Neutralization of the adaptor protein PAG by monoclonal antibody limits murine tumor growth

Marianne Strazza,1 Emily K. Moore,1 Kieran Adam,1 Inbar Azoulay-Alfaguter,2 and Adam Mor1,3

1Columbia Center for Translational Immunology, Columbia University Medical Center, New York, NY 10032, USA; 2Perlmutter Cancer Center, New York University School of Medicine, New York, NY 10016, USA; 3Division of Rheumatology, Department of Medicine, Columbia University Medical Center, New York, NY 10032, USA

The transmembrane adaptor phosphoprotein associated with glycosphingolipid-enriched microdomains 1 (PAG) is phosphorylated in T cells downstream of PD-1 signaling and contributes to the resulting functional inhibition of multiple cellular processes. Furthermore, PAG expression is negatively correlated with survival in multiple human tumors and is a driver of murine tumor growth and immune evasion. Here we develop an antibody that targets the extracellular domain of human PAG, with cross-reactivity to murine PAG. We demonstrate that this antibody binds to extracellular PAG on intact cells and affects T cell activation. Finally, we show that administration of anti-PAG monoclonal antibody in combination with anti-PD-1 antibody to mice bearing MC38 tumors limited tumor growth and enhanced T cell infiltration to tumors.

INTRODUCTION

Immune checkpoint therapy is a relatively new modality in the treatment of cancer. Specifically, PD-1 and PD-L1 targeting antibodies release the breaks on a patient’s T cells, allowing a more robust anti-tumor immune response. It is well established that enhanced T cell infiltration into tumors and activation correlate with better prognosis. It is through increased T cell activation that PD-1 blockade leads to tumor identification and subsequent clearance. Despite great promise for success, the average response rate to PD-1 blockade for most tumors is 23%,1–3 leaving open the opportunity for improvement.

We recently showed that phosphoprotein associated with glycosphingolipid-enriched microdomains 1 (PAG), a member of the transmembrane adaptor protein (TRAP) family, is phosphorylated after PD-1 is ligated by PD-L1 or PD-L2, and that PAG phosphorylation is associated with inhibition of various T cell functions downstream of PD-1.4 Furthermore, we showed that two murine tumors, colon adenocarcinoma MC38 and melanoma B16, exhibited limited growth in PAG knockout (KO) mice, with enhanced sensitivity to PD-1 blockade.5 Through T cell adoptive transfer experiments of PAG KO T cells into wild-type, tumor-bearing mice, we showed that this function of PAG in the context of the tumor immune response is T cell intrinsic.3

Through confocal live imaging we also established that PAG localizes to the point of contact or immune synapse between a T cell and antigen-presenting cell (APC), and that this localization is essential for its function in the PD-1 pathway.3 This leads us to hypothesize that by targeting PAG in vivo through antibody binding we could neutralize its inhibitory function. In this way, combined antibody administration of anti-PAG and anti-PD-1 antibodies to cancer patients could enhance the anti-tumor immune response and overall patient survival. To test this hypothesis, we generated antibodies targeting human PAG in mice and assayed the efficacy of these antibodies with respect to binding and neutralizing PAG function in vivo.

RESULTS

The extracellular portion of PAG is a feasible antibody target

PAG has a short, 16 amino acid extracellular portion or ectodomain that we will target through our immunization strategy. Notably, PAG does not have a signaling peptide that aides in its localization at the plasma membrane leaving open the possibility that the extracellular domain of PAG may be cleaved during protein processing. To first demonstrate that the 16 amino acid extracellular domain is intact on full-length PAG we stably expressed PAG-GFP in A549 lung epithelial cells (which do not express endogenous PAG), enriched for the protein in the cell lysate through immunoprecipitation with anti-GFP antibody-conjugated beads, and performed mass spectrometry. We conclude that the extracellular domain of PAG is not cleaved in protein processing and is present on full-length PAG (Figure 1A). We next used de novo peptide structure prediction4 on the extracellular portion of human and mouse PAG to demonstrate that it has a tertiary structure (Figure 1B). Notably, the predicted structures of human and mouse PAG extracellular portions are highly similar and have a low hydropathy index, indicative of low hydrophobicity. To support the role of PAG in activated T cells, we show that upon stimulation with anti-CD3 and anti-CD28 antibodies, PAG expression is increased and less phosphorylated6 (Figure 1C).

