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RESEARCH

# Extracellular PKM2 modulates cancer immunity by regulating macrophage polarity

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## Abstract

Tumor controls its immunity by educating its microenvironment, including regulating polarity of tumor associated macrophages. It is well documented that cancer cells release PKM2 to facilitate tumor progression. We report here that the extracellular PKM2 (EcPKM2) modulates tumor immunity by facilitating M2 macrophage polarization in tumors. EcPKM2 interacts with integrin $\alpha_v\beta_3$ on macrophage to activate integrin-FAK-PI3K signal axis. Activation of FAK-PI3K by EcPKM2 suppresses PTEN expression, which subsequently upregulates arginase1 (Arg1) expression and activity in macrophage to facilitate M2 polarity. Our studies uncover a novel and important mechanism for modulation of tumor immunity. More importantly, an antibody against PKM2 that disrupts the interaction between EcPKM2 and integrin $\alpha_v\beta_3$ is effective in converting M2 macrophages to M1 macrophages in tumors, suggesting a new therapeutic strategy and target for cancer therapies. Combination of the anti-PKM2 antibody with checkpoint blockades provides enhanced treatment effects.

**Keywords** Pyruvate kinase M2 · Macrophage · Integrin $\alpha_v\beta_3$ · Cancer immunotherapy · Arginase1 · Glycolysis

## Introduction

Abundant infiltration of immune cells in tumor provides evidence of recognition of aberrations in cancer by host immune system. A long puzzling question is "how do cancer cells avoid host immune destruction?" In fact, tumor turns host immuno-responses into cancer promoting factors, meaning tumor infiltration immune cells often facilitate cancer progression and metastasis [1, 2]. Cancer cells educate immune cells in tumor through cross-talk between cancer cells and immune cells and the cells within tumor microenvironment [3]. A key component of tumor associated inflammation is TAMs. Macrophages in tumor come from two sources, tissue-resident macrophage and circulating monocytes recruited to tumor site [4, 5]. Macrophage is activated to different polarized status with two extreme phenotypes, classically activated M1 and alternatively activated M2. The functional roles of M1/M2 macrophages in modulating inflammation response are almost completely opposite with M1 as pro-inflammation while M2 anti-inflammation or pro-regeneration. Macrophage polarization is not completely fixed. M1/M2 are interconvertible dependent on surrounding environment [6, 7]. Macrophages are the most abundant immune cells in tumor. Levels of macrophages directly correlate with cancer progression [4]. TAMs mostly adopt M1 at the early stage of cancer development. However, TAMs in late-stage cancer are predominately M2 subtype [8, 9]. It is well documented that cancer cells educate TAMs to M2 subtype to favor cancer progression, metastasis, and apoptosis resistance. Abundant M2 macrophages also facilitate cancer relapses after eradication of tumor by chemotherapies and/or radiation therapies.

Pyruvate kinase is an enzyme that catalyzes the last reaction in glycolysis. There are four isoforms of pyruvate kinases, L/R and M1/M2, which are expressed in different tissue types or under different physiological conditions. PKM2 can form a homodimer or a homotetramer. The tetramer is active as pyruvate kinase [10, 11], while the dimer is a protein kinase [12–14]. Interestingly, a number of recent studies show that PKM2 is functionally involved in multiple cellular processes in different sub-cellular locations, including metabolism control, transcription regulation, and chromatin packaging [13, 15, 16]. High serum levels of

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PKM2 have long been observed in patients of solid tumors and with various inflammation diseases [17–19], suggesting a potential role of EcPKM2 in modulating inflammation responses in tumor and other inflammation associated pathological conditions. Our previous studies demonstrated that EcPKM2 facilitates tumor growth by promoting angiogenesis [20]. During cutaneous wound repair, PKM2 is released by infiltration neutrophils at the wound site. The released PKM2 facilitates wound repair [21]. We recently reported that PKM2 is not expressed in quiescent fibroblasts. However, upon myofibroblast differentiation stimulated by TGFβ, expression of PKM2 is upregulated in myofibroblasts and secretion of PKM2 is increased [22]. In this report, we present evidence to show that EcPKM2 also facilitates cancer progression by modulating tumor immunity. EcPKM2 promotes alternative activated macrophage (M2) in tumor, thus favor suppression of tumor immunity. EcPKM2 promotes M2 macrophage by interacting with integrin $\alpha_v\beta_3$ on macrophage and consequently activating the integrin signaling, which leads to activating the integrin-FAK-PI3K signaling axis. Activation of integrin-FAK-PI3K results in suppression of PTEN and subsequent upregulation of arginase1 (Arg1) expression and activity. Disruption of EcPKM2 and integrin $\alpha_v\beta_3$ interaction by a PKM2 antibody abolished the effects of EcPKM2 on promoting M2 macrophage and increasing M1 macrophage population in tumors, suggesting a new strategy and target in cancer therapy. Combination of the PKM2 antibody with checkpoint blockades provides enhanced cancer treatment effects.

## Methods

### Recombinant protein expression and purification

The cDNAs that encode human PKM1, PKM2 and G415R were purchased from Adgenes. The cDNAs were subcloned into bacterial expression vectors. The recombinant proteins were expressed and purified from bacterial lysates by a two column procedure as in previous reports [13, 20].

### Cell Line and mouse bone marrow derived macrophages

*Cell lines*: Cell lines Raw264.7, 4T1, and B16.F10 were purchased from ATCC. Cells were cultured according to the vendor's instruction.

*Mouse bone marrow derived macrophage (BMMφ) adopted from* [23]: Briefly, mice were sacrificed and using aseptic technique. Bone marrow was harvested from the tibia and femur bones after the surrounding muscles were removed. To extract the bone marrow, the joints were cut with a scalpel, and the marrow was flushed out of the bones using a 25-gauge needle attached to a 10 ml syringe filled with PBS. All the clumps were gently dissociated and passed through a 70 µm cell strainer. The cell suspension was then centrifuged at 250 g for 5 min at room temperature to pellet the cells. To differentiate into BMMφ, isolated cells were cultured in DMEM with 10% heat inactivated FBS and 10 ng/mL M-CSF1 for 5 days. The differentiated BMMφ is ready for subsequent experiments.

*Patient tissue* analyses were carried out under the guidelines of NIH and adhered to the Declaration of Helsinki. The study was approved by GSU Institutional Review Board (IRB). All tissue samples are de-identified. It falls under IRB exemption 4. Samples were sectioned and analyzed by different staining. Tumor and normal tissues were purchased from US Biomax Inc.

### Animal model and treatments

All animal experiments conformed to the guidelines of the NIH Guide for the Care and Use of Laboratory Animals and were approved by Georgia State University IACUC. At the end of each experiment, the mice were euthanized by $CO_2$ inhalation. Animal euthanasia conformed to the guidelines of the NIH Guide for the Care and Use of Laboratory Animals. *Animal models*: The same procedures similar to our previous reports were followed to generate 4T1 and GEM-NSCLC models [24, 25]. For B16 tumor model, 7–8-week-old C57BL/6 mice were restrained by hand. B16 cells ($4\times10^5$, suspension in 100 µl HBSS) were injected underneath the skin (s.c.). The mice returned to their cages. The tumors typically grow to ~ 200 $mm^3$ in volume in 7–8 days in our hands. *Treatments*: the designated treatments began; (1) for 4T1 model, when tumors grew to average of 200 $mm^3$ in volume (~ 8 days after 4T1 cell inoculation), (2) for B16, 8 days after B16 cell inoculation, and (3) for GEM-NSCLC, 8 weeks after AdV-Cre delivery. Mice were randomly assigned to the treatment groups. The agents as indicated in the figure panels and legends were administered via i.p. injections. Animals were euthanized 2 days after the last dose treatments. The treatment for assessment of survival of B16 tumor mice, treatments (i.p. injection) start 8 days after B16 cell inoculation, PKAb (4 mg/kg, b.i.w), αPD-1 (4 mg/kg, b.i.w) and the combination for 4 weeks in total 9 doses. The mice were maintained in cages for survival assessment.

