Peptide vaccine targeting mutated *GNAS*: a potential novel treatment for pseudomyxoma peritonei

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ABSTRACT

Background Pseudomyxoma peritonei (PMP) is a rare, slow-growing abdominal cancer with no efficacious treatment options in non-resectable and recurrent cases. Otherwise, rare activating mutations in the *GNAS* oncogene are remarkably frequent in PMP and the mutated gene product, guanine nucleotide-binding protein α subunit (Gsα), is a potential tumor neoantigen, presenting an opportunity for targeting by a therapeutic cancer vaccine.

Methods Tumor and blood samples were collected from 25 patients undergoing surgery for PMP (NCT02073500). *GNAS* mutation analysis was performed by next-generation targeted sequencing or digital droplet PCR. Responses to stimulation with Gsα mutated (point mutations R201H and R201C) 30 mer peptides were analyzed in peripheral blood T cells derived from patients with PMP and healthy donors. Fresh PMP tumor samples were analyzed by mass cytometry using a panel of 35 extracellular markers, and cellular subpopulations were clustered and visualized using the visual stochastic network embedding analysis tool.

Results *GNAS* mutations were detected in 22/25 tumor samples (88%; R201H and R201C mutations detected in 16 and 6 cases, respectively). Strong T cell proliferation against Gsα mutated peptides was observed in 18/24 patients with PMP. Mass cytometry analysis of tumor revealed infiltration of CD3 +T cells in most samples, with variable CD4+:CD8 + ratios. A large proportion of T cells expressed immune checkpoint molecules, in particular programmed death receptor-1 and T cell immunoreceptor with Ig and ITIM, indicating that these T cells were antigen experienced.

Conclusion These findings point to the existence of a pre-existing immunity in patients with PMP towards mutated Gsα, which has been insufficient to control tumor growth, possibly because of inhibition of antitumor T cells by upregulation of immune checkpoint molecules. The results form a rationale for exploring peptide vaccination with Gsα peptides in combination with immune checkpoint inhibiton as a possible curative treatment for PMP and other *GNAS* mutated cancers.

BACKGROUND

Pseudomyxoma peritonei (PMP) is a rare, slow-growing abdominal cancer that commonly originates in ruptured appendiceal mucinous neoplasms, seeding tumor cells, and mucin into the peritoneal cavity. The disease is characterized by slow, progressive accumulation of mucinous tumor tissue in the peritoneal cavity, ultimately leading to abdominal compression. Standard-of-care treatment involves cytoreductive surgery (CRS) to remove all visible tumor tissues, followed by hyperthermic intraperitoneal chemotherapy (HIPEC) to eliminate microscopic residual disease.[1–3] This treatment is curative in approximately 50% of the patients, but for patients who cannot be cured by CRS-HIPEC, no efficacious treatment options exist. Responses to systemic chemotherapy are generally poor, and biological agents, such as angiogenesis inhibitors, have been suggested as alternative therapeutic approaches, but so far with little success. In the setting of non-resectable and recurrent disease, PMP is a debilitating and ultimately fatal condition, leaving patients to experience progressively poor quality of life caused by an increasing intraperitoneal tumor burden.

A remarkably high frequency (60%–100%) of otherwise infrequently occurring mutations in the *GNAS* oncogene points to *GNAS* as a potential therapeutic target in PMP.[4] *GNAS* encodes the guanine nucleotide-binding protein α subunit (Gsα), which functions as a molecular switch to control cell growth, survival, and motility. The mutations are located in codon 201 (R201C and R201H) and result in constitutive activation of downstream signaling through the protein kinase A pathway. This activation may explain the massive production of mucin and suggests that mutated *GNAS* is a major oncogenic driver in PMP. Interestingly, the same *GNAS* mutations are found in subgroups of several other cancer entities (overall estimated frequency of 4.4%), making this the

most frequently mutated G protein in cancer, further supporting the potential value of a therapeutic intervention directed at this target.[5 6]

The intracellular location of Gsα combined with the single-codon mutation makes this a difficult target for small-molecule inhibitors and antibodies, which might explain why no targeted therapies exist. A vaccine-based treatment targeting mutated *GNAS* could therefore be a valuable addition to the cancer treatment toolbox with a much broader scope than PMP alone.

Mutations in cancer driver genes may act as neoantigens and are particularly interesting targets for development of therapeutic cancer vaccines, and patients with slow-growing tumors represent an ideal clinical setting. Therefore, a vaccination approach targeting mutated *GNAS* could represent a novel therapeutic opportunity in the hope of providing a cure for PMP. To explore mutated *GNAS* as a target for a cancer vaccine approach, we first questioned if long, synthetic Gsα peptides carrying the relevant mutations are immunogenic by analyzing responses in peripheral blood T cells derived from patients with PMP. We further investigated immune cell infiltration in the PMP tumor microenvironment in tumor samples collected at the time of surgery from the same patients and performed immunoprofiling by mass cytometry on immune cells isolated from tumor specimens.

METHODS
Clinical samples
Patients with suspected PMP were included in the study between April 2018 and May 2020 when admitted for CRS-HIPEC at the Norwegian Radium Hospital, Oslo University Hospital Comprehensive Cancer Center. Tumor and peripheral blood samples were collected at the time of surgery. Tumor distribution on the peritoneal surface was classified by the surgeon according to the Peritoneal Cancer Index (PCI), giving a score between 0 and 39.[7] Residual tumor after CRS was classified using the completeness of cytoreduction (CC) score (CC-0, no residual tumor; CC-1, residual tumor <0.25 cm; CC-2, tumor between 0.25 cm and 2.5 cm and CC-3, tumor >2.5 cm).[7] Complete cytoreduction was defined as CC-0 and CC-1. All PMP cases were evaluated by an expert pathologist and classified according to the Peritoneal Surface Oncology Group International classification.[8] Peripheral blood was collected from anonymous healthy donors for testing of immune responses on informed consent (project ID #2019/121).

