stained with crystal violet.

2.8 | RNA isolation and qRT-PCR analysis

Total RNA isolation and qRT-PCR analysis were performed as previously described.[22] Sequences of specific primers are: *Actb*, forward: 5′-GATCAAGATCATTGCTCCTCCTG-3′, reverse: 5′-AGGGTGTAAAACGCAGCTCA-3′; *Mmp2*, forward: 5′-CATCGTAGTTGGTTGTGGTCG-3′, reverse: 5′-GTCTTCCCCTTCACTTTCCTG-3′; *Ifng*, forward: 5′-AACTGGCAAAAGGATGGTGA-3′, reverse: 5′-GCTGTTGCTGAAGAAGGTAG-3′. mRNA levels were normalized to the mRNA levels of *Actb* and analyzed using the $2^{-\Delta\Delta Ct}$ method.

2.9 | Purity assay

Tumor cells (4×10^5/well) were seeded into 6-well plates and cultured to confluency to reduce the adhesion of PBMCs. After co-culturing with PBMCs (4×10^7/well) for 48 h, the suspended and adherent cells were separated and detected for their expression of CD45, a leukocyte-specific antigen,[23] using flow cytometry. The purity of the tumor cells and the PBMCs is shown (Figure S1) as percent of $CD45^+$ cells.

2.10 | mAb blocking assay

PBMCs or tumor cells were pretreated with anti-NKG2D mAb (BD Biosciences) or anti-NKG2DL mAbs (R&D system) for 4 h. After washing with PBS, tumor cells (4×10^5/well) were cocultured with PBMCs (4×10^7/well) in 6-well plates for 48 h and then collected.

2.11 | mTOR and/or STAT3 inhibition assay

GL261 cells (4×10^5/well) were cultured in medium with rapamycin (50 nmol/L) and/or NSC 74859 (150 μmol/L) (MedChemExpress) for 24 h in 6-well plates and then washed to remove residual rapamycin and/or NSC 74859. After coculturing with PBMCs (4×10^7/well) for 48 h, the adherent tumor cells were collected to test their migration and proliferation capacity.

2.12 | Western blotting

Western blotting was conducted as described previously.[22] Antibodies against GAPDH, JAK2, Phospho-JAK2, Stat3, Phospho-Stat3, AKT or mTOR (Cell Signaling Technology), and horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Proteintech) were used. The blotting was examined using chemiluminescence (TransGen Biotech) and analyzed with ImageJ software (NIH, USA).

2.13 | Co-immunoprecipitation

Cell lysates were incubated with anti-phosphoserine mAb (Abcam) at 4°C overnight, and then, Protein G Agarose Beads (Cell Signaling Technology) were added and incubated at 4°C for 2 h followed by western blotting analysis.

2.14 | Flow cytometry

Fluorescence-conjugated antibodies against mouse CD45, CD8a, CD314, NK1.1, H-2Kb molecules and isotype IgG (BD Biosciences), the antibodies against mouse Rae1, H60, MULT1, isotype IgG (R&D Systems) and Alexa Fluor 647 goat anti-rat (Bioss) secondary antibodies were used. All stained cells were analyzed using Accuri C6 (BD Biosciences).

2.15 | Statistical analysis

All calculations and statistical analyses were performed using GraphPad Prism 5.0 for Windows software. Survival rates for mice were compared using the Kaplan-Meier test. Comparisons between groups were conducted using analysis of unpaired *t* tests. Differences were considered statistically significant for *P*-values <.05.

3 | RESULTS

3.1 | Effect of in vivo growth on NKG2DL expression of GL261 cells

As reported, glioma cells were identified as expressing various NKG2DLs in vitro but still developed into deadly tumors with high recurrence,[9-14] suggesting that glioma cells could change their NKG2DL expression in vivo to escape killing by NKG2D$^+$ immune cells. To verify this, we selected GL261 cells, a glioma cell line cell, and subcutaneously (s.c.) or intracranially (i.c.) inoculated these cells into mice. After 24 d, GL261 cells were recovered from the formed tumors, and immediately tested for their NKG2DL (Rae1, H60 and MULT1) expression using flow cytometry (Figure 1A). Results showed that either 90%, none, or 1% of parental (in vitro-cultured) GL261 cells expressed Rae1, H60, or MULT1, but in contrast about 90%, 28%, or 25% of s.c. and i.c. recovered GL261 cells expressed Rae1, H60, or MULT1, respectively (Figure 1B). Moreover, compared with the parental GL261 cells, the expression levels of Rae1, H60, and MULT1 on i.c. or s.c. recovered GL261 cells were 2.3-, 28-, and 22-fold increased or 2.3-, 43-, and 30-fold increased, respectively (Figure 1C). These results suggested that the Rae1 on GL261 cells is constitutive out of the NKG2DLs.