### Macrophage polarization inductions

Raw264.7 or the isolated BMMφ were cultured under standard conditions. To induce M1 polarization, LPS was added

into medium to final concentration of 10 ng/ml. To induce M2 polarization, IL-4 was added into medium to final concentration of 20 ng/ml. The cells were maintained for designated. The treated cells were then harvested for subsequent analyses.

### FACS analyses strategies

*Tissue process for FACS analysis Adopted from* [26]: Briefly, tumors were sliced out from the mice and minced (1 × 1 mm) and digested with collagenase D. After digestion and filtration, the recovered cells were resuspended and washed with FACS staining buffer. The resultant cells were subjected to FACS analysis.

*FACS gating and analysis strategies for macrophages*: (1) Analysis of macrophages in tissue samples, the isolated viable single cells were first gated for $CD45^+$ cells to identify leukocytes. Macrophages were then selected based on $F4/80^+$ expression (the FACS gating for macrophage selection please see supplementary Fig. 1A). The selected macrophages were then FACS sorted via $CD206^+$ for M2 macrophages and $iNOS^+$ for M1 macrophages. (2) His-tag for rPKM1 or G415R binding analysis, the isolated viable single cells were first gated by $CD45^+$ $F4/80^+$ macrophage identification. The selected macrophages were then FACS sorted via $His^+$ for binding to his-tagged proteins. (3) Analysis with cultured cells, viable cultured cells were gated for $F4/80^+$ cells to identify macrophages, followed by analysis for $CD206^+$ cells to identify M2 macrophages, by $CD86^+$ for M1 macrophages.

*FACS gating and analysis strategies for T cells from tissue samples*: The isolated viable single cells were first gated for $CD3^+$ cells to identify T-cells. The isolated T cells were analyzed by $CD4^+$ and $FoxP3^+$ as $T_{reg}$ cells $CD8^+$ as CD8 + CTL.

*The procedures for His-tag Co-precipitation, Western blotting, cell attachment assay, and Flow cytometry* were adopted from our previous reports [20, 21, 27, 28].

### Tissue section and cell staining

*IHC and IF*: IHC and IF staining procedures were similar to those of previous reports [21, 27].

*PI3K and Arg1 activity Assays* were performed by the procedures similar to our previous reports [21, 22, 27]

### Statistical calculations

Statistical analyses were carried out using the GraphPad Prism 6.0 software. Kaplan–Meier survival curves were calculated using survival time for each mouse from all treatment groups. Statistical analyses in the survival experiments were performed by log-rank (Mantel-Cox) test. All in vitro experiments were carried out at least 5 times. For image quantifications, FACS, and other analyses, statistical significance was assayed by unpaired Student's t-test. In all figures, $*P < 0.05$; $**P < 0.01$, $***P < 0.001$; n.s. denotes not significant. All data are presented as mean ± s.e.m. or as box plots.

## Results

### EcPKM2 in patient tumors and PKM2 is secreted by cultured cancer cells

High levels of PKM2 are presented in blood circulation of cancer patients, and the levels of circulating PKM2 correlated with tumor progression [19, 29]. ELISA analyses of culture medium of cancer cells demonstrated that PKM2 is released from cultured cancer cells (Fig. 1A). To further confirm presence of EcPKM2 in tumor, we carried out IHC staining of tissue samples from cancer patients using an in-house developed rabbit monoclonal antibody against PKM2, IgGPK [21]. High levels of PKM2 were detected in cancer patient tissues. The EcPKM2 was apparent in the staining. As control, almost no PKM2 staining was observed in adjacent normal tissues (Fig. 1B). Our results indicate that PKM2 is expressed intracellularly and released to extracellular space of tumor of cancer patients, and PKM2 is secreted by cancer cells.

### EcPKM2 interacts with integrin $\alpha_v\beta_3$ on macrophage and promotes M2 polarity

We previously reported that EcPKM2 interacts with integrin $\alpha_v\beta_3$ on angiogenic endothelial cells and myofibroblasts and activates the integrin signaling [20, 22]. It was demonstrated that integrin $\alpha_v\beta_3$ is highly expressed in macrophages [30–32]. We confirmed the integrin expression on macrophages by probing integrin $\alpha_v\beta_3$ expression in cultured Raw264.7 (Raw) cells and bone marrow-derived macrophages (BMMφ) from healthy mice (Fig. 1C). We reasoned whether EcPKM2 interact with the integrin $\alpha_v\beta_3$ on macrophages. To this end, we first carried out cell attachment assay with Raw and BMMφ cells using culture plate coated with recombinant PKM2 (rPKM2) or PKM1 (rPKM1) that expressed and purified from bacterial *E. Coli.* or BSA. Clearly, more cells attached to rPKM2 compared to rPKM1 and BSA coated plates (Fig. 1D). The cell attachment is integrin $\alpha_v\beta_3$ dependent, as antibody against integrin $\alpha_v\beta_3$ LM609 blocked the Raw cells attaching to the rPKM2 coated plate, while IgG did not (Fig. 1E). Co-precipitation (by His-tag pull-down) of His-rPKM2 with integrin $\alpha_v\beta_3$ from extracts made from BMMφ, confirmed the interaction of PKM2 with the integrin (Fig. 1F). To