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Correspondence: Marianne Strazza, Columbia University Medical Center, 650 W 168 Street, BB-1708, New York, NY 10032, USA.
E-mail: ms5800@cumc.columbia.edu
Correspondence: Adam Mor, Columbia University Medical Center, 650 W 168 Street, BB-1708, New York, NY 10032, USA.
E-mail: am5121@cumc.columbia.edu

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As we have previously stated, PAG functions downstream of PD-1 in T cells following stimulation. Additionally, when a T cell and APC interact, the immune synapse is formed between the two cells. We have previously demonstrated that PAG and PD-1 both polarize to the immune synapse. To determine if PAG and PD-1 are co-localized within close proximity following PD-1 ligation, we used the proximity ligation assay (PLA) in combination with the PD-L2-overexpressing Raji B cell-Jurkat T cell co-culture system in the presence of superantigen staphylococcal enterotoxin E (SEE). PLA uses primary antibodies from different host species directed against two endogenously expressed proteins followed by secondary antibodies, termed PLA probes, which are tagged with a short sequence specific DNA tag. If the two proteins are within a proximity of 40 nm or less, the PLA probes are close enough to interact and the DNA strands can participate in rolling circle DNA synthesis. The resulting amplified DNA circle is then bound by fluorescent-labeled complementary oligonucleotide probes, and these fluorescent clusters are indicative of proteins within proximity when viewed by fluorescent microscopy. We demonstrate that endogenous PAG and PD-1 are within 40 nm of each other following PD-1 ligation, when PAG and PD-1 polarize to the immune synapse (Figure 1D).

To test our hypothesis that we can exclude PAG from the immune synapse and disrupt co-localization with PD-1, we expressed full-length PAG-GFP or full-length PAG conjugated to human IgG Fc (200 amino acids) at its N terminus and expressed extracellularly (Fc-PAG-GFP) in Jurkat T cells, to mimic antibody binding and the potential resulting steric hindrance (Figure 1E). We then co-cultured these transfected Jurkat T cells with Raji B cells and imaged the cells for PAG localization. We observed that PAG-GFP was enriched at the contact site more than Fc-PAG-GFP (Figures 1F and 1G). Furthermore, we observed that Fc-PAG-GFP was excluded from the immune synapse more often than PAG-GFP (Figures 1F and 1G). To correlate PAG exclusion from the synapse to T cell function, we measured IL-2 secretion from T cell-Raji cell conjugates that express either PAG-GFP or Fc-PAG-GFP and revealed impaired ability of PD-1 to inhibit cytokine secretion (Figure 1H). On the basis of these observations, we would propose that antibody bound to PAG could lead to mislocalization and dysfunction of PAG in the context of the immune synapse.

To develop antibodies to the extracellular portion of PAG, we immunized Balb/C and B6-129 mice with amino acids 1–16 of human PAG in combination with keyhole limpet hemocyanin (KLH), a large, copper-containing protein that is a highly immunogenic T cell antigen. Two boosters of BSA-PAG were administered. The primary serum screen was done using ELISA. Hybridoma libraries were made by fusing myeloma cells with B cells derived from the spleens of positive serum mice. The resulting immortalized B cells (hybridomas) produce monoclonal antibodies. Hybridoma supernatants were first screened using ELISA and then by flow cytometry binding to intact cells (Figure S1).

**PAG antibody clone 7M16A binds to human PAG**

Following clonal expansion of the hybridoma library, we screened hybridoma supernatants using ELISA against human, mouse, and cynomolgus PAG peptides and narrowed in on 2 human binding clones and 1 human non-binding clone (Figure 2A). To test the ability of the hybridoma supernatants to bind PAG on intact cells, we stained spleenocytes from WT or PAG KO mice (Figure S2A). We found that 7M16A stained WT but not PAG KO splenocytes (Figure 2B), suggesting specificity for PAG and the ability to bind endogenously presented PAG protein. To determine if clone 7M16A binds to the extracellular domain of human PAG on intact cells, we generated A549 cells that stably express PAG-GFP for this secondary screen. We confirmed the level of PAG-GFP expression using western blot (Figure 2C) and that it is expressed at the plasma membrane (Figure 2D). We then stained non-permeabilized A549 cells with clones 7M16A, 4F23A, and 8D04A. We observed PAG-specific surface staining with 7M16A by flow cytometry (Figures S2B and S2E). We then confirmed protein binding using a dose-response ELISA and found that clone 7M16A binds to human PAG, while clones 8D04A and 4F23A were found not to bind (Figure 2F).