Analysis of tumor *GNAS* and *KRAS* mutations
Fresh tumor samples were collected at the time of surgery from 22/25 cases. Samples were immediately snap frozen and stored at −80°C until further processing. The tumor content was assessed in H&E-stained frozen sections. Regardless of tumor content, available samples were homogenized and disrupted using TissueLyzer LT (Qiagen, Hilden, Germany). DNA was extracted using the AllPrep DNA/RNA/miRNA Universal Kit, automated with the use of QIAcube (Qiagen). For 10 PMP cases, no fresh tumor tissue was collected at CRS-HIPEC (n=3) or no mutation was detected in fresh-frozen samples with no or very low tumor cell content (n=7). In these cases, DNA was additionally extracted from the formalin fixed, paraffin-embedded routine pathology samples of the peritoneal disease or the primary appendiceal tumor after microdissection, using the QIAcube and AllPrep DNA/RNA formalin-fixed paraffin-embedded (FFPE) Kit. DNA purity was measured using NanoDrop (Thermo Fisher Scientific, Waltham, Massachusetts, USA); median absorbance ratio 260/280 was 1.83 (min–max 1.51–2.55), and concentrations were determined with the Qubit fluorometer (Thermo Fisher Scientific). Targeted next-generation sequencing was performed with the Ion GeneStudio S5 system and the Oncomine Comprehensive Assay V.3 (Thermo Fisher Scientific), covering single-nucleotide variants and indels from 161 unique genes. The median coverage of called variants was 4929, enabling detection of variants down to 1% allele frequency. Variants were called, annotated, and filtered with Ion Reporter Software V.5.10 (Thermo Fisher Scientific) and manually reassessed using Integrative Genomics Viewer. In DNA from the formalin-fixed samples, the presence of *GNAS* and *KRAS* mutations was assessed using the digital droplet PCR (ddPCR) system from BioRad (BioRad, Hercules, California, USA). The ddPCR Mutation Assay (GNAS R201H (assayID: dHsaMDV2516792) and GNAS R201C (assayID: dHsaMDV2510562)) were used for detection of R201H and R201C mutations, respectively. For detection of *KRAS* G12D, G12V, and G13D mutations, the KRAS G12D (assayID: dHsaMDV2510596), KRAS G12V (assayID: dHsaMDV2510592), and KRAS G13D (assayID: dHsaMDV2510598) assays were used. Premix preparation, droplet generation, and thermal cycling were performed according to the manufacturer's instructions. The fluorescence intensity in droplets was detected by a QX200 Droplet Reader (BioRad). For both assays, a 'no template control' and a positive control were included for quality control. QuantaSoft V.1.7.4 analysis software and QX Manager Software (BioRad) was used for data acquisition and analysis. Only tests providing >13.000 droplets were considered valid.

T cell proliferation assays
Peripheral blood mononuclear cells (PBMCs) from patients with PMP and healthy donors were isolated and frozen and later thawed for testing as previously described.[9] PBMCs were stimulated once in vitro with 10 µM Gsα mutated peptides at 2×10^6 cells/mL in X-Vivo 15 medium (Lonza, Basel, Switzerland), adding 20 U/mL IL-2 (Clinigen, Burton on Trent, UK) and 5 ng/mL IL-7 (Bio-Techne, Minneapolis, Minnesota, USA) on day 3. The Gsα peptides (aa 186–215) contained point mutations R201H and R201C (ProImmune, Oxford, UK) and were of indicated lengths (30 amino acids). After

12–14 days, T cells were tested in proliferation assays; prestimulated T cells were seeded at 5×10^4 cells per well in 96-well round-bottomed plates. The same number of irradiated (30 Gy), autologous PBMCs was added for use as antigen-presenting cells and Gsα mutated peptides as well as the wild-type (WT) peptide sequence were added at 10 µM. Staphylococcal superantigen SEC3 (0.1 µg/mL) was added as a positive control. Proliferation was measured on day 3 after labeling with 3.7×10^4 Bq ^{3}H-Thymidine (Montebello Diagnostics AS, Oslo, Norway) overnight before harvesting. All conditions were tested in triplicate. The Stimulatory Index (SI) was defined as proliferation with peptide divided by proliferation without peptide and SD were calculated. SI ≥2 was considered as a positive response.

Interferon gamma (IFN-γ) enzyme-linked immunospot (ELISPOT) assays

If cell numbers were sufficient with either freshly peptide prestimulated or thawed prestimulated T cells, IFN-γ ELISPOT assays (CTL Europe GmbH, Bonn, Germany) were performed. T cells and autologous PBMCs were plated onto plates precoated with antihuman IFN-γ at 1:1 ratio (0.5–1.0×10^5 cells) and 10 µM of 30 mer Gsα peptides (WT, R201C, and R201H) were added. Wells with no stimulus served as a negative control, while cells stimulated with 0.1 µg/mL SEC3 served as a positive control. Plates were incubated for 24 hours prior to addition of detection reagents and substrate following the manufacturer's instructions. The plates were imaged and the spot counts determined using an automated ELISPOT analyzer ImmunoSpot S6ULTIMATE (Cellular Technology, Shaker Heights, USA). Peptide-specific spot counts were determined by subtracting the mean spot number of no peptide-stimulated coincubated T cells and PBMC control wells for each patient from the number of spots in the Gsα peptide (WT, R201C, and R201H) stimulated wells. As there were insufficient cell numbers to perform titrations (except for one patient), saturated well counts for SEC3-positive controls where spots were too numerous to count (TNT) were set to >1000 spots or TNTC. Raw data included images for each well for visual quality checking (online supplemental figure 1).