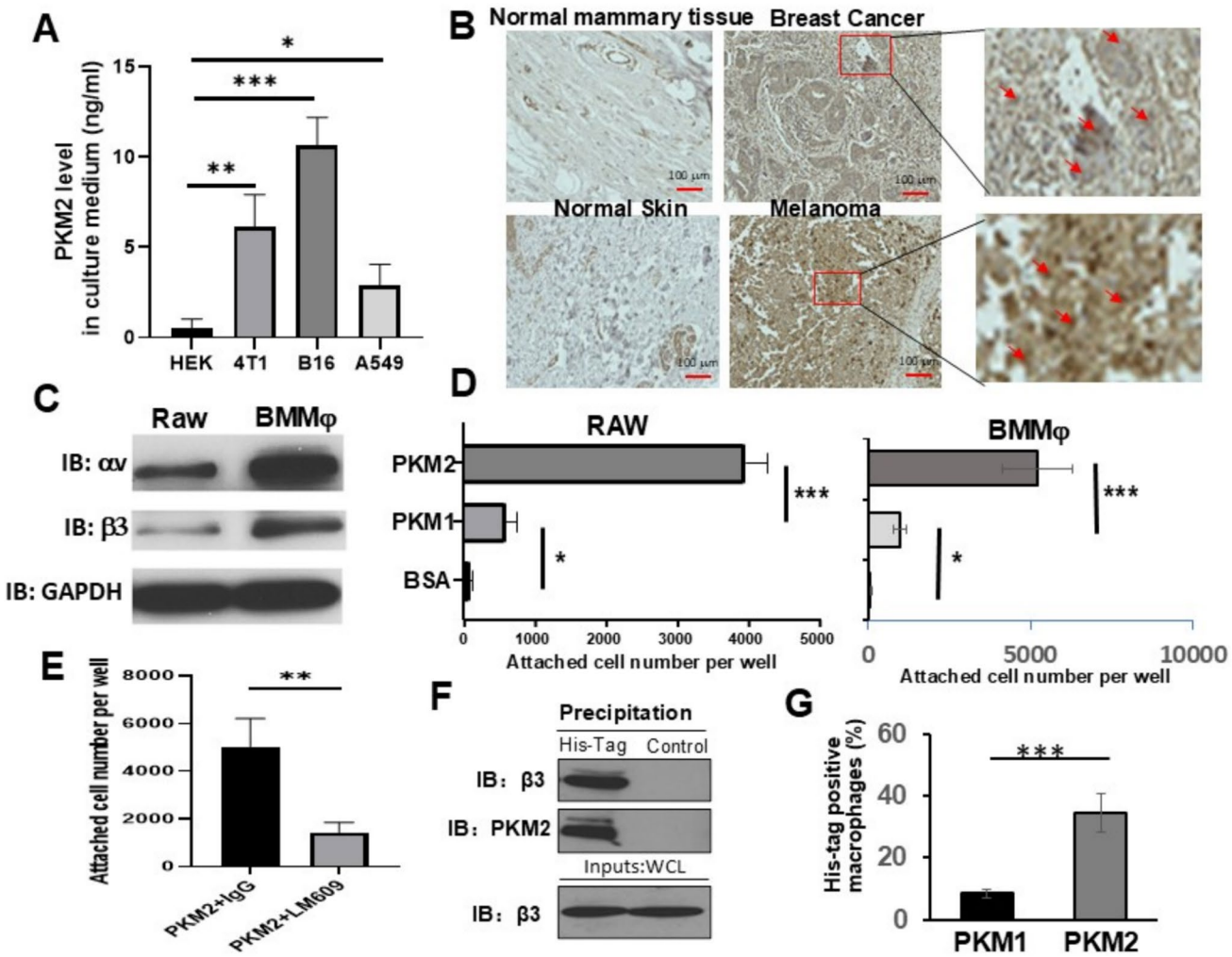


**Fig. 1** Extracellular PKM2 interacts with integrin αvβ3 on macrophages in tumors. **A** The levels of PKM2 in the culture medium (ng/ml) of indicated cell lines were measured by ELISA. **B** Representative images of IHC staining of PKM2 in indicated tumor tissues and adjacent normal tissues from patients (n = 12). On the right are images with higher magnification of the call out of red boxes. The red arrows indicate examples of extracellular PKM2. **(C)** Cellular levels of integrin αv and β3 in Raw and BMMφ cells are measured by immunoblot (IB: αv and IB: β3). IB: GAPDH is a loading control. **D** Attachment of Raw (left) and BMMφ (right) cells to BSA, PKM1, and PKM2 coated plates, and **E** attachment of BMMφ cells to PKM2 coated plates in the presence of IgG (as control) or anti-integrin $\alpha_v\beta_3$ antibody LM609 were measured by cell counting. The cell attachments in (D) and (E) are presented as attached cells per well. **F** Co-precipitation of His-tagged PKM2 with integrin β3 from cell extracts was measured by His-tag pull-down followed by immunoblot of indicated antibodies (IB: β3 or IB: PKM2). Control is the His-tag pull-down from cell extracts without addition of bait His-tag PKM2 to the extracts. IB: β3 in the input of whole cell lysate (Inpust:WCL) represents the total extracts used for His-tag pull-down. **G** Binding of His-tagged PKM2 or PKM1 to macrophage in 4T1 tumors was analyzed by FACS using anti-His-tag antibody (n = 6). Macrophages were first sorted by FACS using $CD45^+/F4/80^+$. The resultant population was FACS analyzed by His-$tag^+$ sorting and is presented as % of His-tag positive cells in the total population of $CD45^+/F4/80^+$. Error bars in **A**, **D**, and **E**, represent mean ± S.E.M. from 5 independent experiments, and in (**G**) is standard deviations of calculation of 6 mice. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

verify that rPKM2 interacts with macrophage in tumor, macrophages were FACS sorted from the 4T1 tumors from the tumor-bearing mice that were treated with His-rPKM1 or His-rPKM2. The macrophages were then analyzed via FACS using anti-his-tag antibody. The his-rPKM2 interacted with macrophage much stronger compared to his-PKM1 in the tumor (Fig. 1G). We conclude from our results that EcPKM2 interacts with integrin $\alpha_v\beta_3$ on macrophages in tumors.

Our next question is what the functional role(s) of the interaction between EcPKM2 and integrin $\alpha_v\beta_3$ on macrophage is. Careful examination of morphology of Raw cells that were treated by different agents, including rPKM2, rPKM1, LPS, or IL-4, revealed that the cells

treated with rPKM2 resembled IL-4 treated cells in morphology (Fig. 2A). It is well known that IL-4 activates M2 macrophage, while LPS stimulates M1 polarization. M2 macrophages secrete IL-10 and express cell surface marker CD206 [4, 33]. Thus, we examined secretion of IL-10 in culture medium of Raw and BMMφ cells that were treated with LPS, rPKM1, rPKM2, or IL-4. The cells treated with rPKM1, or LPS did not secrete IL-10, while the IL-4 and rPKM2 treated cells secreted high levels of IL-10 (Fig. 2B, C). FACS analysis of Raw and the BMMφ cells that treated with LPS, rPKM1, rPKM2, or IL-4 indicated that macrophages expressed high levels of CD206 upon IL-4 or rPKM2 treatment, while CD206 expression was almost undetectable in LPS or rPKM1 treated macrophages (Fig. 2D, E). It is well documented that expressions of iNOS and CD86 are important molecular signatures of M1 macrophage polarity. FACS analysis of M1 macrophage marker CD86 on Raw cells showed that PKM2 treatment decreased $CD86^+$ cell population (Fig. 2F), while iNOS levels in Raw cells were reduced by rPKM2 but not rPKM1 (Fig. 2G). We further tested whether EcPKM2 could convert M1 macrophage to M2 subtype. To this end, Raw and BMMφ cells were incubated with LPS in the presence or absence of rPKM1 or rPKM2. Secretion of TNFα into culture medium was measured as an indication of M1 macrophage polarity [34]. Interestingly, the addition of rPKM2 reduced TNFα in