3.2 | Effect of Rae1 on NKG2DL expression and tumor development of GL261 cells

To observe whether Rae1 could drive in vivo induction of NKG2DLs and, therefore, influence tumorigenicity of GL261 cells, we first established a Rae1-deficient GL261 cell line (Rae1$^{-/-}$ GL261 cells) as a cell model by knocking out *Rae1d* and *Rae1e* genes, being the only 2 copies of *Rae1* in the C57BL/6 genome,[24] in GL261 cells, using the CRISPR/Cas9 system. Rae1$^{-/-}$ GL261 cells were confirmed by detection of Rae1 expression (Figure 2A). Compared with Rae1$^{+/+}$ GL261 cells, Rae1$^{-/-}$ GL261 cells displayed decreased susceptibility to splenocyte killing (Figure 2B) and increased MHC-I expression (Figure S2). To examine the effect of Rae1 on NKG2DL expression and tumorigenicity of GL261 cells, parental Rae1$^{+/+}$ and Rae1$^{-/-}$ GL261 cells were inoculated s.c. or i.c. into mice and then cells recovered (Figure 2C). Results showed that s.c. or i.c. inoculation of Rae1$^{-/-}$ GL261 cells failed to affect Rae1 expression, but increased H60 expression by 7- or 4-fold, and increased MULT1 expression by 6- or 7-fold, respectively (Figure 2D). The s.c. recovered Rae1$^{+/+}$ GL261 cells, but not the i.c. recovered Rae1$^{+/+}$ GL261 cells, generated 1.5-fold more migrated cells (Figure 2E). Both the s.c. and i.c. recovered Rae1$^{-/-}$ GL261 cells were increased in number (Figure 2F). Next, tumor-bearing mice were observed for their tumor development and survival rate. On day 16 after s.c. inoculation or day 12 after i.c. inoculation, the mice inoculated with Rae1$^{-/-}$ GL261 cells developed nonvisible or smaller tumors than mice inoculated with Rae1$^{+/+}$ GL261 cells. On day 38 after s.c. inoculation or day 24 after i.c. inoculation, the mice inoculated with Rae1$^{-/-}$ GL261 cells developed similar or larger sized tumors (Figure 2G). Furthermore, mice i.c. inoculated with Rae1$^{-/-}$ GL261 cells were shorter lived than mice i.c. inoculated with Rae1$^{+/+}$ GL261 cells. In contrast, survival rates of mice s.c. inoculated with Rae1$^{+/+}$ or Rae1$^{-/-}$ GL261 cells were similar (Figure 2H). Together, the results suggested that Rae1 deletion in GL261 cells could reduce NKG2DL expression and therefore inhibit growth of s.c. inoculated tumors in the early stages.

3.3 | Role of Rae1 on GL261 cells in recruitment of immune cells to the tumor site

To study whether Rae1 on GL261 cells could affect recruitment of immune cells to the tumor site and therefore affect early tumor size in mice, we examined the proportion of infiltrated immune cells in the s.c. or i.c. tumor sites of the mice inoculated with Rae1$^{+/+}$ or Rae1$^{-/-}$ GL261 cells (Figure 3A). Histopathologically, on day 12, the number of immune cells was 3-fold larger in the i.c. or s.c. tissues inoculated with Rae1$^{+/+}$ GL261 cells than that in the tissues inoculated with Rae1$^{-/-}$ GL261 cells (Figure 3B). For flow cytometry analysis, on days 4, 8, and 12 post inoculation, the numbers of NKG2D$^+$ immune cells in i.c. or s.c. tissues inoculated with Rae1$^{+/+}$ GL261 cells were more than in tissues inoculated with Rae1$^{-/-}$ GL261 cells (Figure 3C). Lack of Rae1 in GL261 cells could markedly reduce infiltration of CD8$^+$ T cells and NK1.1$^+$ cells into s.c. tumor sites, but not to i.c. tumor sites (Figure 3D,E). These results revealed that Rae1 on GL261 cells could be required for recruitment of NKG2D$^+$ cells to the tumor sites.

3.4 | Effect of Rae1 on the activation of malignancy-related molecules in GL261 cells

To examine the effect of Rae1 on malignancy of GL261 cells in vivo, Rae1$^{+/+}$ and Rae1$^{-/-}$ GL261 cells were inoculated i.c. or s.c. into mice and cells recovered on day 24 to detect the activation of malignancy-related molecules (Figure 4A). As MMP2, mTOR and STAT3 signaling is associated with metastasis of tumor cells[25-31] and mTOR and STAT3 signaling could activate E2F, which can upregulate NKG2DLs,[32-34] we determined the mRNA levels of *Mmp2* and phosphorylation of mTOR and STAT3 signaling in recovered Rae1$^{+/+}$ and Rae1$^{-/-}$ GL261 cells. The results showed that expression levels of *Mmp2* in s.c. tumor cells were significantly decreased when Rae1 was knocked out (Figure 4B). Phosphorylation of STAT3 was increased in i.c. or s.c. recovered Rae1$^{+/+}$ and Rae1$^{-/-}$ GL261 cells (Figure 4C-E). Phosphorylation of mTOR was increased in s.c. recovered Rae1$^{+/+}$ GL261 cells, but not in i.c. recovered Rae1$^{+/+}$ GL261 cells or in i.c. or s.c. recovered Rae1$^{-/-}$ GL261 cells (Figure 4F-H). These results suggested that the Rae1 may promote GL261 cell malignancy in vivo by activating mTOR signaling.