Mass cytometry (cytometry by time of flight (CyTOF)) analysis of immune cells from PMP tissues

Fresh tumor tissue was disaggregated with Collagenase II (Sigma-Aldrich, St Louis, Michigan, USA) and DNase I (Sigma-Aldrich) after cutting tumor into small pieces. The single-cell suspension was then washed and erythrocytes were lysed by adding ACK Lysis Buffer (Thermo Fisher Scientific) before a second wash. The single cells were frozen in fetal bovine serum (Gibco, Thermo Fisher Scientific) containing 12% dimethyl sulfoxide (Sigma-Aldrich) and stored in liquid nitrogen. Briefly, single cells from biopsies were thawed, washed, and resuspended in MaxPar cell staining buffer (Fluidigm, San Francisco, California, USA), before staining with Cell-ID cisplatin

(Fludigm) for 5 min, then washed and stained with extracellular antibodies for 30 min. The samples were then fixed with 1.6% paraformaldehyde and permeabilized in 99% methanol (Sigma-Aldrich). Samples were stored at –80°C in methanol for up to 4 weeks. After removing methanol, all samples were incubated with iridium cell tracker (Fluidigm) for 20 min, washed and resuspended in water with 10% of calibration beads (Fluidigm). Samples were filtered immediately before sample acquisition on a CyTOF 2 (Fluidigm) instrument at the OUS Flow Cytometry core facility. Analysis was performed using Cytobank Cellmass (cytobank.org). A panel of 35 extracellular markers was used (see online supplemental table 1). Typical gating strategy was applied as follows: (1) EQ-140 versus Ir-191 to exclude calibration beads, (2) Ir-191 versus Ir-193 to gate singlets, (3) Ir-191 versus event length to gate intact singlet, and (4) cisplatin versus CD45-89Y to gate live lymphocytes. All further analyses were carried out on this population. Cellular subpopulations were clustered and visualized using the (visual stochastic network embedding (viSNE) analysis tool (based on the t-distributed stochastic neighbor embedding clustering algorithm). The clustering was based on the expression of CD45RA, CD19, CD11b, CD4, CD20, CD21, IgD, CD14, CD8a, CD3, HLA-DR, CD56, CD16, CCR6, CD25, PD-L1, PD-L2, lymphocyte-activation gene 3 (LAG-3), TIM-3, T cell immunoreceptor with Ig and ITIM (TIGIT), programmed death receptor-1 (PD-1), CCR7, CD28, CTLA-4, ICOS, CXCR3, CXCR5, CXCR4, CD161, CD127, NKG2D, CD38, and CD33 and ran with the following parameters: 1000 iterations, 30 perplexities and 0.5 theta.

Flow cytometry analysis of immune cells from PMP tissues

Single cells from biopsies were thawed, washed and resuspended in staining buffer (phosphate buffered saline with 2% fetal calf serum) at 0.2–0.5×10^6 per tube. Ten microlitres of aggregated gamma-globulin (Fc receptor block) was added and left for 15 min at room temperature (RT). Antibodies were added and the samples were incubated at RT for another 20 min in the dark. Antibodies used were CD3 FITC (OKT3; eBioscience, San Diego, California, USA), CD4 BV421 (OKT4; BioLegend, San Diego, California, USA), CD8 BV605 (RPA-T8, BioLegend), CD39 APC (eBioA1, eBiosciences), CD69 PE-Cy7 (FN50, BioLegend), TIGIT PE-Cy7 (MBSA43, eBioscience), and PD-1 PE-Cy7 (eBioJ105, eBioscience)

Cells were then washed in staining buffer prior to direct acquisition on a FACS Canto II (BD Biosciences). Data were analyzed using FlowJo software (Treestar, Ashland, USA). The gating strategy was the following: the lymphocytes were selected from the scatter gate in forward scatter (FSC) versus side scatter (SSC) dot plot, then CD4 and CD8 gates were set in a CD4 versus CD8 dot plot before gating out CD39 and CD69 single or double positive populations for CD8 + or CD4+ cells in a CD39 versus CD69 dot plot. Cells were also stained for PD-1 and TIGIT to confirm that the percentages of

positive cells were similar to what was obtained in mass cytometry.

Statistical analysis

Paired, two-tailed t-tests were used to compare T cell proliferation against the WT Gsα peptide with each of the other conditions. Paired, two-tailed t-tests were also used to compare T cell IFN-γ production against the WT Gsα peptide with each of the other conditions for each patient. All statistical analyses were performed using GraphPad Prism V.8 software (GraphPad Software, San Diego, California, USA).