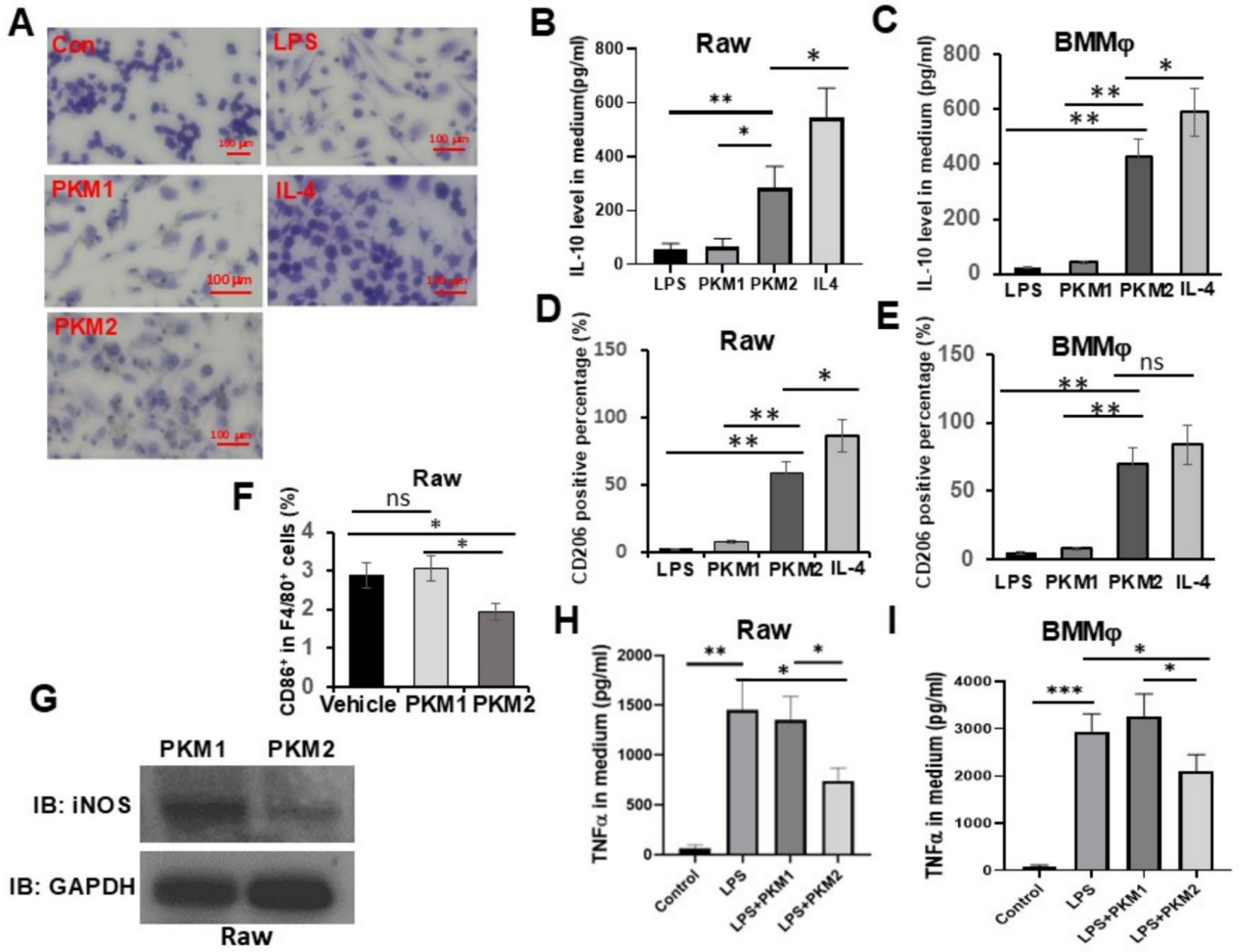


**Fig. 2** EcPKM2 promotes M2 macrophage polarity. **A** Representative phase contrast microscopy images of RAW cells treated by the indicated agents (scale bar 100 μm). **B** and **C** Levels of IL-10 in culture medium of RAW (B) and BMMφ (C) cells that were treated with the indicated agents were measured by ELISA. The IL-10 levels are presented as pg/ml culture medium. **D**, **E** $CD206^+$ Raw (D) or BMMφ (E) cells that were treated with the indicated agents were analyzed by FACS. Results are presented as % of CD206 positive in total cell population. **F** FACS analyses of $F4/80^+$ and $CD86^+$ M1 macrophages. **G** Levels of iNOS (IB: iNOS) in Raw cells were analyzed by immunoblot. The cells were treated with PKM1 or PKM2. **H**, **I** Levels of TNFα in culture medium of RAW (H) and BMMφ (I) cells that were treated with the indicated agents were measured by ELISA. The TNFα levels are presented as pg/ml culture medium. Error bars in B, C, D, E, F, H, and I represent mean ± S.E.M. from 5 independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

culture medium of both Raw and BMMφ that treated with LPS (Fig. 2H, I), suggesting that EcPKM2 may be capable of converting M1 macrophages to M2 polarity.

## EcPKM2 increased M2 macrophages and decreased M1 macrophages in tumors

To test whether EcPKM2 promotes M2 macrophage in vivo, we employed the breast cancer orthotopic 4T1 tumor model. The mice carrying 4T1 tumor were treated with G415R, a PKM2 mutant with higher solubility and dimer ratio [35], or rPKM1 when the tumor grew to an average size of ~ 200 $mm^3$ in volume (Fig. 3A). Animals were euthanized 2 days after the last dose treatments. Tumor growth was measured, and tumors were weighted. Clearly, G415R but not rPKM1 increased tumor growth rate, size, and weight (Fig. 3B–D). Immune cells in tumor analyzed via Flow Cytometry and Fluorescence-Activated Cell Sorting (FACS). Figure 3E shows an example of FACS plots of macrophage polarity analysis. FACS analyses gated by $F4/80^+$ and subsequent sorting via $CD206^+$ $iNOS^+$ indicated that G415R treatment increased percentage of M2 and decreased percentage of M1 in the total macrophage population in 4T1 tumor compared to the rPKM1 group (Fig. 3F). It is well documented that M2 macrophage induce $CD4^+$ $T_{reg}$ cells in tumor [36]. Evidently, $CD4^+$ $T_{reg}$ cells ($CD4^+/FoxP3^+$) increased in the tumors of G415R treated mice (Fig. 3G). On the other hand, G415R decreased cytotoxic $CD8^+$ T-cells in the tumor (Fig. 3G). TAM polarity plays a vital role in cancer metastasis [2, 37]. Examination of metastatic nodules in lungs revealed that G415R treatment increased both metastatic nodular number and size compared to the groups treated with rPKM1 (Fig. 3H, I). We asked whether G415R treatment affected macrophage polarity in lung metastatic tumors. FACS analyses showed that G415R increased M2 macrophage percentage in lung metastatic tumors compared to the rPKM1 treated group (Fig. 3J). Similarly, G415R treatment also increased $T_{reg}$ cells in the lung metastatic tumors (Fig. 3K).

EcPKM2 promotes M2 macrophage by interacting with and activating integrin $\alpha_v\beta_3$. We reasoned that antibody disrupting the PKM2 and integrin $\alpha_v\beta_3$ interaction would exert opposite effects in tumor. We used an in-house developed monoclonal anti-PKM2 antibody (Previous IgGPK, changed the name to PKAb here) that disrupts PKM2 and integrin $\alpha_v\beta_3$ interaction [20, 21]. Tumor bearing mice were treated with PKAb or rabbit IgG as a control. PKAb treatment led to tumor growth inhibition and smaller tumors (supplementary Fig. 1B) and less and smaller metastatic nodules in lungs compared to the IgG treatment group (Fig. 3L–N). PKAb led to high M1 and low M2 macrophage populations (Fig. 3O) and decreased $CD4^+$ $T_{reg}$ cells (Fig. 3O, P) in primary tumors. FACS analyses of M2 macrophage and $T_{reg}$ in lung metastatic tumors revealed that PKAb treatment decreased M2 macrophage percentage and $T_{reg}$ in metastatic tumors (Fig. 3 Q&R). We conclude from our experiments that EcPKM2 promotes M2 macrophage, while antibody disrupt EcPKM2 and integrin $\alpha_v\beta_3$ interaction abrogates the effects of EcPKM2 on polarity of TAMs.

To test the commonality of the role of EcPKM2 in modulating TAM polarity in tumor, we analyzed the effects of rPKM2 with two additional murine cancer models: (1) Orthotopic xenograft of B16 cells, a murine melanoma cell line. (2) DuPage, M., and co-workers developed a genetic engineering mouse (GEM) lung cancer model (Ref to as GEM-NSCLC). The GEM-NSCLC mice spontaneously develop lung cancer 3 weeks after intratracheal intubation delivery of adenoviral coded Cre recombinase (AdV-Cre) due to specific expression of $Kras^{G12D}$ and $Trp53^{R172H}$ in the lung [25, 38]. The B16 tumor-bearing mice and the GEM-NSCLC mice were treated with rPKM1 or G415R; 8 days post tumor inoculation for B16, or 8 weeks after delivery of AdV-Cre for GEM-NSCLC respectively (Fig. 4A). Animals were euthanized 2 days after the last dose treatments. The B16 tumor size in G415R treated mice was larger than that in rPKM1 treated mice (Fig. 4B). The tumor nodule number and size in G415R treated GEM-NSCLC mice was higher and larger than those in rPKM1 treated mice (Fig. 4C). FACS analyses demonstrated that G415R treatment increased M2 macrophage percentage and $T_{reg}$ cells in tumors of both B16 and GEM-NSCLC mice (Fig. 4D–G). Conversely, PKAb treatment led to smaller B16 tumor in weight (Fig. 4J). Importantly, PKAb decreased M2 macrophage percentage and $T_{reg}$ cells (Fig. 4K–N) in tumors of both models. Examination of macrophage and $T_{reg}$ in lung metastatic tumors of the treated B16 tumor bearing mice showed that G415R increased M2 macrophage percentage and $T_{reg}$ cells in lung metastatic tumor (Fig. 4H, I), while PKAb reduced M2 macrophage percentage and $T_{reg}$ cells in B16 lung metastatic tumor (Fig. 4O, P). These results further confirmed our conclusion that EcPKM2 promotes M2 macrophage polarity in both primary and metastasis tumors, while antibody that disrupt the interaction between EcPKM2 and integrin $\alpha_v\beta_3$ promotes M1 macrophage polarity.