3.5 | Role of NKG2DL-NKG2D interaction on activation of mTOR and STAT3 signaling in GL261 cells

Upon observation that in vivo growth increased NKG2DL expression, migration capacity, and mTOR phosphorylation of GL261 cells, we

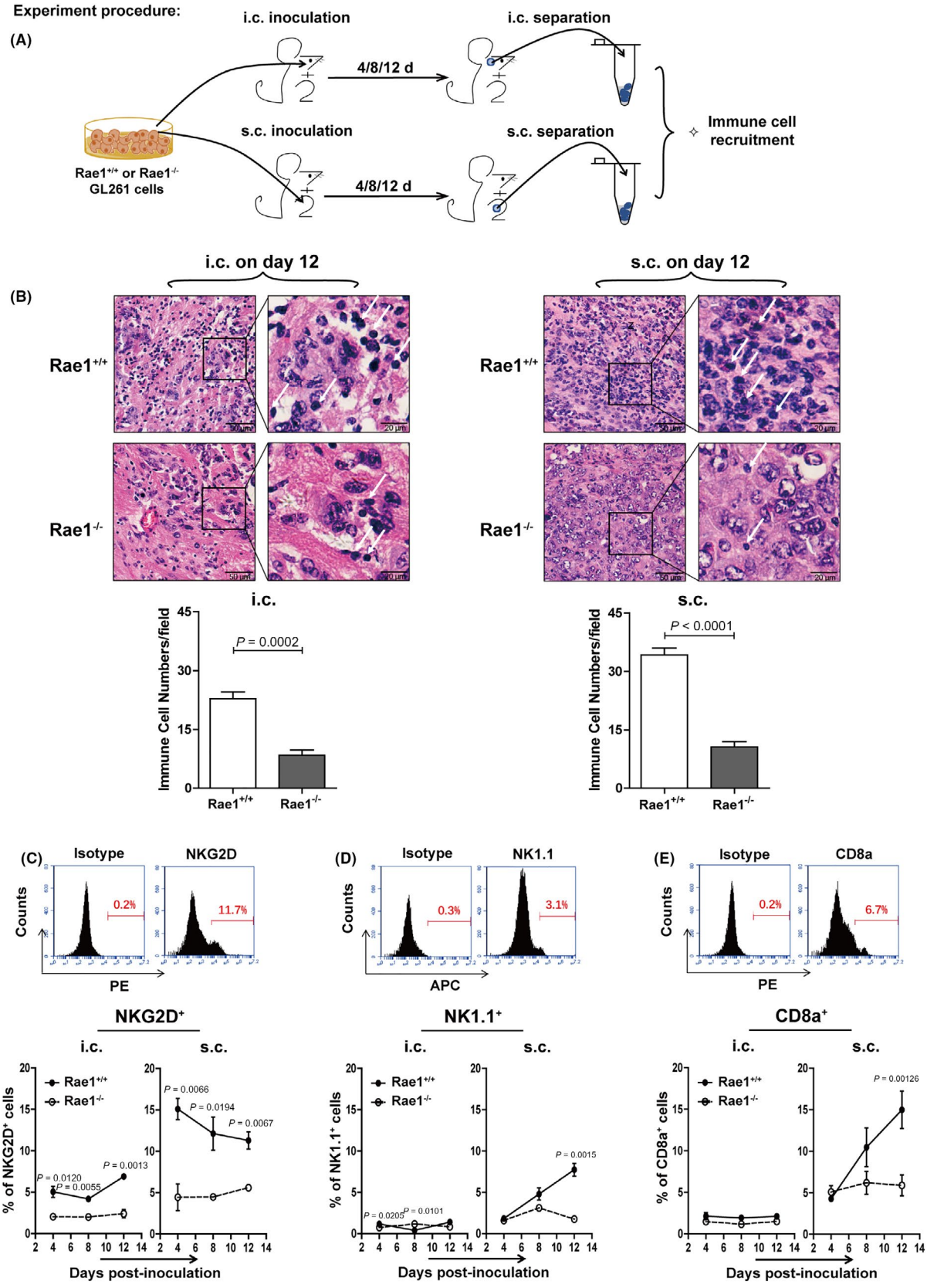

FIGURE 3 Effect of Rae1 on immune cells recruited by in vivo inoculated GL261 cells. As shown in (A), the Rae1$^{+/+}$ and Rae1$^{-/-}$ GL261 cells were inoculated subcutaneously (s.c.) or intracranially (i.c.) into mice on day 0. On day 12, the i.c. and s.c. tissues were isolated and observed for recruited immune cells (B). On days 4, 8, and 12, the percentages of NKG2D^{+} cells (C), NK1.1^{+} cells (D), and CD8a^{+} cells (E) in the tissues were determined by flow cytometry. Data are represented as mean ± SD (n = 3)

