Melanoma-specific antigen-associated antitumor antibody reactivity as an immune-related biomarker for targeted immunotherapies

Annika Rähni,1,2, Mariliis Jaago1,2, Helle Sadam1,2, Nadežda Pupina1, Arno Pihlak1, Jürgen Tuvikene1,2,3, Margus Annuk4, Andrus Mägi5, Tõnis Timmusk1,2, Amir M. Ghaemmaghami6 & Kaia Palm1,2

Abstract

Background: Immunotherapies, including cancer vaccines and immune checkpoint inhibitors have transformed the management of many cancers. However, a large number of patients show resistance to these immunotherapies and current research has provided limited findings for predicting response to precision immunotherapy treatments.

Methods: Here, we applied the next generation phage display mimotope variation analysis (MVA) to profile antibody response and dissect the role of humoral immunity in targeted cancer therapies, namely anti-tumor dendritic cell vaccine (MelCancerVac®) and immunotherapy with anti-PD-1 monoclonal antibodies (pembrolizumab).

Results: Analysis of the antibody immune response led to the characterization of epitopes that were linked to melanoma-associated and cancer-testis antigens (CTA) whose antibody response was induced upon MelCancerVac® treatments of lung cancer. Several of these epitopes aligned to antigens with strong immune response in patients with unresectable metastatic melanoma receiving anti-PD-1 therapy.

Conclusions: This study provides insights into the differences and similarities in tumor-specific immunogenicity related to targeted immune treatments. The antibody epitopes as biomarkers reflect melanoma-associated features of immune response, and also provide insights into the molecular pathways contributing to the pathogenesis of cancer. Concluding, antibody epitope response can be useful in predicting anti-cancer immunity elicited by immunotherapy.

Plain language summary

Immunotherapy treatments, which utilize the patient’s own immune system to fight cancer, have become a standard treatment of cancer. However, for many patients’ immunotherapy does not work. During the immune response the body produces proteins called antibodies. This study characterized the antibodies produced following treatment with two different types of immunotherapies that treat skin cancer, to gain insights into how the immune system responds in different individuals. Our results demonstrate that multiple proteins that are present in patients with skin cancer are specifically targeted by the immune system during skin cancer specific immunotherapy. Our results should help further anti-cancer drug development.

1 Protobios Llc, Tallinn, Estonia. 2 Department of Chemistry and Biotechnology, Tallinn University of Technology, Tallinn, Estonia. 3 dxlabs Llc, Tallinn, Estonia. 4 EGeen International Inc., Mountain View, CA, USA. 5 Tartu University Hospital, Tartu, Estonia. 6 Immunology and Immuno-Bioengineering Group, School of Life Science, Faculty of Medicine and Health Sciences, University of Nottingham, Nottingham, UK. Email: kaia@protobios.com
knowledge of the immunosuppressive tumor micro-environment has markedly improved within the last decade (reviewed in ref. 1). To achieve immunogenicity, tumor cells must express antigens capable of eliciting immune activation. The identification of applicable tumor antigens is indispensable for the development of effective cancer immunotherapy. Most known tumor antigens are considered canonical if derived from protein-coding regions in contrast to noncanonical antigens that include sequences outside protein-coding regions or that are generated by antigen-processing2. Melanoma cells are considered highly immunogenic with well-described tumor-associated antigens (TAAs)3, including cancer-testis antigens (CTAs)4 and neo-antigens carrying novel epitopes of self-antigens. Some well-known examples include carcinoembryonic antigen (CEA), B melanoma antigen 1 (BAGE), G antigens (GAGEs), cancer/testis antigen 1 (CTAG1; also known as NY-ESO1), and melanoma-associated antigens (MAGEs) (Rev in ref. 5). The antigenic repertoire is a critical factor for immunosurveillance and cancer progression6. However, most studies have focused on the role of T cells in these battles6, while considerably less is known about B cell response9. Humoral response against cross-reactive auto-antigens has been detected in different cancers10. A burst of recent publications is pointing to the role of antibodies contributing to tumor control11 as cancer-associated autoimmunity targeting non-malignant tissues may reflect favorable disease outcome12. On the other hand, the reasons underlying the immunogenicity of the tumor, or the lack of it, are not well understood13. The antitumor immunity can result from many factors including MHC genetic variation, tumor mutational load, tissue microenvironment13, but also by cell stress, reactivation of embryonic or gonadal transcription, epigenetic instability, aberrant RNA splicing, and others14,15. For example, it is argued that the capture of either apoptotic or necrotic cells by macrophages and dendritic cells in the tumor microenvironment may lead to immune suppression or stimulate inflammatory pathways contributing to antitumor cytotoxicity16.

Discoveries in cancer biology have led to new strategies in awakening tumor immunogenicity, including checkpoint blockade, adoptive cellular therapy, and cancer vaccines, underscoring the role of the immune system in waging the war on cancer tissue. Among these are monoclonal antibodies that target cancer immune checkpoint inhibitors (ICIs) including anti-CTLA-4, anti-PD-1, and anti-PD-L1/2 antibodies that are able to restore anticancer immunity and are widely used for the management of various cancers, including melanoma17. Immunogenicity of CTAs has led to the use of melanoma-associated antigens as promising candidates for novel cancer treatments18,19. In addition to monoclonal antibodies, cancer vaccines, in particular those based on dendritic cells (DCs) as vectors for antigen delivery, are a major focus of current developments20. To date, personalized neoantigen-based DC vaccines are evolving and have shown clinical success in melanoma and other solid tumors21.