## EcPKM2 activates integrin-FAK-PI3K signaling axis and upregulates Arg1 in macrophage

An open question was how the interaction between EcPKM2 and integrin $\alpha_v\beta_3$ regulated macrophage polarity. We previously showed that EcPKM2 interacted with integrin $\alpha_v\beta_3$ and activated the integrin signaling and downstream PI3K signaling axis in myofibroblasts and angiogenic endothelial cells [20, 22]. Thus, a reasonable speculation would be that

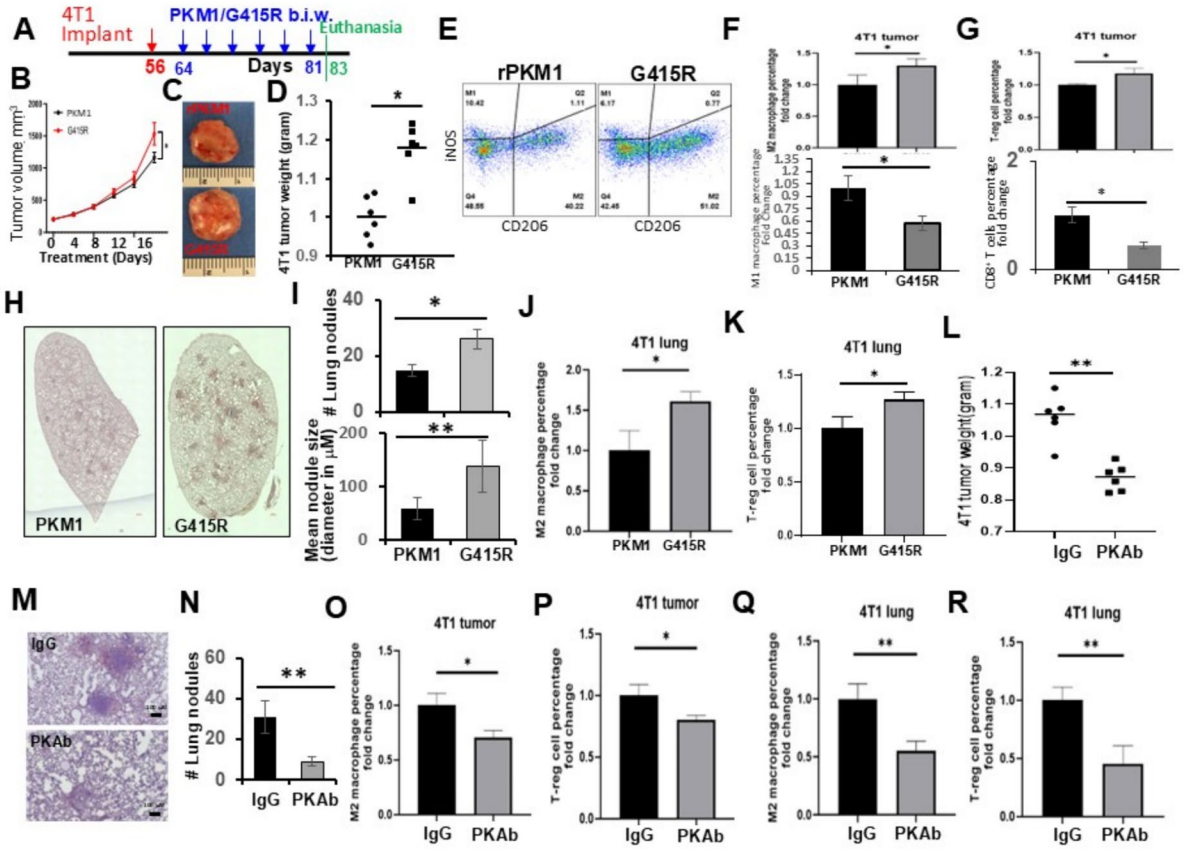


**Fig. 3** Effects of extracellular PKM2 on tumor immunity. **A** The scheme illustrates G415R or PKM1 (blue arrows) treatment regimen of 4T1 mice. **B–D** Tumor growth curve (**B**), representative pictures (**C**), and weight **D** of 4T1 tumors from mice treated with indicated agents (n = 6). Tumor weights were measured by weighting tumors cut out from the treated mice. **E** Representative FACS plots of iNOS$^+$ and CD206$^+$ populations in 4T1 tumors treated by indicated agents. The cells collected from tumor were first sorted with CD45$^+$ and F4/80$^+$ FACS gating. **F**, **G** FACS analyses of; CD206$^+$ M2 macrophages (F, Upper), iNOS$^+$ M1 macrophages (F, Bottom), CD4$^+$ and FoxP3$^+$ $T_{reg}$ cells (G, Upper), CD8$^+$ cytotoxic T-cells (G, Bottom) in 4T1 tumors from mice treated with indicated agents. in FACS analyses, for macrophage, gated by CD45$^+$ and F4/80$^+$, for T-cells gated by CD3$^+$. **H** Representative images of whole lungs of treated 4T1 mice treated with indicated agents (n = 10). **I** Quantitative measurements of both number (Upper) and size (Bottom) of lung metastatic nodules in lung of the treated mice treated with indicated agents (n = 10). **J**, **K** FACS analyses of F4/80$^+$ and CD206$^+$ M2 macrophages (J) and CD4$^+$ and FoxP3$^+$ Treg cells (**K**) in lung metastatic tumors from 4T1 mice treated with indicated agents. **L** Tumor weight of 4T1 tumors from mice treated with indicated agents (n = 6) was measured by weighting tumors cut out from the treated mice. **M** Representative images of lungs sections of treated 4T1 mice treated with indicated agents (n = 10). **N** Quantitative measurements of the number (#) of lung metastatic nodules in lung of the treated mice treated with indicated agents. **O–R** FACS analyses of F4/80$^+$ and CD206$^+$ M2 macrophages (**O**, **Q**) and CD4$^+$ and FoxP3$^+$ $T_{reg}$ cells (**P**, **R**) in tumors (**O**, **P**) or lung metastatic tumor (**Q**, **R**) from 4T1 mice treated with indicated agents. Quantities of M1/M2 macrophages (**F**, **J**, **O**, **Q**) or $T_{reg}$/CD8-T cells (**G**, **K**, **P**, **R**) are presented as fold changes of percentage of total population of macrophages or T cells respectively. Scale Bar in (**H**) 300 mm, **M** 100 mm. Error bars in **B**, **F**, **G**, **J**, **K**, **O**, **P**, **Q**, and **R** represent mean ± S.E.M. from 5 independent experiments, and in **I** and **N** represent mean ± S.E.M. from 10 mice. *$P < 0.05$, **$P < 0.01$

EcPKM2 activates the same integrin-FAK-PI3K signaling axis in macrophage. To test the speculation, we first examined activation of FAK. Evidently, FAK was activated upon addition of rPKM2 to culture medium of Raw as shown by the FAK phosphorylation (Fig. 5A). The activation of FAK was diminished by the antibodies PKAb or 23C6, a mouse monoclonal antibody against integrin $\alpha_v\beta_3$ (Fig. 5B). It is well established that activation of integrin signaling FAK leads to activation of downstream PI3K [39]. Thus, we examined the activation of PI3K in the Raw cells upon rPKM2 treatments. Immunoblot demonstrated that PI3K was activated as shown by the PI3K phosphorylation (Fig. 5A). PI3K activity assay confirmed the activation of PI3K by PKM2 in the cells (Fig. 5C). The activation of PI3K by PKM2 was abrogated by a PI3K inhibitor and by a FAK inhibitor (Fig. 5A, C). It is well established that PI3K—PTEN pathway regulates Arg1 expression in macrophages and subsequently modulate macrophage polarity [40, 41]. We reasoned that EcPKM2 may modulate macrophage polarity by the same FAK-PI3K-PTEN signaling pathway