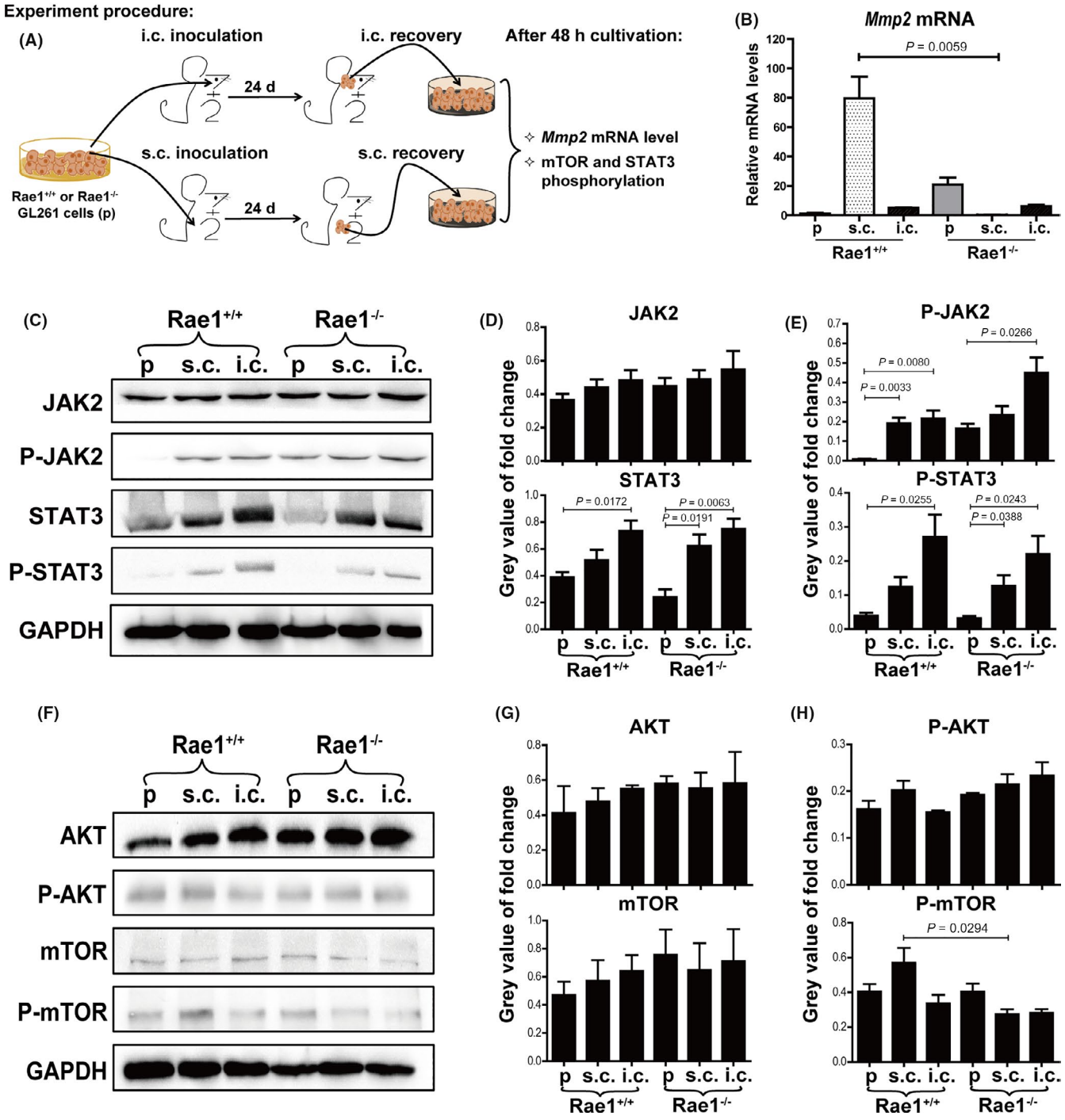

FIGURE 4 Effect of Rae1 on activation of malignancy-related signaling in GL261 cells. As shown in (A), parental Rae1$^{+/+}$ and Rae1$^{-/-}$ GL261 cells (p) were inoculated subcutaneously (s.c.) or intracranially (i.c.) into mice and recovered to detect *Mmp2* mRNA levels (B) by qRT-PCR and activation of STAT3 (C), and mTOR signaling (F) by western blotting. Expression and phosphorylation of STAT3 signaling (D, E) and mTOR signaling (G, H) were analyzed. Data are represented as mean ± SD (n = 3)

next assessed whether in vitro NKG2DL-NKG2D interaction could also "educate" GL261 cells. To select cells for conducting the in vitro experiment, we first determined the relative numbers of NKG2D$^+$ cells in mouse lymph node (LN) cells, splenocytes, and PBMCs. As shown in Figure S3, 16%, 22% or 18% NKG2D$^+$ cells were detected in LN cells, splenocytes, or PBMCs, respectively. As NKG2D$^+$ cells in PBMCs circulate throughout the body and, therefore, are more likely to come into contact with the tumor cells in various tissues, we cocultured Rae1$^{+/+}$ GL261 cells and Rae1$^{-/-}$ GL261 cells with PBMCs. After 48 h, GL261 cells were either immediately assessed for their NKG2DL expression and migration capacity or cultured in medium for another 24 h and then assessed again. As shown in Figure S4, PBMCs induced H60/MULT1 expression and migration of Rae1$^{+/+}$ GL261 cells, but not Rae1$^{-/-}$ GL261 cells. Expression remained for