RESULTS
Patients

Twenty-five patients with PMP were included in the study, 16 women and 9 men, with a median age of 55 years (min–max 32–74) (table 1). Of these, 23 had appendiceal primary tumors (classified as low-grade mucinous neoplasm, n=17; high-grade mucinous neoplasm, n=3; mucinous adenocarcinoma, n=2; and not classified, n=1), while two patients had primary mucinous adenocarcinomas of the ovary. On histological examination of the peritoneal disease, 22 cases were classified as low-grade PMP (of these, 9 contained acellular mucin only), 2 as high-grade, and 1 as high-grade with signet-ring cells. The median PCI was 25 (min–max, 2–39). For 20 patients, complete cytoreduction was achieved (CC0/1), and these

Table 1 Key clinicopathological parameters, mutational status and T cell proliferation score for patients with PMP

Case number	Sex	Age	Primary tumor	PCI	HIPEC	Histological classification Primary tumor	Peritoneal disease	*GNAS* mutation	*KRAS* mutation	Proliferation score according to peptide Wild type	R201C	R201H
434	M	55	Appendix	32	Yes	LAMN	LG	R201H	G12D	Not performed*		
440	F	47	Appendix	3	Yes	LAMN	LG†	R201H	G12D	1.5	4.4	5.7
445	F	70	Appendix	7	Yes	LAMN	LG	R201C	G13D	4.4	20.9	2.7
510	F	73	Appendix	27	Yes	LAMN	LG	R201H	G13D	2.9	7.4	18.8
519	F	60	Appendix	2	Yes	LAMN	LG†	R201C	NMD	0.9	1.3	1.2
522	M	38	Appendix	11	Yes	HAMN	LG	R201H	G12D	1.0	0.9	1.6
523	F	61	Appendix	39	No	MA	PMCA-S	NMD	NMD	0.6	2.9	1.6
530	F	56	Appendix	38	No	HAMN	LG	R201C	G12V	9.4	16.8	16.1
534	M	48	Appendix	10	Yes	LAMN	LG†	R201H	G13D	1.6	53.4	9.4
537	F	39	Ovary	29	Yes	MA	LG	R201C	G12D	24.6	58.8	46.6
543	F	72	Appendix	28	Yes	LAMN	LG	R201H	G12V	3.2	5.2	27.0
547	M	48	Appendix	39	Yes	LAMN	LG†	R201H	G12V	1.1	1.1	0.6
549	M	32	Appendix	13	Yes	LAMN	LG†	R201H	G12D	0.9	4.6	0.5
558	F	37	Ovary	6	Yes	MA	LG	R201H	G12D	1.4	1.0	0.2
559	M	54	Appendix	24	Yes	LAMN	LG†	NMD	NMD	5.4	35.4	0.3
563	F	50	Appendix	23	Yes	MA	HG	R201H	G12D	6.2	15.3	10.4
570	F	70	Appendix	2	Yes	HAMN	LG†	R201H	G13D/ G12D	1.1	16.8	13.6
572	M	46	Appendix	30	Yes	LAMN	LG†	R201C	NMD	2.4	10.2	28.3
575	M	72	Appendix	35	No	LAMN	LG	R201C	G12V	0.9	1.1	1.2
576	F	55	Appendix	19	Yes	LAMN	LG	R201H	G12V	1.0	4.7	3.5
581	M	39	Appendix	37	Yes	LAMN	LG	R201H	G12D	0.6	2.3	0.7
583	F	74	Appendix	21	Yes	LAMN	LG	NMD	G12D	0.8	1.1	1.1
585	F	53	Appendix	25	Yes	LAMN	LG†	R201H	G12D	1.4	0.6	3.8
586	F	69	Appendix	32	No	NA‡	HG	R201H	G12D	1.1	1.0	2.2
588	F	59	Appendix	32	No	LAMN	LG	R201H	G12D	1.0	0.7	2.1

*Blood samples not available, CyTOF analysis only.
†Only acellular mucin detected at microscopy of resected peritoneal tumor.
‡Primary tumor suspected to be appendiceal on radiology, no surgical specimen available.
CyTOF, cytometry by time of flight; F, female; HAMN, high-grade mucinous neoplasm; HG, high-grade; LAMN, low-grade mucinous neoplasm; LG, low-grade; M, male; MA, mucinous adenocarcinoma; NMD, no mutation detected; PCI, Peritoneal Cancer Index; PMCA-S, peritoneal mucinous carcinomatosis with signet-ring cells; PMP, pseudomyxoma peritonei.

patients received mitomycin C-based HIPEC, while in 5 cases, palliative procedures were performed and no HIPEC was given.

Tumor mutation analysis

GNAS mutations were detected in samples from 22/25 patients (88%), while in three cases, no mutation was found. The R201H and R201C mutations were detected in 16 and 6 cases, respectively (table 1). *KRAS* mutations, which are also commonly present in PMP, were detected in 21/25 samples (84%), with no mutations in four cases (G12D, n=12; G13D, n=3; G12V, n=5; both G12D and

G13D, n=1). In most cases, the two genes were comutated (in 20 of the 25 analyzed cases); in two cases, no mutations were detected in either gene, and in three cases, only one gene was mutated (*GNAS* only, n=2; *KRAS* only, n=1).