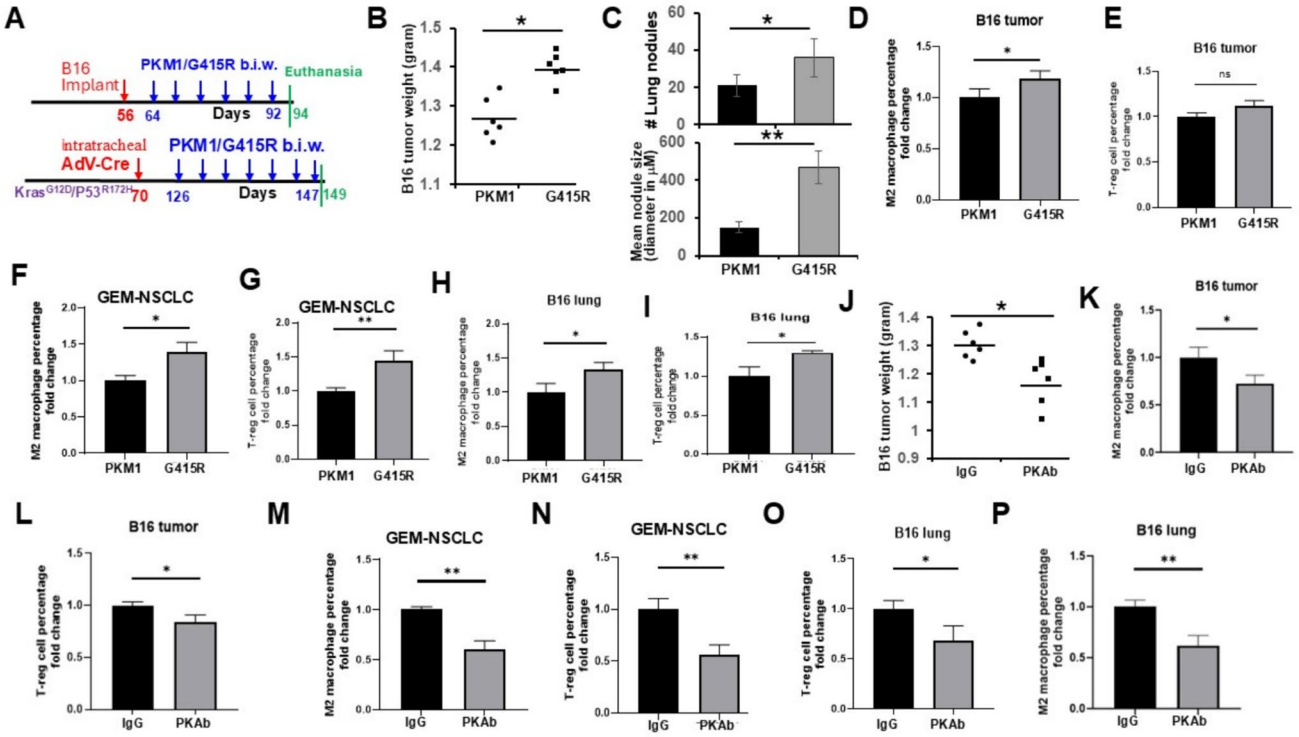


**Fig. 4** Effects of extracellular PKM2 and anti-PKM2 antibody PKAb on tumor immunity. (A) The scheme illustrates G415R or PKM1 (blue arrows) treatment regimen of B16 (Upper) and GEM-NSCLC (Bottom) mice. **B**, **J** Tumor weight of B16 tumor bearing mice treated with indicated agents (n = 6) was measured with weighting of sliced tumors from the treated mice. **C** Quantitative measurements of both number (Upper) and size (Bottom) of lung metastatic nodules in lung of the B16 mice treated with indicated agents (n = 10). **D, E, F and G** FACS analyses of F4/80$^+$ and CD206$^+$ M2 macrophages (**D**, **F**) and CD4$^+$ and FoxP3$^+$ $T_{reg}$ cells (**E**, **G**) in B16 tumors **D**, **E** and GEM-NSCLC tumors **F**, **G** from mice treated with indicated agents. **H** and **I** FACS analyses of F4/80$^+$ and CD206$^+$ M2 macrophages (**H**) and CD4$^+$ and FoxP3$^+$ Treg cells (**I**) in lung metastatic tumors from B16 mice treated with indicated agents. Quantities of M2 macrophage (**D**, **F**, **H**) or $T_{reg}$ cell (E, G, I) are presented as fold changes of percentage of macrophages or $T_{reg}$ in total population of macrophages or T cells respectively. **K** and **L**, **M**, and **N** FACS analyses of F4/80$^+$ and CD206$^+$ M2 macrophages (**K**, **M**) and CD4$^+$ and FoxP3$^+$ $T_{reg}$ cells (**L**, **N**) in B16 tumors **K**, **L** and GEM-NSCLC tumors **M**, **N** from mice treated with indicated agents. **O**, **P** FACS analyses of CD4$^+$ and FoxP3+ Treg cells (**O**) and F4/80+ and CD206+ M2 macrophages (**P**) in lung metastatic tumors from B16 mice treated with indicated agents. Quantities of M2 macrophage (**K**, **M**, **O**) or $T_{reg}$ cell (**L**, **N**, **P**) are presented as fold changes of percentage of macrophages or $T_{reg}$ in total population of macrophages or T cells respectively. Error bars in **D**–**P** represent mean ± S.E.M. from five independent experiments, and in **C** represent mean ± S.E.M. from 10 mice. ns: statistically non-significant, $^*P < 0.05$, $^{**}P < 0.01$

and subsequently regulates the Arg1 expression/activity. We probed Arg1 levels and activity in the rPKM2 treated Raw and BMMφ cells. Clearly, addition of rPKM2 but not rPKM1 into culture medium upregulates Arg1 (Fig. 5A, D). Arg1 activity assay revealed that rPKM2 increased arginase activity (Fig. 5E). The effects of rPKM2 on Arg1 activity were abrogated by addition of the antibody 23C6 or the FAK inhibitor FI14 into culture medium (Fig. 5F). Knockdown of integrin β3 in Raw cells also abolished the effects of PKM2 in regulation of Arg1 (Fig. 6A, B), suggesting that upregulation of Arg1 and increase in arginase activity in macrophage by EcPKM2 were mediated through integrin $\alpha_v\beta_3$. We further analyzed the PTEN levels and activity in Raw and BMMφ. Evidently, the addition of rPKM2 but not rPKM1 into culture medium reduced PTEN in the cells (Fig. 6C), suggesting that EcPKM2 might play a role in downregulation of PTEN in macrophage to promote M2 subtype. To verify the role of EcPKM2 in regulating Arg1 expression and promoting M2 polarity via suppression of PTEN expression, PTEN was exogenously expressed in Raw via adenoviral vector carry either null or PTEN (AdV-Null/AdV-PTEN) (Fig. 6D). Exogenous expression of PTEN downregulated Arg1 levels (Fig. 6E) and decreased arginase activity in the rPKM2 treated cells (Fig. 6F).