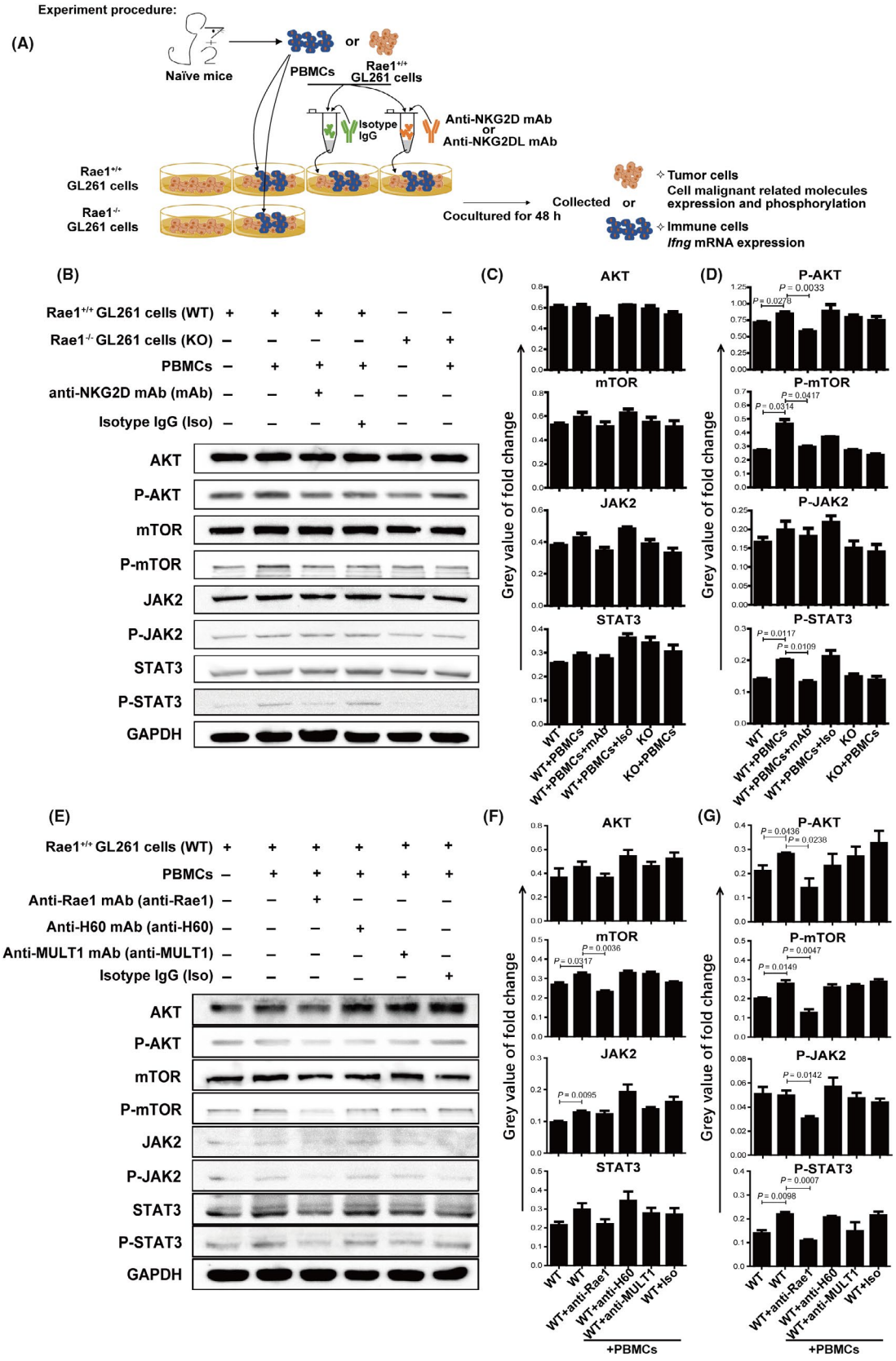

FIGURE 5 Effect of NKG2DL-NKG2D interaction on the activation of mTOR and STAT3 signaling in GL261 cells. As shown in (A), GL261 cells were cocultured with PBMCs for 48 h in the presence of anti-NKG2D mAb or anti-NKG2DL mAb. Expression and phosphorylation of AKT, mTOR, JAK2 and STAT3 in GL261 cells of the NKG2D blocking system (B-D) and the NKG2DL blocking system (E-G) were detected. Data are represented as mean ± SD (n = 3)