To test the functional impact of 7M16A, we stimulated primary human CD3 T cells with anti-CD3 antibody and anti-CD28 antibody alone or in combination with 7M16A anti-PAG antibody and quantified IL-2 secretion over 48 h. Given that PAG is inhibitory to T cell activation, the neutralization of PAG is expected to lead to enhanced T cell activation, and accordingly, the addition of 7M16A increases IL-2 secretion beyond anti-CD3 and anti-CD28 antibodies alone (Figure 2G). To determine how 7M16A binding affects the enrichment of PAG and PD-1 at the immune synapse, we stably expressed both PAG-GFP and PD-1-SNAP + SNAP-AF647 in Jurkat T cells (PD-1-SNAP-AF647) to visualize the localization of these proteins.
A

B

C

D

E

F

G

H

I

(legend on next page)
These cells were pre-treated with 7M16A prior to co-culture with Raji B cells. We observed that PAG-GFP was enriched at the immune synapse in the majority of cells, but when 7M16A is introduced, PAG-GFP is excluded from the center of the immune synapse in the majority of cells (Figure 2H). We observed that when both proteins were co-expressed, PD-1-SNAP-AF647 was also enriched outside of the immune synapse in the majority of cells (Figure 2I).

On the basis of these experiments, we conclude that clone 7M16A (Tables 1–3) binds to PAG and interferes with its localization and inhibitory function in vitro.

Binding PAG and PD-1 limits tumor growth and enhances T cell infiltration

We have previously shown that two murine tumor models (MC38 and B16F10) exhibit limited growth and enhanced T cell infiltration in the absence of PAG, suggesting that PAG is contributing to limited T cell immune response. To determine if systemic administration of anti-PAG antibody alone or in combination with anti-PD-1 antibody affects tumor growth, we used the MC38 murine tumor model. First, we implanted MC38 murine colon adenocarcinoma cells subcutaneously and monitored tumor growth daily until tumors reached 60–75 mm³. At this point, antibodies were delivered through intraperitoneal (i.p.) injection every four days for a total of four doses. The combined administration of anti-PAG clone 7M16A and anti-PD-1 antibodies resulted in inhibited tumor growth relative to untreated and anti-PD-1 alone (Figure 3A). Additionally, tumors from mice treated with 7M16A alone or in combination demonstrated delayed growth kinetics (Figures 3B and 3C), with a longer growth time for the few tumors that did exceed 500 mm³ (Figure 3D). Immunohistochemistry of these tumors revealed increased CD3⁺ T cell infiltrates in the tumors from mice treated with anti-PAG clone 7M16A and anti-PD-1 compared with untreated mice (Figures 4A and 4B). More in-depth immune phenotyping of the tumor infiltrating lymphocytes suggests additional differences in CD4⁺Ki67⁺CD8⁺TCF1⁺Helios⁺ T cell subset distribution secondary to 7M16A and anti-PD-1 treatment (Figure S3).

DISCUSSION

PAG expression within tumors has been previously identified to correlate negatively with patient outcome in colon adenocarcinoma, renal cell carcinoma, melanoma, acute myeloid leukemia, invasive breast, cervical squamous, and testicular cancers. Additionally, we have demonstrated that PAG is phosphorylated downstream of PD-1 engagement, and that genetic deletion of PAG in mice limits tumor growth. During T cell-APC interaction, PAG is enriched at the immune synapse. The exclusion of molecules from the immune synapse by size has been previously reported. In one detailed study using a range of sizes of dextran molecules, it was established that the movement of dextran molecules ≤ 4 nm in and out of the immune synapse was unrestricted, but the movement of 10–13 nm dextran molecules was greatly reduced, and dextran molecules above 32 nm were nearly completely excluded. This study went further to show that monoclonal antibodies are excluded from the immune synapse. This led us to design a strategy to target PAG localization to alter PD-1 signaling and T cell function.