## PKAb enhances efficacy of checkpoint blockades

Addition of checkpoint inhibitors to various chemotherapies represents an important advancement in the treatment of cancers of different types [42, 43]. It is well documented that macrophage and polarity play a critical role in modulating cancer immunity by secreting pro- or anti-inflammatory

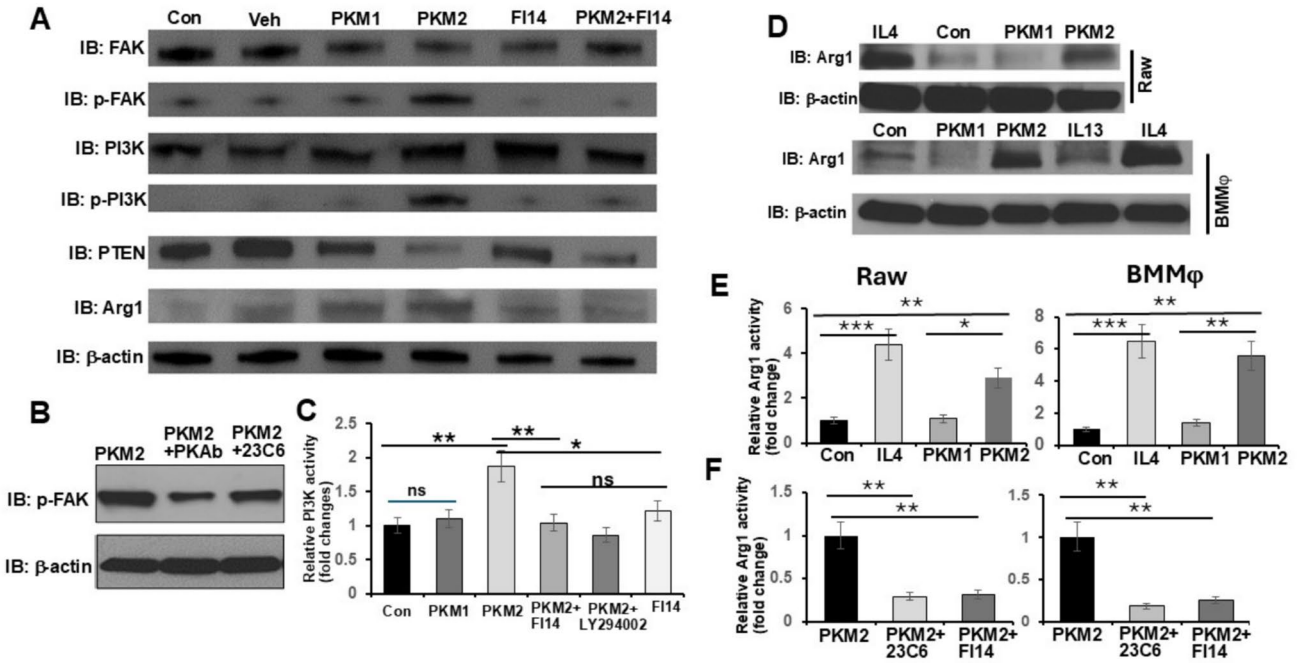


**Fig. 5** EcPKM2 activates FAK-PI3K signal axis and subsequently Arg1 in macrophage. **A**, **B** Levels of FAK (IB: FAK), phosphor FAK (IB: p-FAK), PI3K (IB: PI3K), phosphor PI3K (IB: p-PI3K, PTEN (IB: PTEN), and Arg1 (IB: Arg1) in Raw cells were analyzed by immunoblot. The Raw cells were treated with the indicated agents. Con and Veh are control (without treatment) or treated with vehicle respectively. **C** PI3K activity in Raw cells was measured with PI3K activity kit. The cells were treated with indicated agents. The PI3K activity is presented as relative PI3K activity (fold changes) by defining the activity of untreated control cells (con) as 1. **D** Levels of Arg1 (IB: Arg1) in Raw (upper) and BMMφ (Bottom) cells were analyzed by immunoblot. The cells were treated with the indicated agents. **E**, **F** Arg1 activity in Raw (**E**, **F**, Left panel) and BMMφ (**E**, **F**, Right panel) cells was measured with Arg1 activity kit. The cells were treated with indicated agents. The Arg1 activity is presented as relative Arg1 activity (fold changes) by defining the activity of untreated control cells (con) as 1 (E) or the PKM2 treated cells as 1 (**F**). IB: β-actin is a loading control in (**A**), (**B**), and (**D**). Error bars in (**C**), (**E**), and (**F**) represent mean ± S.E.M. from five independent experiments. ns: statistically non-significant, $*P<0.05$, $**P<0.01$, $***P<0.001$

soluble factors, such as cytokines/chemokines [4]. The anti-PKM2 antibody PKAb that disrupts the interaction between EcPKM2 and integrin $\alpha_v\beta_3$ promotes M1 macrophage polarity in tumor. It would be intriguing to test anti-cancer effects of PKAb in combination with checkpoint blockade. B16 is a murine melanoma model that is well tested with checkpoint blockade treatment. We therefore used this model to test efficacy of a combination of PKAb + anti-PD-1 antibody (αPD-1). Tumor bearing mice were treated with PKAb, αPD-1, and PKAb + αPD-1. PKAb and αPD-1 alone provided marginal or modest benefits on the animal overall survival and tumor growth. The combination provided strong anti-cancer effects (Fig. 6G).

## Discussion

Our study here uncovered an unexpected role of EcPKM2 in modulating cancer immunity by regulating macrophage polarity. EcPKM2 regulates macrophage polarity by interacting and activating integrin $\alpha_v\beta_3$ on macrophage in tumor. It is well documented that PKM2 can be released from cancer cells of different cancer types [19, 29]. We previously demonstrated that PKM2 can also be released from neutrophils and myofibroblasts [21]. Thus, neutrophils in tumor and cancer associated fibroblasts (CAF) that are phenotypically similar to myofibroblasts can also be the sources for EcPKM2 release. The released EcPKM2 acts on several targets in tumor to promotes cancer progression. (1) EcPKM2 facilitates tumor angiogenesis by promoting proliferation and migration of angiogenic endothelial cells [20]. (2) EcPKM2 may acts on cancer cells to promote metastasis [29]. (3) EcPKM2 protects myofibroblasts from apoptosis and facilitates collagen production in myofibroblasts [22]. Given CAFs are phenotypically similar to myofibroblasts, it is likely that EcPKM2 may exert similar effects on CAFs. (4) Herein, we demonstrate furthermore that EcPKM2 polarize macrophage to M2 subtype by interacting and activating integrin $\alpha_v\beta_3$ on macrophage to modulate cancer immunity. In consistence with these cancer promotion actions of EcPKM2 on multiple targets, it is well documented that