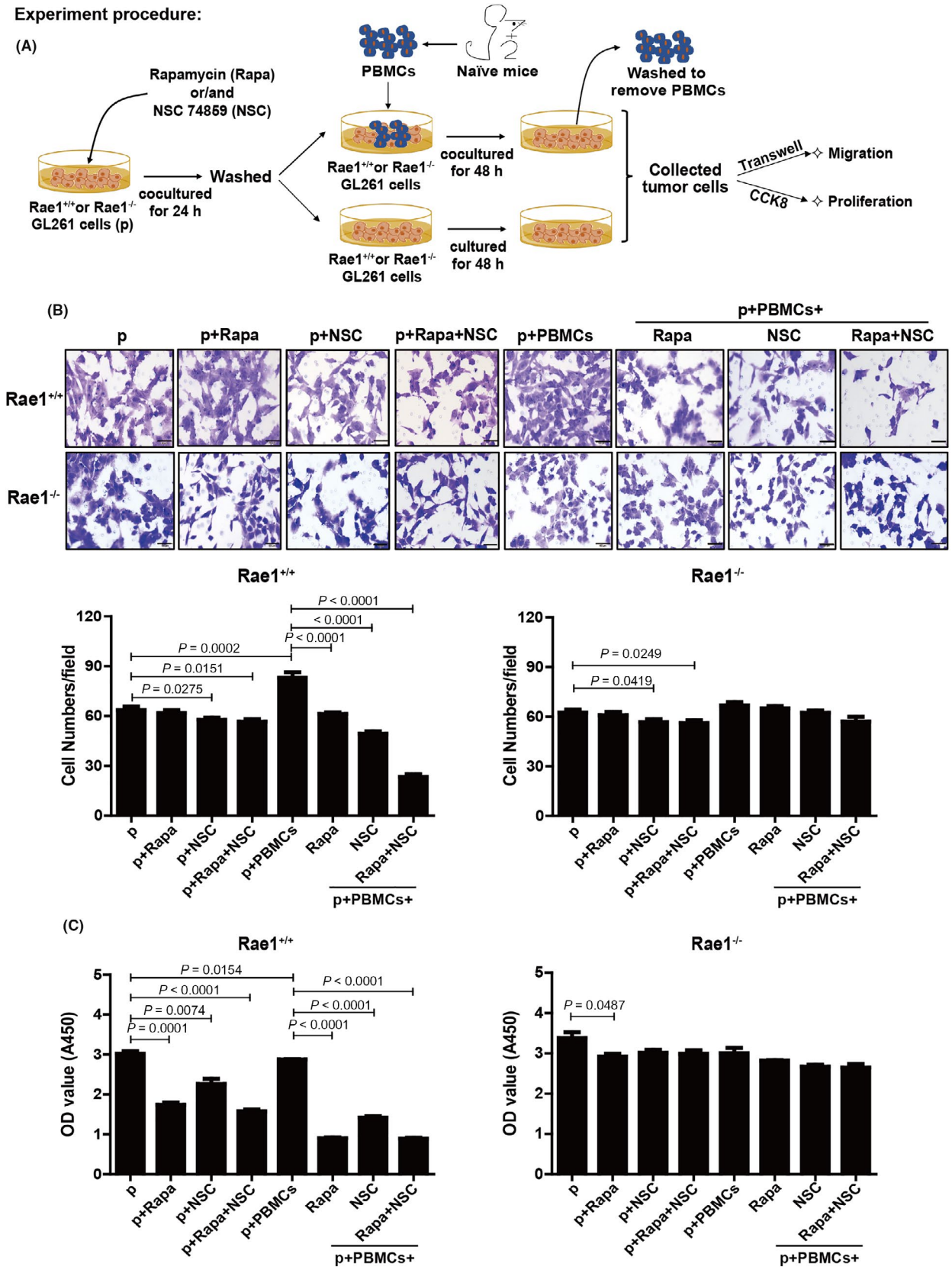

FIGURE 6 Effect of mTOR and STAT3 inhibition on GL261 cell migration and proliferation in vitro. As shown in (A), Rae1$^{+/+}$ GL261 cells or Rae1$^{-/-}$ GL261 cells (p) were cultured with rapamycin or/and NSC 74859 for 24 h, and then cultured for 48 h in the presence or absence of PBMCs. After removing the PBMCs, the GL261 cells were tested for their migration (B) and proliferation (C). Scale bars, 100 μm. Data are represented as mean ± SD (n = 3)