Immune stimulation of T cells from peripheral blood samples

PBMC samples were available from 24 of the 25 patients and from 10 healthy donors. When stimulated with the mutated peptides, proliferative T cell responses against one or both peptides were observed in 18/24 PMP samples (table 1 and figure 1A). A trend towards
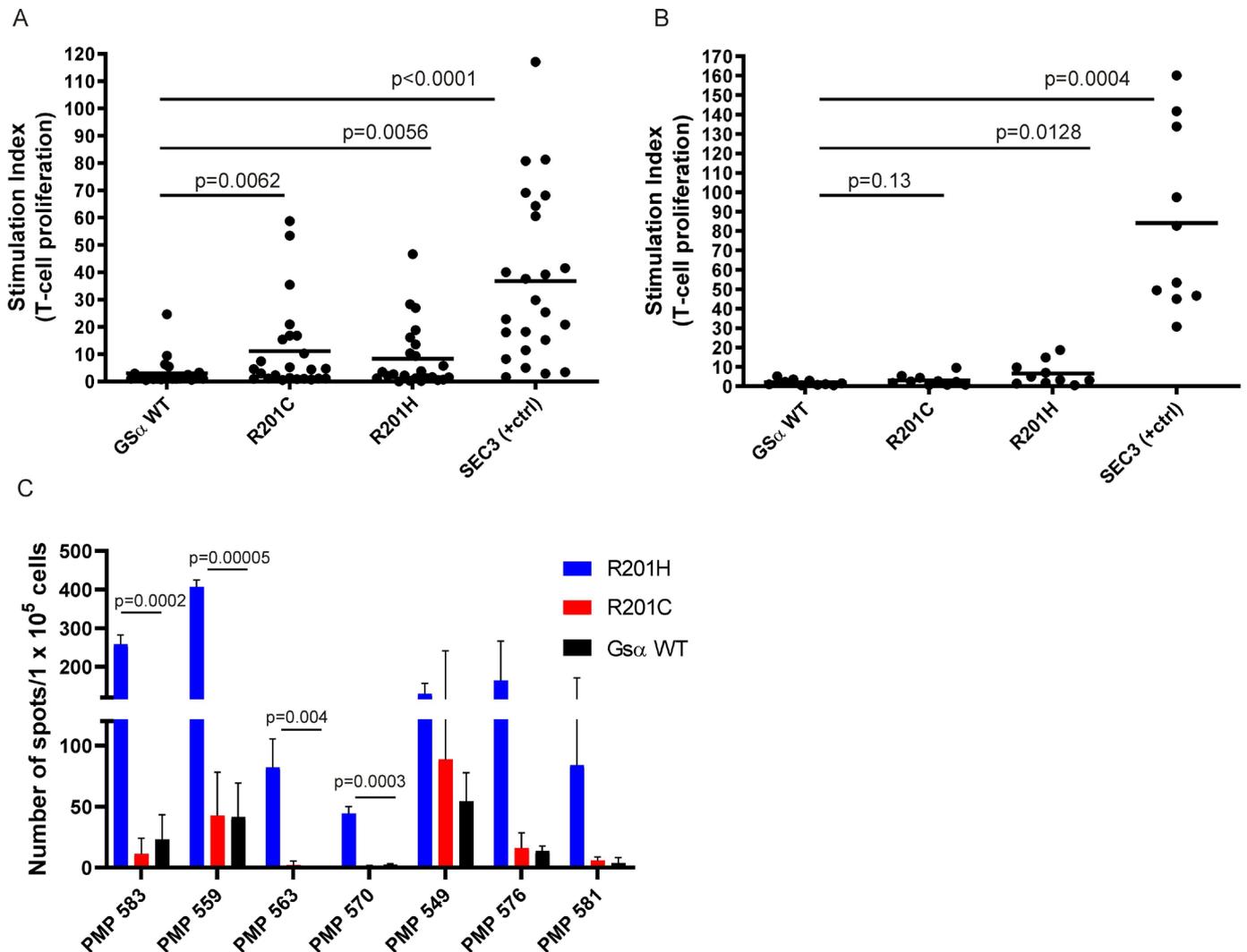
Figure 1 Mutated Gsα peptides are immunogenic in patients with PMP and healthy donors. (A) T cell reactivity (proliferation) in blood samples from patients with PMP (n=24). (B) T cell proliferation in healthy donors (n=10). Peripheral blood mononuclear cells were prestimulated with mutated Gsα 30-mer peptides (R201C and R201H). After 12–14 days, the T cells were restimulated with the mutated and WT Gsα peptides for 2 days before radioactive ³H-thymidine was added and the proliferation measured. A Stimulation Index of ≥2 (above background) was considered positive. SEC3 superantigen was included as a positive control. Paired, two-tailed t-tests were used to calculate the statistical significance of T cell proliferation against WT Gsα peptide versus other conditions. (C) T cell reactivity (IFN-γ production) in blood samples from patients with PMP (n=7). PBMCs were prestimulated with mutated Gsα 30 mer peptides (R201C and R201H). After 12–14 days, the T cells were either tested directly (patients 559, 563, and 583) or previously prestimulated T cells were thawed (patients 549, 570, 576, and 581) and tested for IFN-γ production in response to the mutated and WT Gsα peptides. SEC3 superantigen was included as a positive control. Statistically significant responses against Gsα peptide R201H compared with WT are indicated. Gsα, guanine nucleotide-binding protein α subunit; IFN-γ, interferon gamma; PBMC, Peripheral blood mononuclear cell; PMP, pseudomyxoma peritonei; WT, wild type.
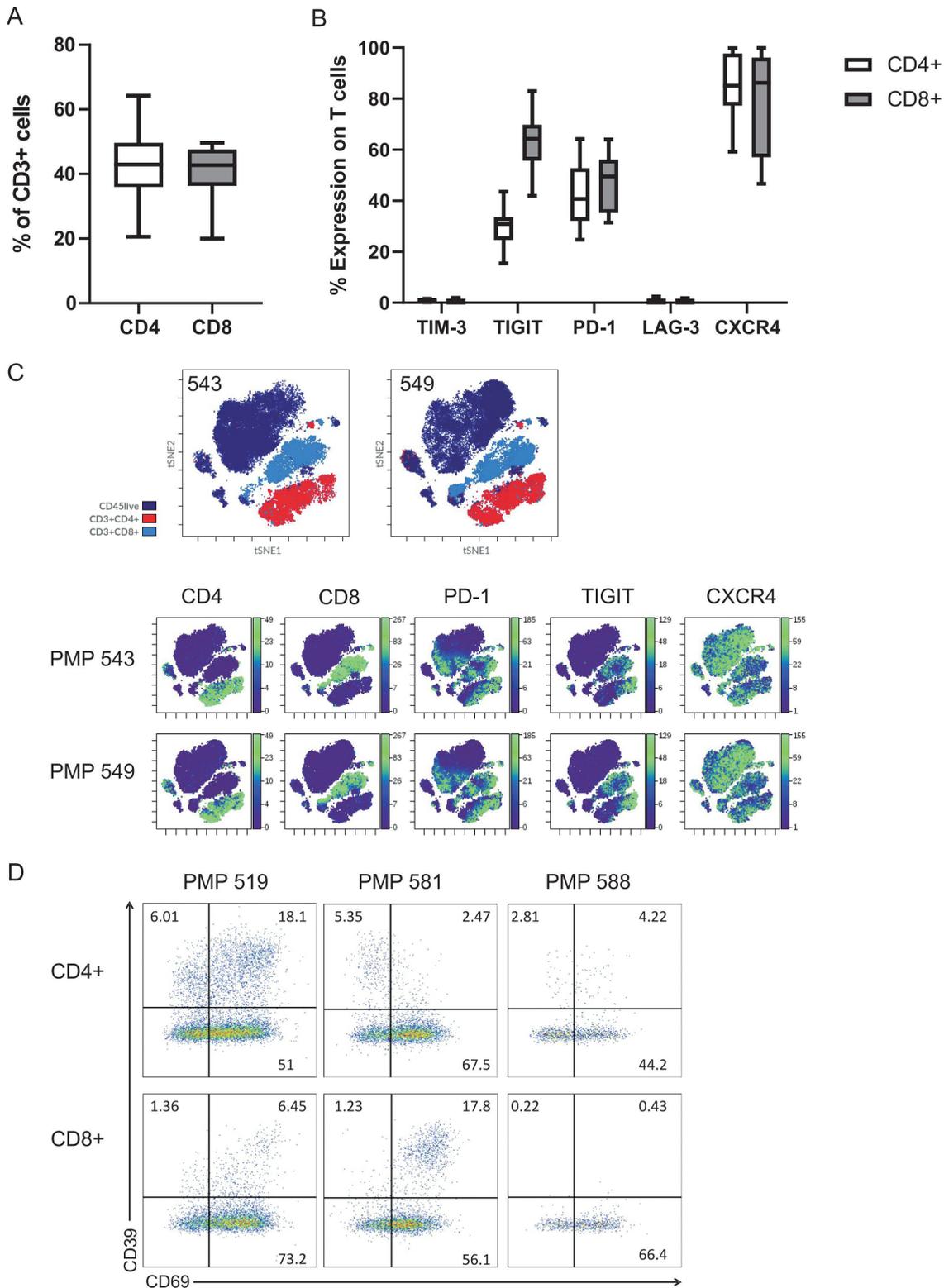