In this study we apply this principle of size exclusion and describe a novel strategy of using a monoclonal antibody to change the localization of a T cell surface protein in the context of the immune synapse (Figure 5). Antibodies have been previously shown to alter the localization of a protein on the cell surface, and in fact the synaptic autoimmune disorder anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis is characterized by auto-antibodies that target NMDAR in the brain and cause removal of the receptor from the neurological synapse. When a T cell and APC come together, forming the immune synapse, the precise localization of each protein contributes to overall signaling and function. Overall, the immune synapse architecture can be broken down into circular regions referred to as the central, peripheral, and distal supramolecular activation clusters (SMACs), and the relative proximity between individual proteins within these regions is critical to cellular function. Leveraging the strict organization of the immune synapse for immunotherapeutic intervention is currently of high interest in the field, and numerous approaches are being explored. The success of a monoclonal antibody targeting PAG provides a strong rationale to apply the same strategy to other signaling proteins. Specifically, we have shown that SLAMF6 modulates T cell function through a mechanism dependent on proximity to the T cell receptor, making SLAMF6 a promising protein candidate for size exclusion antibody targeting. The phosphatase CD45 has been previously shown to be excluded from the immune synapse on the basis of size, raising the possibility of inducing size exclusion by bispecific antibodies targeting CD45 and a signaling protein of interest, such as PAG, SLAMF6, or PD-1 itself. This has recently been achieved with an engineered version of PD-1 that cross-links to CD45 and inhibits PD-1 signaling.

Figure 2. Clone 7M16A binds to the extracellular domain of PAG

(A) ELISA with cloned PAG peptide (human, mouse, or cynomolgus) using hybridoma supernatants. (B) 7M16A staining of non-permeabilized wild-type C57BL/6 or PAG knockout (KO) murine splenocytes shows specificity of binding. (C) Western blot of A549 cells made to stably overexpress PAG-GFP. (D) Western blot of fractionated cell lysates showing that PAG-GFP is expressed at high levels at the cell membrane. Confocal imaging showing the localization of PAG-GFP at the plasma membrane. (E) 7M16A staining of non-permeabilized A549 cells shows binding to PAG-GFP on the surface. (F) ELISA with immobilized human PAG peptide using purified monoclonal antibodies. (G) ELISA of IL-2 secretion from primary human CD3⁺ T cells stimulated as indicated for 48 h. n = 3; “p ≤ 0.05 and **p ≤ 0.01. (H and I) Jurkat T cells stably express PAG-GFP with or without PD-1-SNAP incubated with Raj B cells in the presence of SEE. Cells were imaged using confocal microscopy to assess the location of PAG and PD-1 relative to the immune synapse. White arrow indicates the center of the immune synapse where PAG was excluded with 7M16A pretreatment. White star indicates the point in the cell with enrichment of PD-1 either at or away from the immune synapse. Quantification of the number of cells per phenotype. *p ≤ 0.05.
Table 1. PAG antibody clone 7M16A CDR analysis

| LP sample name | Chain type | CDR-1 | CDR-2 | CDR-3 |
|----------------|------------|-------|-------|-------|
| AVS-4378HCPT.1 | HC         | YTFTSYV | IPYNGDT | ARYKYGQFAY |
| AVS-4378LCP.1  | LC         | ENYSN  | AAT   | QHFWGTPWT |

Through this study, we have successfully generated and purified an antibody that binds human and mouse PAG and alters murine tumor growth in combination with anti-PD-1. Of note, this study of murine tumor growth was designed to establish the ability of this anti-PAG antibody to alter tumor growth and enhance anti-PD-1 response in a situation of poor response to anti-PD-1 antibody alone. For this reason, we initiated anti-PAG antibody administration prior to the previously published three-dose, 200 μg regimen of anti-PD-1 antibody, therefore increasing the tumor size at initiation of anti-PD-1 antibody and somewhat diminishing the MC38 response to anti-PD-1 antibody.

The next steps for this PAG antibody include affinity maturation and humanization, as well as assessing the mechanism of neutralization. During this process, we will also assess the potency of T cell activation by combined neutralization of PAG and PD-1, with particular focus on the potential for cytokine release syndrome (CRS). Identifying more precise causes for CRS in patients that receive immunotherapy is an essential area of ongoing research. On the basis of the current understanding of the field, anti-PD-1 treatment in combination with an additional factor is more likely to result in high-grade CRS, with few reports of CRS following anti-PD-1 therapy alone.21,22 One specific case study of esophageal cancer concluded that CRS was induced in a patient receiving anti-PD-1 only after additionally receiving therapeutic radiation.23 Bispecific antibodies targeting various T cell molecules are an important area of future solid tumor therapeutic design, and research is emerging that bispecific antibodies that target the T cell receptor benefit from low-affinity CD3 binding.24 The lesson that can be learned from this is that T cell activation by checkpoint inhibitors should also be carefully optimized such that maximum activation is unlikely to yield maximum benefit. The improved affinity, humanized antibody will be tested for function in a humanized mouse model along with human anti-PD-1 to test efficacy as well as safety.