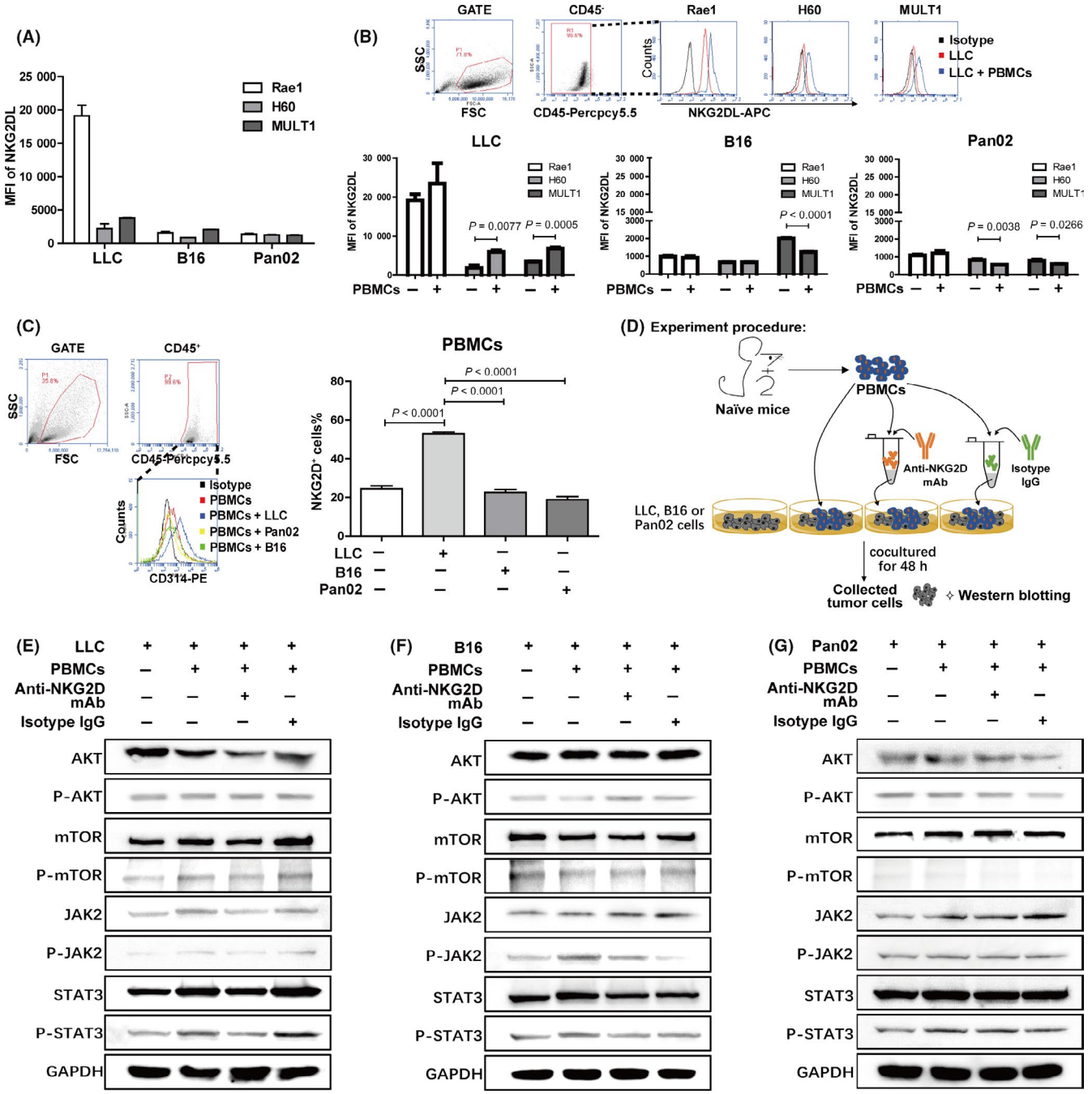

FIGURE 7 Effect of Rae1-NKG2D interaction on expression of NKG2DLs and activation of mTOR and STAT3 in LLC, B16, and Pan02 cells. LLC, B16, and Pan02 cells were investigated for their NKG2DL expression when cultured in medium (A) or when cocultured with PBMCs (B) for 48 h. The percentage of NKG2D$^+$ cells (C) in the cocultured PBMCs was also determined. As shown in (D), tumor cells were cocultured with PBMCs in the presence or absence of anti-NKG2D mAbs for 48 h and then collected to detect the expression and phosphorylation of AKT, mTOR, JAK2, and STAT3 in LLC cells (E), B16 cells (F), and Pan02 cells (G). Data are represented as mean ± SD (n = 3)

a short time after removal of PBMCs, suggesting that the change in the phenotype was not immediately reversible. Furthermore, the NKG2DL-NKG2D blocking system was used to observe whether the NKG2DL-NKG2D axis participated in activation of mTOR and STAT3 in tumor cells (Figure 5A). The results of western blotting showed that anti-NKG2D mAb blockade reduced PBMC-induced phosphorylation of STAT3 and mTOR in Rae1$^{+/+}$ GL261 cells, but not in Rae1$^{-/-}$ GL261 cells (Figure 5B-D). Furthermore, anti-Rae1 mAb blockade, but not anti-H60/MULT1 mAb blockade, reduced the PBMC-induced phosphorylation of STAT3/mTOR in Rae1$^{+/+}$ GL261 cells (Figure 5E-G) and levels of *Ifng* mRNA in PBMCs (Figure S5). These results suggested that NKG2DL-NKG2D interaction, especially Rae1-NKG2D interaction could increase the malignancy of GL261 cells by activating mTOR and STAT3 signaling.

3.6 | Effect of mTOR and STAT3 activation through NKG2DL on migration and proliferation of GL261 cells

To clarify the possible effect of mTOR and STAT3 activation through NKG2DL on GL261 cells, we cultured $Rae^{+/+}$ and $Rae1^{-/-}$ GL261 cells with rapamycin, an mTOR inhibitor,[35,36] or with NSC 74859, a STAT3 inhibitor,[37,38] or both, to inhibit activation of mTOR or/and STAT3 in cells. Next, $Rae1^{+/+}$ GL261 cells and $Rae1^{-/-}$ GL261 cells were cultured in the presence or absence of PBMCs and assessed for their migration and proliferation (Figure 6A). As shown in Figure 6B, the presence of PBMCs reduced the migration of $Rae1^{+/+}$ GL261 cells treated with mTOR inhibitors, STAT3 inhibitors or both, but did not affect the migration of $Rae1^{-/-}$ GL261 cells treated with mTOR and/or STAT3 inhibitors; the migration of $Rae1^{+/+}$ GL261 cells without mTOR or STAT3 inhibitor treatment was increased. Similarly, as shown in Figure 6C, the presence of PBMCs reduced proliferation of $Rae1^{+/+}$ GL261 cells treated with mTOR inhibitors, STAT3 inhibitors or both, but did not significantly affect proliferation of $Rae1^{-/-}$ GL261 cells treated with mTOR and/or STAT3 inhibitors. The proliferation of $Rae1^{+/+}$ GL261 cells without mTOR or STAT3 inhibitor treatment was barely reduced. These results suggested that Rae1 could be required for mTOR or STAT3 activation enhanced migration of GL261 cells.

3.7 | Effect of NKG2DL-NKG2D interaction on activation of mTOR and STAT3 signaling in other tumor cells

To verify whether Rae1-NKG2D interaction could also induce NKG2DL expression on other tumor cells and proportion of $NKG2D^+$ immune cells, we cocultured LLC cells, B16 cells, and Pan02 cells for 48h with or without PBMCs. Adherent tumor cells or PBMCs were assessed for NKG2DL or NKG2D expression, respectively. The presence of PBMCs increased H60 and MULT1 expression levels on Rae1 high-expressing LLC cells and decreased MULT1 or H60 and MULT1 expression on NKG2DL low-expressing B16 or Pan02 cells, respectively (Figure7A,B). PBMCs cocultured with LLC cells, but not with B16 cells or Pan02 cells, contained increased $NKG2D^+$ cells (Figure7C). Furthermore, mTOR and STAT3 activation of tumor cells in the NKG2D blockade system was detected (Figure7D). Quantitative western blotting analysis showed that anti-NKG2D mAb blockade reduced PBMC-induced phosphorylation of mTOR and STAT3 in LLC cells (Figures7E and S6A), but not in B16 cells (Figures7F and S6B), and failed to reduce PBMC-induced phosphorylation of STAT3 in Pan02 cells (Figures7G and S6C). Together, these results suggested that the $NKG2D^+$ immune cells, through NKG2DL-NKG2D interaction, could increase tumor cell malignancy by inducing H60/MULT1 expression and activation of mTOR/STAT3 signaling.