Figure 2 Immune checkpoint molecules/exhaustion markers are upregulated on tumor infiltrating T cells in patients with PMP (n=18) analyzed by mass cytometry. (A) Percentage of CD4 + and CD8+ T cells (CD3+) in tumor samples from patients with PMP. The number of positive cells found in patient samples ranged between 766 and 69920 for CD3+, 284–14368 for CD4 + and 309–13960 CD8 + T cells. (B) Percentage of T cells expressing immune checkpoint molecules TIM-3, TIGIT, PD-1, LAG-3, and chemokine receptor CXCR4. (C) viSNE clustering analysis of PMP tumor from two representative patients showing CD4 + and CD8+ T-cell populations in combination with immune checkpoints PD-1 and TIGIT and chemokine receptor CXCR4. (D) Expression of activation/exhaustion markers CD39 and CD69 on T cells from PMP biopsies. Three patients (519, 581, and 588) had sufficient biopsy material for analysis of CD39 and CD69 expressions on T cells by flow cytometry. Top panels show the expression in CD4 + T cells and bottom panels show CD8 + T cell populations. LAG3, lymphocyte-activation gene 3; PD-1, programmed death receptor-1; PMP, pseudomyxoma peritonei; TIGIT, T cell immunoreceptor with Ig and ITIM.
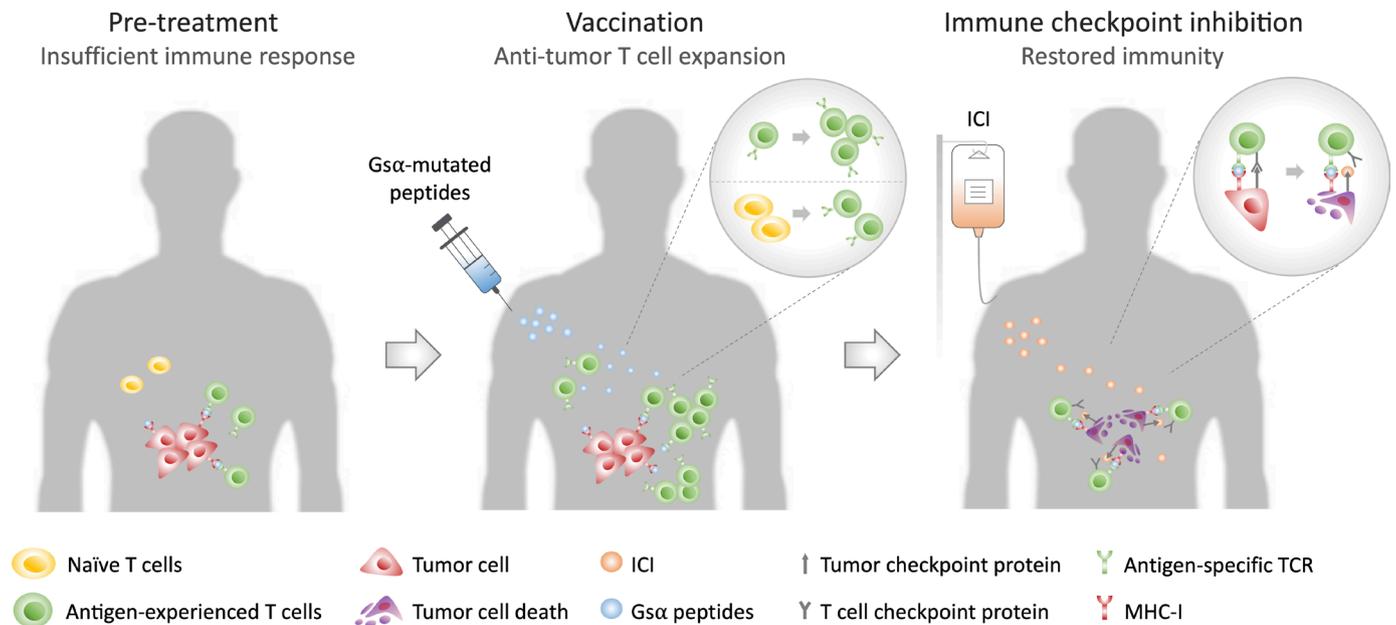
Figure 3 Pseudovax hypothesis and therapeutic concept. Mutated Gsα peptides act as neoantigens and elicit a spontaneous, antitumor-specific T cell response in patients with PMP, but this is not sufficient to control tumor growth. We hypothesize that vaccination with mutated Gsα peptides will amplify the existing response and induce de novo responses of naïve T cells, resulting in a clonal expansion of T cells recognizing mutated Gsα. The antitumor immune response can then be boosted by adding an ICI to remove the inhibition caused by upregulation of immune checkpoint molecules on tumor-infiltrating T cells, restoring a functional immune response. Gsα, guanine nucleotide-binding protein α subunit; ICI, immune checkpoint inhibitor; MHC-I, major histocompatibility complex class I; PMP, pseudomyxoma peritonei.