Chimeric antigen receptor (CAR) T cells that co-express the CAR and immune-modulating antibodies, including anti-PD-1, have remarkable anti-tumor capacity. If left without limitations, persistent T cell activation will lead to severe immune-related adverse events and CRS. By limiting the expression of anti-PD-1 antibody to IFN-γ secreting CAR T cells, the cells that expressed the antibody exhibited lower PD-1 expression, increased T cell activation, and greater anti-tumor activity against PD-L1-positive tumor cell lines.25 Among patients with relapsed/refractory diffuse large B cell lymphoma previously treated with CD19-directed CAR T cells and subsequently treated with anti-PD-1 antibodies, 40% of patients achieved complete remission.26 A particularly interesting future direction of our study would be to include anti-PAG antibody under this IFN-γ restricted promoter to be co-expressed with anti-PD-1 antibody in CAR T cells and enhance efficacy.

Materials and Methods

General reagents

RPMI (catalog no. SLM-140) 1640 medium, DMEM (catalog no. SLM-020), Dulbecco’s PBS (DPBS), penicillin/streptomycin (P/S; catalog no. P4333), and fetal bovine serum (FBS; catalog no. F4135) were purchased from Life Technologies. Lymphoprep was purchased from Stemcell Technologies (catalog no. 07811). Superantigen staphylococcal enterotoxins was purchased from Toxin Technology (catalog no. ET404).

DNA constructs

peGFP-N1-PAG and peGFP-N1-FC-PAG constructs were generated by PCR sub-cloning and site-directed mutagenesis (catalog no. 210518; Agilent). pCDH-PAG-GFP was generated by PCR cloning. PHR-PD-1-SNAP was generously provided by Enfu Hui (University of California, San Diego).

Antibodies

Antibodies used include those against RhoGDI (polyclonal; catalog no. ab175243; Abcam), PD-1 (clone D4W2J; catalog no. 86163T; Cell Signaling), and EGFR (clone EP38Y; catalog no. ab52894; Abcam), PD-1 (clone D4W2J; catalog no. 86163T; Cell Signaling), and PAG (clone MEM-255; catalog no. SM3069P; OriGene).

Cell culture and transfection

Primary murine splenocytes were isolated by mechanical disruption of spleens from 10- to 12-week-old mice to generate a single-cell suspension. A549 human lung epithelial carcinoma cell line and HEK 293T human embryonic kidney epithelial cells (catalog nos. CRM-CCL-185 and CRL-3216; American Type Culture Collection [ATCC]) were maintained in 5% CO2 at 37°C in DMEM.

Table 2. PAG antibody clone 7M16A heavy chain sequence AVS-4378HCPT.1

| Nucleotide sequence | Amino Acid sequence |
|---------------------|---------------------|
| GAGTTCACGC TGCAGCATGC TGGACCTGAA CTGTAAAGGC CTGGGCGCTTC AGTGAAAGAT GCCTGCAAGG CTTCCTGATA CACATTCACT AGCTATGTTA TCAGCTAGGT GAAGCAAGAG CTTGGGCAAG GCCTGAGGAT GATGGATAT ATTTATCTTT ACAATGATGG TACTAAGTAC AATGAGAAGT TCAAAGGCAA GGCCACACTG ACTTCAGACA AATCCTCCAG CACAGCCTAC ATGGAGCTCA GCAGCCCTGAC TCTGCCGGTCT ATTATGGTCG ATAGTGCTTC AAGATATAAG TATGGTCAGG GGTTTGCTTA CTGGGGGCAA GGGACTCGGG TCACTTGCAC CAGAGCCTAG ATGAGGATCA GAGGACTGCGG TCACTGTGTC TGCAG |
| EVQLQGSGPELVKPGASVFKMSCKASGYFTSTYVMHWVKQKPGQGLEGWIYIPYNQDTKYNFKGKATLTSDKSSSAYMEISSLTS EDASVYYCARYKYGQFAYWGQGTRVTVSA |
supplemented with 10% FBS and 1% penicillin/streptomycin. Jurkat and Raji cells were obtained from the American Type Culture Collection (catalog nos. TIB-152 and CCL-86). These cell lines were maintained in 5% CO₂ at 37°C in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin. Raji and Jurkat cells were used in coculture assays stimulated with SEE (2 μg/mL) for 30 min at 400 × g for 30 min. The lysates were centrifuged for 10 min at 12,000 × g and 4°C. Lysates were then used for immunoprecipitation of PAG-GFP using anti-GFP antibody-conjugated agarose beads (catalog no. D153-8; MBL) according to the manufacturer’s protocol. The PAG-GFP-enriched protein lysates were then separated using Tris-glycine PAGE and stained with Coomassie Brilliant blue, and the band at the correct size was cut. The gel was treated with chymotrypsin, and the enriched proteins were identified using a Fusion Tribrid mass spectrometer and analyzed using Scaffold 4.0 software.