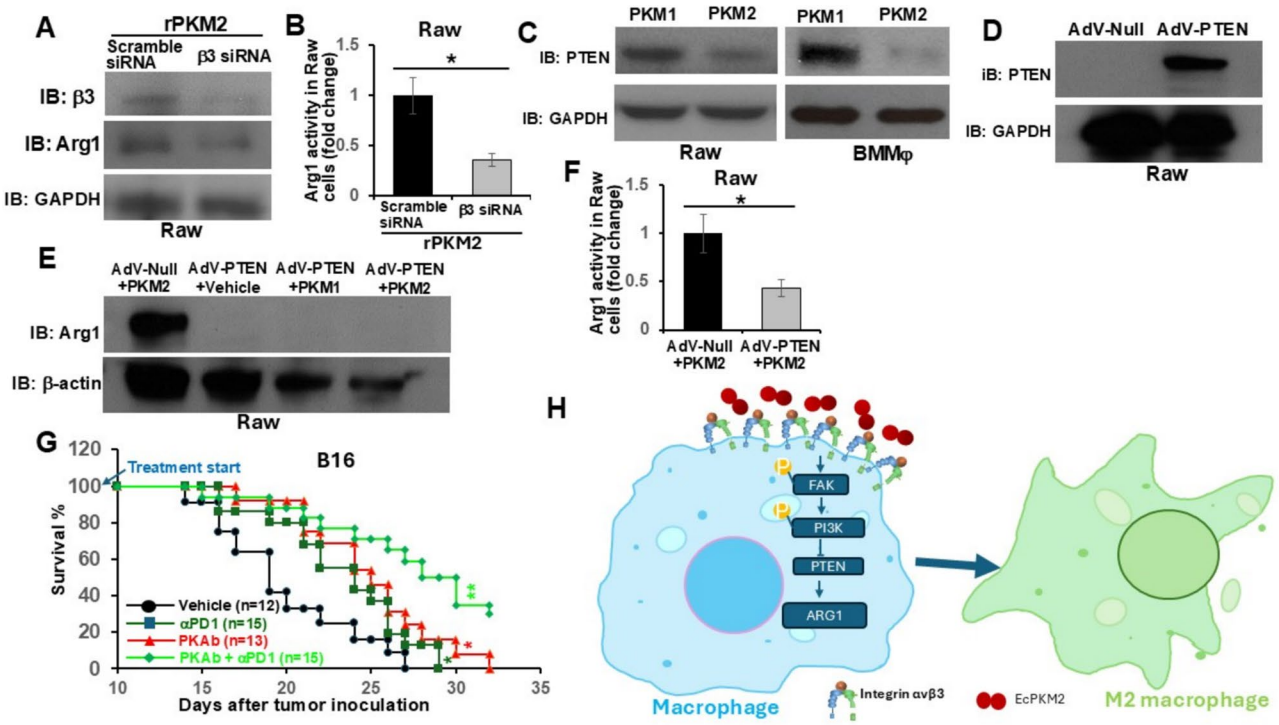


**Fig. 6** EcPKM2 regulates Arg1 levels and activity by suppressing PTEN in macrophage. **A** Levels of integrin β3 (IB: β3) and Arg1 (IB: Arg1) in Raw cells were analyzed by immunoblot. **B** Arg1 activity in Raw cells were analyzed using Arg1 activity kit. Integrin β3 in Raw cells was knocked down by β3 siRNA or the cells were treated with scrambled siRNA. The Raw cells were treated with PKM2. The Arg1 activity is presented as relative activity (fold change) by defining the activity of PKM2 and scrambled siRNA treated cells as 1. **C** Levels of PTEN (IB: PTEN) in Raw (Left) and BMMφ (Right) cells were analyzed by immunoblot. The cells were treated with either PKM1 or PKM2. **D** Levels of PTEN (IB: PTEN) in Raw cells were analyzed by immunoblot. PTEN was exogenously expressed using PTEN expressing adenoviral vector (AdV-PTEN) or the cells were infected with virus carry empty vector (AdV-Null). **E** Levels of Arg1 (IB: Arg1) in Raw cells were analyzed by immunoblot. PTEN was exogenously expressed using PTEN expressing adenoviral vector (AdV-PTEN) or the cells were infected with virus carry empty vector (AdV-Null). The cells were treated with vehicle, PKM1 or PKM2. **F** Arg1 activity in Raw cells were analyzed using Arg1 activity kit. PTEN was exogenously expressed using PTEN expressing adenoviral vector (AdV-PTEN) or the cells were infected with virus carry empty vector (AdV-Null). The cells were treated with PKM2. The Arg1 activity is presented as relative activity (fold change) by defining the activity in AdV-Null/PKM2 cells as 1. **G** Kaplan-Meier survival analysis of B16 tumor bearing mice treated with indicated agents. Treatment started 8 days after B16 cell inoculation. The n is group size. **H** Cartoon schematically illustrates the functional role of extracellular PKM2 in modulation of macrophage polarity. Error bars in (**B**) and (**F**) represent mean ± S.E.M. from five independent experiments. $*P<0.05$, $**P<0.01$.

levels of PKM2 in patient blood circulation correlate well with patient disease prognosis.

Along with our previous observations that EcPKM2 facilitates tumor growth [20], wound healing [21], and organ and tissue fibrosis by promoting angiogenesis and fibrogenesis, we propose a theme that EcPKM2 may bridge the inflammatory response to a later proliferation/regeneration phase (fibrogenesis/angiogenesis) during tissue injury repair and cancer progression. During wound repair, PKM2 is released from infiltrating neutrophils. The released PKM2 promotes angiogenesis and myofibroblasts differentiation to facilitates wound repair and regeneration [21]. While in tumor, cancer cells, neutrophils, and CAFs release EcPKM2 to promote "regeneration", which consequently facilitates proliferation/survival and metastasis. Interestingly, integrin $\alpha_v\beta_3$, the EcPKM2 target, is highly expressed in angiogenic endothelial cells, myofibroblasts/CAFs, and activated macrophages. A common physiological role of these three cell types is in "bridge the inflammatory response to a later proliferation phase (fibrogenesis/angiogenesis) during tissue injury repair and cancer progression".

Role of macrophage polarity in cancer progression is well documented. TAMs mostly adopt M1 at the early stage of cancer development. It is believed that M1 macrophages facilitate tumorigenesis and early cancer development/progression due to its pro-inflammatory roles [5]. Cancer cells at early-stage secret less PKM2. Tumors at early stage may have less associated CAFs. Thus, early-stage tumors may have less EcPKM2, which may provide a partial explanation that TAMs in early-stage tumor are mostly adopt a M1 subtype. However, TAMs in late-stage cancer are predominately

M2 subtype [8]. Cancer cells educate TAMs to M2 subtype to favor cancer progression, metastasis, and apoptosis resistance. Abundant M2 macrophages also facilitate cancer relapses after eradication of tumor by chemotherapies and/or radiation therapies. Release of EcPKM2 by cancer cells and CAFs may confer in-part of this educational course for TAMs by cancer cells and tumor microenvironment. Along with cancer progression, tumor immunity, angiogenesis, cancer cell proliferation/survival, metastasis, and stroma interactions are highly coordinated. An interesting scenario is that cancer cells and the cells in tumor microenvironment coordinately release EcPKM2 to orchestrate the effects and responses in favor of growth and "regeneration".

In consistence with our previous report [22], EcPKM2 exert its action by interacting with integrin $\alpha_v\beta_3$ and activating the integrin signaling on TAMs. Activation of integrin signaling by EcPKM2 triggers activation of FAK-PI3K signaling axis. This signal axis suppresses PTEN to upregulate and activate Arg1 (Fig. 6H). The role of PTEN expression in regulation of macrophage polarity is well documented [44]. It was well demonstrated that the same PI3K signaling axis plays a critical role in macrophage polarity via activation of AKT—mTOR [45]. It will be interested to explore whether the FAK-PI3K singling axis activated by EcPKM2 also activates downstream AKT-mTOR in addition to PTEN downregulation and Arg1 upregulation.

Another supportive evidence for the role of EcPKM2 in modulating macrophage polarity is that a neutralization antibody that disrupts the EcPKM2 and integrin $\alpha_v\beta_3$ interaction shifts the M2 macrophages back to M1 macrophages in tumor. Targeting macrophage in tumor, especially macrophage polarity, has drew increased attention as a promising anti-cancer strategy [4]. Our results demonstrated that the anti-PKM2 antibody PKAb enhanced efficacy of immune checkpoint blockade therapies in pre-clinical models, suggesting a potential application in cancer treatment. An advantage of targeting PKM2 as a potential cancer therapy agent is that the PKM2 neutralization molecule simultaneously targets tumor angiogenic endothelial cells, TAM polarity, and possibly CAFs. The multi-target effects may bring unique advantages in cancer treatment. High levels of circulating PKM2 closely associated with cancers and various inflammatory diseases. PKM2 in blood circulation is almost no detectable or detected in very low levels in normal healthy individuals, suggesting safety of neutralization circulating PKM2. Furthermore, levels of serum PKM2 in patients can be served as an easy assessment marker for potential treatment prediction.

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**Author contributions** ZR Liu conceptualized, planned, and coordinated the study. ZR Liu wrote the paper. G. Peng and B. Li conducted most of experiments, data analyses, and participated in paper writing; Y. Yuan H. Han, Y. Huang, and F. Mishra prepared recombinant PKM1/PKM2 G415R expression & purification and macrophage FACS. All authors discussed the results and commented on the paper.

**Data availability** No datasets were generated or analysed during the current study.

## Declarations

**Conflict interest** The authors declare no competing conflict of interests



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