a preferential response against one of the peptides (proliferation value >20% larger than towards the other peptide) was noted in 15 of the 18 responding cases; 9 and 6 samples favoring the R201C and R201H peptides, respectively. The WT peptide also elicited responses in the PMP samples in 8 of the 24 samples analyzed, but in all cases, the response to one or both mutant peptides was stronger. Concordance between the mutation detected in the tumor samples and a preferential response towards the corresponding peptide was observed in 7 of the 15 cases, where a preferential response was noted. For the healthy donors, responses were noted in 7 of the 10 analyzed samples (figure 1B and online supplemental table 2). The responses in healthy donors were generally of lower magnitude than in the patients with PMP. To further investigate the immunogenicity of the Gsα peptides, prestimulated T cells from seven patients with sufficient cell numbers were tested for IFN-γ production in ELISPOT assays (figure 1C and online supplemental figure 1). These T cells were either thawed or freshly prestimulated T cells were used. The assay clearly showed higher responses for peptide R201H. Five of the patients harbored this specific mutation, whereas no mutation was detected for the last two (patients 559 and 583). The patients had previously shown somewhat higher or similar proliferative T cell responses to the R201C peptide.

Analysis of T cell subsets in PMP samples

Successful preparation of single cell suspensions from PMP samples with subsequent staining and analysis by CyTOF was achieved in 18/25 cases. The results clearly showed that most patients had CD3 + T cell infiltration (figure 2A). The CD4+:CD8 + T cell ratio was variable and in some cases did not make up 100% of the CD3 + population. Some of these CD3 + cells could be CD8−CD4− NKT cells or double-negative T cells, also described to be immune suppressive.[10] A large part of the infiltrating T cells seemed, however, to be antigen experienced as they expressed varying levels of immune checkpoint inhibitor molecules (figure 2B). The most predominantly expressed checkpoints were PD-1 and TIGIT domains, whereas TIM-3 (T cell immunoglobulin mucin-3) and LAG-3 were seen at very low levels in a few patients. PD-1 levels were similarly expressed by CD4 + and CD8+ T cells with an average expression of 40%–50%. In contrast, TIGIT was predominantly expressed by CD8 + T cells (average of 60%), whereas the average expression on CD4 + T cells was around 35%, indicating that these T cells have seen their cognate antigen in vivo.[11 12] viSNE clustering analysis from two representative patients with high levels of PD-1 and TIGIT expression is shown in figure 2C. The top plots show cells gated on live, CD45 + cells with clear CD8 + and CD4+ populations. The same clusters showed increased expression of PD-1 and TIGIT in particular; however, here in these patients CD4 + T cells exhibited the highest intensity of TIGIT staining. Some T cell populations expressed both TIGIT and PD-1. Interestingly, mass cytometry analysis showed a high percentage (69%–99%) of CXCR4 (C-X-C chemokine receptor type 4) expression on infiltrating T cells, which may contribute to T cell homing to tumor. CXCR4 expression has been

implicated in metastasis of several cancers, including in colorectal cancer,[13] but is also important in lymphocyte trafficking. Its ligand-stromal cell-derived factor-1 (SDF1 and CXCL12) is produced in the tumor microenvironment.[14] To further investigate T cells carrying these checkpoint molecules, we performed flow cytometry on biopsies from three patients with sufficient cell numbers available and stained T cells for expression of CD39 and CD69 (figure 2D). All patients displayed CD69+ CD4 + and CD8+ T cell populations and CD8 + T cells expressed less CD39+. The results were more variable with respect to T cell populations that were either CD39 + or CD39+CD69+, indicating that these markers may be interesting to investigate in a larger patient cohort.