4 | DISCUSSION

NKG2DLs have been considered a "danger signal" on the surface of cells to alert and activate the immune system to find and eliminate NKG2DL-expressing cells such as virus-infected cells or tumor cells. Many malignant tumors express high levels of NKG2DLs and therefore could be targeted by $NKG2D^+$ immune cells.[6,7,39,40] Research in this area has mostly focused on the function of infiltrated $NKG2D^+$ immune cells and has been less concerned about how NKG2DLs on tumor cells influence tumor development. In this study, we found that in vivo growth "educated" Rae1 high-level expressing GL261 cells (Figure 1B) to markedly increase the expression of 2 other NKG2DLs (H60 and MULT1) (Figure 1C). Moreover, when cultured with murine PBMCs, GL261 cells or LLC cells, cells that expressed high levels of Rae1 and with barely detectable H60 and MULT1 levels (Figures 1B and 7A), could also be induced to dramatically upregulate their H60 and MULT1 levels (Figures S4 and 7B). As this expansion was correlated with Rae1 expression in vivo, as well as the migration and proliferation capacities of GL261 cells, we presumed that Rae1 on tumor cells may also be a molecule that promotes tumor growth under certain circumstances. Consistently, Rae1-deficient GL261 cells developed smaller tumors at early stages (Figure 2G). However, noticeably, mice i.c. inoculated with Rae1-deficient GL261 cells survived for a shorter time than mice s.c. inoculated with these cells (Figure 2H), even though migration and proliferation capaciteis of the cells were found to be similar (Figure 2E,F). It is clear that a brain tumor developed from the $Rae1^{-/-}$ GL261 cells caused death more quickly than its counterpart extra-cranial tumor, as brain tumors, albeit small, may predominantly affect cells that are vital for survival.[41]

Interestingly, the results presented here implied that NKG2DLs on tumor cells may play a direct or indirect role in promoting tumor development. For the direct role, Rae1 on GL261 cells drives growth (Figure 2G) and migration of tumor cells (Figure 2E), and activation of mTOR/STAT3 signaling both in vivo (Figure 4) and in vitro (Figure 5). Inhibition of mTOR and/or STAT3 could eliminate PBMC-induced migration of GL261 cells (Figure 6). Noticeably, mTOR phosphorylation was increased in Rae1-expressing GL261 cells recovered from s.c. tissues but not in cells recovered from i.c. tissues (Figure 4F). This discrepancy could be attributed to the varied microenvironment, including subcutaneous adipocytes that are absent in brain tissues and could induce mTOR phosphorylation in variety cancer cells.[42,43] Furthermore, glioma-derived GL261 cells growing in the brain may have limited chance to contact cells such as adipocytes from which they receive signals for mTOR activation. For the indirect role, Rae1 on GL261 cells triggered immune cells to release IFN-γ (Figure S5), which could activate the JAK2-STAT3 and AKT-mTOR pathways in tumor cells[18,19] and therefore increase tumorigenicity. Nevertheless, Rae1 on the i.c. and s.c. recovered GL261 cells had little effect on activation of the JAK2-STAT3 pathway (Figure 4C), implying that the microenvironment in vivo may shape their tumorigenic capacity. Intriguingly, JAK2 phosphorylation was detected in parental Rae1-defitient GL261 cells, implying that JAK2 is constitutively phosphorylated. This phenomenon has been observed in myeloproliferative neoplasms with a JAK2 mutation[44-48] that led to constitutive phosphorylation of JAK2[45-48] and is possibly necessary to maintain the

malignant phenotype. This possibility could be strengthened by research showing that inhibition of JAK2-mediated phosphorylation decreased the activity of downstream signaling by mTOR, thereby reducing glioma proliferation.[49]

Here, it was found that Rae1 deficiency increased MHC-I expression on GL261 cells (Figure S2), implying that, in addition to upregulating NKG2DLs (Figure 1), Rae1 could also downregulate MHC-I molecules on GL261 cells. The weakened inhibitory signal induced by MHC-I molecules and the enhanced activating signal induced by NKG2DLs make tumor cells more susceptible to NKG2D$^+$ immune cell-mediated killing.[50] In comparison, *Rae1* gene deletion made GL261 cells less susceptible to killing by splenocytes (Figure 2B). Together, Rae1-deficent GL261 cells, although expressing a higher level of MHC-I molecules, were unable to induce sufficient inhibitory signals to completely abolish killing by NKG2D$^+$ immune cells. Convincingly, a similar phenomenon has been reported previously, showing that NKG2D$^+$ immune cells did participate in immunity against MHC-I molecule-expressing tumor cells.[51]

To date, information on the mechanism of rapid growth of NKG2DL high-expressing tumor cells in the NKG2D$^+$ immune cell sufficient host has been and is still lacking. Based on our research, we confirmed that NKG2DLs on tumor cells could promote tumor development both directly and indirectly. Although the indirect effect of NKG2DLs on tumor promotion needs to be further studied, the role of NKG2DLs both as an accomplice to malignant tumors and as an indispensable requisite for recruitment of immune cells should be noted. Compared with NKG2DLs on tumor cells, mTOR and STAT3 in tumor cells might be better targets for immunotherapy.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

ORCID

Liying Wang https://orcid.org/0000-0002-9180-5829

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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