**Western blotting**
If stimulated, Jurkat T cells were stimulated with plate-bound 5 μg/mL anti-CD3 antibody (clone UCHT1) and 1 μg/mL anti-CD28 antibody (clone CD28.2) for 18 h. Cells were then collected and placed on ice, resuspended in ice-cold PBS, and centrifuged for 5 min at 400 × g and 4°C. The cell pellets were resuspended in cold RIPA lysis buffer, containing complete Mini, EDTA-free protease inhibitors. The cells were placed on a rotator, and lysis was carried out at 4°C for 30 min. The lysates were centrifuged for 10 min at 12,000 × g and 4°C. Where fractionation is noted, clarified cell lysates were fractionated using ultracentrifugation at 100,000 × g. The resulting supernatant (S100) contains soluble, cytosolic proteins, and the pellet (P100) contains cell membrane associated proteins. Samples were prepared with 2 × Laemmli buffer, boiled at 95°C for 10 min and run on SDS-PAGE. Following protein transfer for 30 min at 25 V, the nitrocellulose membrane was blocked with 5% BSA in PBS containing 0.05% Tween 20 (PBST) and blotted overnight with primary antibody prepared in PBST containing 2.5% BSA. The membrane was developed using IRDye secondary fluorescent antibody and acquired on an Odyssey CLx Imaging system.

**Proximity ligation assay**
The proximity ligation assay kit, Duolink In Situ Red was obtained from Sigma-Aldrich (catalog no. DUO92101-1KT). WT Jurkat T cells were treated with 2 μg/mL PHA overnight to induce PD-1 expression. The next day, PD-1 expression was confirmed on flow cytometry, and cells were resuspended in OPTI-MEM. Coverslips were washed and coated with 0.01% poly-L-lysine for 5 min at room temperature. Jurkat cells were added to the slide and allowed to adhere for 30 min, while Raji B cells were pre-loaded with 5 μg/mL SEE in OPTI-MEM for 30 min, both at 5% CO₂ at 37°C. Without washing, Raji cells were added to the coverslip in a 1:1 ratio. Cells were incubated for 30 min at 5% CO₂ at 37°C to allow immune synapses to form. Cells were then fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 for 10 min. Finally, cells were subjected to the manufacturer’s protocol for Duolink PLA. Images were acquired using the LSM 900 and analyzed using ImageJ (NIH).

**Immunoprecipitation and mass spectrometry**
A549 cells stably expressing PAG-GFP were lysed in cold RIPA lysis buffer, containing complete Mini, EDTA-free protease inhibitors (Roche). The cells were placed on a rotator and lysis was carried out at 4°C for 30 min. The lysates were centrifuged for 10 min at 12,000 × g and 4°C. Lysates were then used for immunoprecipitation of PAG-GFP using anti-GFP antibody-conjugated agarose beads (catalog no. D153-8; MBL) according to the manufacturer’s protocol. The PAG-GFP-enriched protein lysates were then separated using Tris-glycine PAGE and stained with Coomassie Brilliant blue, and the band at the correct size was cut. The gel was treated with chymotrypsin, and the enriched proteins were identified using a Fusion Tribrid mass spectrometer and analyzed using Scaffold 4.0 software.

**Immunization strategy**
Balb.C and B6-129 mice were immunized using 1–16 amino acid human PAG-KLH to stimulate antibody production. Antibody secreting plasma cells were then isolated from mice with positive serum binding to peptide in the first ELISA screen and fused with myeloma cells to generate hybridomas. Hybridomas were selected in HAT medium and sorted into clones. Clones were selected on the basis of ELISA screening (primary) and flow cytometry screening (secondary) for positive binders. Selected hybridoma clones were then expanded to produce purified monoclonal antibodies.