DISCUSSION

The proliferative response that was observed when T cells from patients with PMP were stimulated with mutated Gsα peptides was exceptionally strong, suggesting the presence of a pre-existing immune response against mutated Gsα. The mutated Gsα peptide inducing the strongest IFN-γ response did not always correlate with the preferred peptide for the proliferative response but was consistently stronger for the mutated peptides compared with the WT peptide. However, proliferative responses and IFN-γ ELISPOT responses could not be consistently compared as very few patients could be tested for IFN-γ production. In healthy donors, the proliferative responses were much less pronounced but detectable in some cases. As T cells from healthy donors would not be expected to have been previously primed against mutated protein in vivo, the detection of Gsα-specific T cell responses in these samples demonstrates the strong immunogenicity of the peptides. In some cases, the WT peptide also elicited proliferative responses in patients with PMP samples, although of lesser magnitude than the mutated peptides. Similar observations have been made in other vaccine trials where the peptides in question carried point mutations only. For instance, in a cancer trial of pancreatic patients vaccinated with a mix of mutated KRAS peptides, a persisting cross-reactivity (>10 years postsurgery and vaccination) against the WT peptide, which was not included in the vaccine, was seen in two patients.[15] No autoimmune adverse effects were observed, and it was speculated that this effect could actually have contributed to the strong memory T cell responses that were observed against the vaccine. Other studies on mutated KRAS epitopes have also confirmed in vitro reactivity against WT KRAS, in addition to the mutated epitope by T cell clones or TCR-modified T cells, and this could indeed be similar for Gsα peptides carrying point mutations.[16–18] Our findings suggest that mutated Gsα acts as a shared (public) tumor neoantigen, resulting in immune responses that are readily detectable in patients with PMP. Because the described GNAS mutations are extremely frequent in patients with PMP, a vaccine would not have to be individually adapted for each patient as is

the case for private neoantigens. Also, a strategy directed at Gsα would be tumor specific, since GNAS is mutated only in cancer cells, and no immune tolerance would be expected to develop, as is often seen for 'self-antigens' overexpressed in the tumor. Moreover, since GNAS is considered to be a driver oncogene, its expression is not likely to be lost by the tumor cells.[19] Not only is GNAS a potentially ideal vaccination target, but also PMP seems to be an ideal disease for a vaccination strategy. Because PMP is a slow-growing cancer, vaccine treatment could be administered in the recurrent setting but also as adjuvant treatment before or after CRS-HIPEC. The adjuvant setting has previously been shown to be an appropriate situation for a therapeutic cancer vaccine strategy, where vaccination could consolidate the effect of surgery and prevent disease relapse.[20] Taken together, these points demonstrate the high potential of Gsα as a candidate vaccine-targeted neoantigen in PMP.[21]

When the immune microenvironment of the same patients with PMP was interrogated, we found that for most of the PMP cases, infiltrating T cell populations could be detected and characterized. The only previous study of immune cells in PMP was performed by immunohistochemical staining of samples from 14 cases and reported varying infiltration of T cells (CD3+), B cells (CD20+), and macrophages (CD68+) in a low percentage of tumors.[22] We chose to focus our analysis on the T cell subsets, as T cells can recognize specific neoantigens and would be the target cells for a peptide vaccine. Mass cytometry analysis showed that both CD4 + and CD8+ T cells infiltrated the PMP biopsies, and both T cell populations expressed PD-1 and TIGIT checkpoint receptors, suggesting that these T cells are antigen experienced and likely tumor reactive. Our preliminary results also suggest that additional markers of antigen-experienced T cells, such as CD39 and CD69, would be interesting to investigate further. The detection of strong T cell responses against mutated Gsα peptides (R201C or R201H) in the circulation of the majority of patients screened indicates that such antigen priming has indeed taken place in patients. PD-1 receptor engagement has an inhibitory effect on T cell effector functions, and high levels of PD-1 have been associated with T cell exhaustion and a dysfunctional phenotype. The inhibition can be caused by tumor intrinsic mechanisms or by cells or factors in the tumor microenvironment such as macrophages.[23 24]

Based on the current analyses of the immune cells in PMP and recent experience from previous vaccine trials, it is therefore likely that a Gsα peptide vaccine should be combined with immune checkpoint inhibition (ICI). Multiple preclinical studies have shown synergy between therapeutic vaccination and ICIs, and several ongoing clinical studies are currently evaluating this.[25] Indeed, a recent study reported circulating CD8 + T cells double positive for PD-1 and TIGIT to be an early marker of therapeutic response to anti-PD-1 therapy in melanoma and Merkel cell carcinoma.[26] From the perspective of currently known predictors of response to ICI, PMP is not

an upfront ideal candidate for ICI monotreatment. In a total of 183 investigated cases in six individual studies, only one case was identified as MSI.[27–32] Also, although a small number of cases have been extensively sequenced, there is no indication that PMP tumors have high tumor mutational burden.[33] In this context, identification of the Gsα mutations as highly immunogenic points to an opportunity for patients with PMP to potentially benefit from immunotherapy in a combination treatment approach.

CONCLUSION

Taken together, our results suggest that Gsα peptides are highly immunogenic and may be used to reinforce a pre-existing immunity in patients with PMP and induce de novo immunity against mutated Gsα. Analysis of the immune microenvironment of PMP tumor samples show that tumor infiltrating T cells are likely to have been antigen exposed, based on the expression of immune checkpoint molecules. These results form the rationale for the planned Pseudovax trial (protocol in preparation), which will explore peptide vaccination with Gsα peptides in combination with ICI in a first-in-man, signal-finding clinical trial (trial concept outlined in figure 3). In the longer term this treatment strategy may provide a possibility to offer a curative treatment to patients with PMP and for patients suffering from other *GNAS*-mutated cancers.

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