**IL-2 ELISA**
To determine the concentration of secreted IL-2 following 48 h antibody stimulation, the human IL-2 ELISA kit (catalog no. 570409; BioLegend) was used according to the manufacturer protocol. Primary human CD3⁺ T cells were purified from peripheral blood using RosetteSep CD3⁺ Negative Selection (catalog no. 15021; Stemcell) followed by Lymphoprep separation. T cells were stimulated with plate-bound anti-CD3 (clone UCHT1) and anti-CD28 (clone CD28.2) antibodies (10 μg/mL) and anti-PAG antibody clone 7M16A (50 μg/mL) for 48 h prior to supernatant collection and ELISA.

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**Table 3. PAG antibody clone 7M16A light chain sequence AVS-4378LCP.1**

| Nucleotide sequence | Amino Acid sequence |
|---------------------|---------------------|
| GAGATCCAGA TGACTCACTGC TCCAGGCTC CTATCGTATG CTGTGGGGAG AACTGTCACC ATCACACGTG GCCACAGTG GAATATTAG ATGAAATTTG CATGATATCA CGAGAAACAG GAAATACCTC CTCAGGCTCT CGTCTATGG CACAAGA ACTCAGATGG TGGCCATCA AGGTGACGT GCAGTGGATC AGGCACACAG TATCGGCTCA AGATCAACAG CCTGCAGTCT CAAGATTTG GAGATTATTA CTGTCACCAT TTGTGCGGTA CTCCTGGTGC ACTGTCAGTG GACCCAAAGC TGGAATAATC AA |
| DIQTMSPSASLVSVEVTITCRRASENIYSNLAWYQQKPQKSLVYAATNLADGVPSRFSGGSQTLKINSLQSEDFFGYCQ HPWGTPWTFGGGTKLEIK |
Primary ELISA was done for screening binding of hybridoma supernatants to human, murine, and cynomolgus PAG (2 mg/mL). In the second ELISA for confirmation of binding of purified monoclonal antibodies to human PAG, 96-well half-area high-binding ELISA plates (Corning 3690) were coated with 1 mg/mL of human PAG protein in 1/20 TBS at room temperature for 2 h. The plate was washed with TBS containing 0.05% Tween 20 and blocked with 2% BSA in TBS for 1 h at room temperature. After 1 h, the plate was washed and diluted PAG antibodies in 1/20 TBS were dispensed to respective wells and incubated for 1 h at room temperature. The plate was washed and incubated with peroxidase-AffiPure F(ab')2 goat anti-mouse IgG (catalog no. 115-006-003; Jackson ImmunoResearch) at 1:5,000 dilution for 1 h at room temperature. After the plate was washed, 3, 3', 5', 5' tetramethyl benzidine (TMB) substrate (catalog no. 421101; BioLegend) was added to each well. The reaction was stopped by adding 2N H2SO4. Absorbance was measured on plate reader (H1; Biotek Synergy) and results calculated as $A = \frac{A_{450\text{nm}}}{A_{570\text{nm}}}$. 

**Flow cytometry**

Non-permeabilized cells were stained with the primary antibody clones in FACS buffer (HBSS without Ca²⁺/Mg²⁺, FBS [3%], NaN₃ [0.02%], and CaCl₂ [2.5 mM]), then washed and stained with
fluorescently conjugated secondary antibody (goat anti-mouse; catalog no. 115-006-003; Jackson ImmunoResearch). Stained cells were then fixed in 1% paraformaldehyde. Splenocytes were pre-treated with Fc receptor block (catalog no. 422301; BioLegend). PAG-GFP-expressing cells were fixed in 1% paraformaldehyde. Events were recorded using FACSCanto (BD) and analyzed using FlowJo software (version 10.8.1).

**Raji-Jurkat conjugate microscopy**

Raji cells were stained with Tag-It Violet (BioLegend) according to the manufacturer's protocol, then pre-incubated with SEE (100 ng/mL) for 1 h at 5% CO₂ at 37°C. When PD-1-SNAP-expressing Jurkat cells were included in the assay, the cells were pre-stained with SNAP-AF-647 (NEB) according to the manufacturer's protocol. When 7M16A was included, Jurkat T cells expressing PAG-GFP or PAG-GFP and PD-1-SNAP were pre-incubated with 7M16A (1 µg/mL) for 1 h at 5% CO₂ at 37°C. Both Raji and Jurkat cells were then individually diluted to 4 × 10⁶ cells/mL in serum-free medium and combined in a 1:1 ratio immediately before imaging on poly-L-lysine coated image plates (MatTek). Conjugates were then imaged continuously for 30 min. Images were acquired using the LSM 900 and analyzed using ImageJ (NIH).

**Mice and tumor cell lines**

Animal studies were approved by the Columbia University institutional animal care and use committee (IAUC). Balb/C and B6-129 mice used for immunizations were sourced by LakePharma. Male, 6- to 12-week-old C57BL/6 (B6) wild-type or PAG knockout (PAG KO) mice were used in all animal studies. The WT and PAG KO mice in all studies were littermates, assuring a homogeneous genetic background. The murine colon adenocarcinoma (MC38) colon carcinoma cells were a gift from Ben Néel of New York University. Prior to use, MC38 cells were authenticated using simple sequence length polymorphism (SSLP). The MC38 cells were maintained in DMEM supplemented with heat-inactivated fetal bovine serum (10%) and penicillin/streptomycin (1% 10,000 U/mL stock) and grown at 37°C with 5% CO₂. Cells were passaged prior to storage and thawed and passaged twice prior to implantation for all described tumor experiments. All cell lines were determined to be free of mycoplasma (catalog no. LT07-318; Lonza).

**Tumor models**

MC38 (1 × 10⁶) cells were implanted subcutaneously in the right hind flank of 6- to 10-week-old mice. Tumor growth was monitored using electronic calipers and calculated using the formula: V = length × width² × 0.52. When tumor volume reached 60–70 mm³, the first dose of 7M16A was administered by i.p. injection (treatment day 0). 7M16A was then administered every 4th day for a total of 4 doses. Anti-PD-1 (200 µg; BioXcell clone RPM1-14) was initiated on treatment day 4 and administered by i.p. injection every 4th day for a total of 3 doses. For immunohistochemistry, tumors were fixed in 10% neutral buffered formalin then paraffin embedded and cut into 5 µm sections. Slices were stained with anti-CD3 (Clone SP7, Abcam, catalog No. ab16669), and bound antibody was detected with peroxidase-based staining.

**Tumor infiltrating lymphocyte flow cytometry**

MC38 (1 × 10⁶) cells were implanted subcutaneously in the right hind flank of 6-10-week-old mice. Tumor growth was monitored using electronic calipers and calculated according to the formula: V = length × width² × 0.52. When tumor volume reached 40–50 mm³, the first dose of 7M16A was administered by i.p. injection (treatment day 0). 7M16A was then administered every 4th day, for a total of 3 doses. Anti-PD-1 (200 µg; clone RPM1-14; BioXCell) was initiated on treatment day 4 and administered by i.p. injection every 4th day.
for a total of 2 doses. Tumors were harvested on day 14 for single-cell isolation. Harvested tumors were diced into small pieces and incubated in digestion medium containing Collagenase H (C8051; Sigma-Aldrich) and DNase (10104159001; Roche) in RPMI at 37°C for 30 min. Following digestion, tumors were pressed through 70 μm mesh strainers to generate a single-cell suspension. Cells were then overlaid on Lymphoprep and centrifuged at 800 × g for 10 min without break to remove any dead cells. Cells were then collected from the cell interface and stained for flow cytometry. Following Fc receptor block, cells were stained for surface proteins with fluorescently conjugated antibodies (TCR-B, CD103, CD44, CXCR3, KLRG1, PD-L1, CD45, Sca-1, Ly6C, CD206, NK1.1, B220, PD-1, Nrp1, CD4, CD39, IA-IE, ST2, CD8, CD62L, CD11c, ICOS, RORγt, CD69, CD11b, F4/80, and CD38), then fixed and permeabilized (True-Nuclear Transcription Factor Buffer Set; BioLegend) for intracellular staining (Ki67, iNOS, TOX, Ly6G, Helios, FoxP3, and TCF-1). Events were recorded using CytomX Aurora 5L, and analyzed using FlowJo software (version 10.8.1).

Statistics
Values are reported as mean ± SEM. Statistical analyses were performed using the Mann-Whitney test. All statistical analyses were performed using GraphPad Prism 9.

Data availability
Data reported in this study will be shared by the lead contact upon request.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omitm.2022.10.012.

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AUTHOR CONTRIBUTIONS
Conceptualization, M.S. and A.M.; Formal Analysis, M.S., E.K.M., and I.A.-A.; Funding Acquisition, A.M.; Investigation, M.S., K.A., E.K.M., and I.A.A.; Methodology, M.S., E.K.M., and I.A.-A.; Project Administration, M.S. and A.M.; Supervision, A.M.; Visualization, M.S.; Writing – Original Draft, M.S.; Writing – Review & Editing, M.S. and A.M.

DECLARATION OF INTERESTS
The authors declare no competing interests.
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