Biomarkers associated with clinical prognosis of the cancer and/or severe immune-related adverse effects (irAEs) of the drugs are areas of active investigation. Different biomarkers have been tackled with variable success, such as levels of PD-L122, genetic mutations23, inflammatory cytokines24, and the presence of tumor-infiltrating lymphocytes (reviewed in ref. 25). Tumor infiltrating B lymphocytes contribute to anti-tumor immunity by promoting antibody response to tumor antigens26,27. High titer antibodies against melanoma differentiation antigens (TRP1/TYRP1, TRP2/TYRP2, gp100, MelAN/MART1) were observed in responder group of melanoma patients treated with ICI mAbs (monotherapies with Nivolumab, Pembrolizumab or Ipilimumab, or the combination of Nivolumab and Ipilimumab)28,29. However, pre-treatment autoantibody profiles in melanoma patients were reported to predict ICI treatment-associated toxicity30. Connectedly, DC vaccines also stimulate robust antibody response31,32 and in some cases, this is associated with prolonged recurrence-free survival32. Despite big hopes, clinical benefit of immunotherapies has remained limited only to a subset of patients34,35 and it is currently undetermined whether increase or decrease in immune response to specific tumor antigens is beneficial to the patient36,37.

Here, we explore the use of a high precision approach called mimotope variation analysis (MVA), a next generation random peptide phage display method to delineate cancer therapy-associated antibody immune response at epitope resolution. We hypothesize that the pre-existing and treatment-induced antibodies against specific antigen targets could reflect the response elicited by anti-tumor drug and that this response could be predictive of cancer immunogenicity and thus, sensitivity to immune therapy. We generate data to test this hypothesis by immunoprofiling analysis of the anti-melanoma antibody response in the sera samples from the phase II clinical trial of patients with non-small cell lung cancer (NSCLC) receiving autologous DC therapy based on allogenic melanoma cell lysate (MelCancerVac)38,39. We correlate the findings on melanoma-specific antigen profiles with those from a group of patients with unresectable metastatic melanoma receiving anti-PD1 (pembrolizumab) treatment as a part of their standard-of-care. We verify the melanoma-antigen specificity using MVA-based competition, and further determine a three-epitope biomarker signature of melanoma-specific antibody response elicited by both immunotherapies. Our results demonstrate the relevance of antibody epitope profiling to better understand the fine line separating beneficial immunosurveillance from harmful autoimmunity in the anticancer immune response elicited by different types of therapy.

Methods

Study population. The present study analyzed samples from a total of 119 individuals from 2 different clinical cohorts of NSCLC and melanoma patients and their appropriate controls, whose clinical characteristics are shown in Table 1 and Supplementary Table 1. The study was conducted in accordance with the guiding principles of the Declaration of Helsinki and the study participants gave informed consent before enrollment.

The NSCLC patient cohort (n = 24) included longitudinal study of patients diagnosed with advanced NSCLC, who participated in the phase II clinical trial evaluating the effectiveness of MelCancerVac® vaccine38,39 (Supplementary Table 2). The clinical trial, completed at the time of this study, was designed and carried out by Dandrit Biotech A/S and approved by European Medicines Agency (https://www.clinicaltrialsregister.eu/ct-search/trial/2006-002202-54/DK). Out of the 24 study participants, 6 NSCLC patients donated blood samples before vaccination (group: MelVac-CTRL) and after receiving MelCancerVac® (group: MelVac), while 18 NSCLC patients had not received any doses of the vaccine at the time of sample donation (group: NSCLC).

The melanoma group comprised of patients with unresectable and metastatic melanoma (n = 5, ICD-10: C43; group: PEM-Mel), who received KEYTRUDA® (anti-PD-1 monoclonal antibody pembrolizumab, Schering-Plough Labo NV) immunotherapy as a part of standard-of-care. Serum samples of melanoma patients were collected 3 weeks after the first immunotherapy treatment, when patients came to receive the second dose (European Medicines Agency guidelines for KEYTRUDA therapy) and were provided by EGeen International (Mountain View CA, USA; ethical permit: 236/T-5).

Control groups included subjects with no history of cancer (n = 10, group: CTRL-NSCLC), with approvals for recruitment to
Table 1 Description of clinical cohorts.

| Sub-cohort                  | Group                      |
|-----------------------------|----------------------------|
|                             | CTRL-NSCLC                 |
|                             | NSCLC patients with        |
|                             | pembrolizumab therapy      |
|                             | (n = 85)                  |
|                             | Healthy controls           |
|                             | (n = 80)                  |
|                             | 85.5 ± 10.7               |
|                             | 42/53/0                   |
|                             | MelVac-CTRL / MelVac CTRL  |
|                             | (n = 6)                   |
|                             | MelCancerVac® therapy      |
|                             | (n = 80)                  |
|                             | NSCLC patients receiving   |
|                             | pembrolizumab treatment    |
|                             | (n = 10)                  |
|                             | PEM-Mel                   |
|                             | melanoma patients         |
|                             | receiving pembrolizumab    |
|                             | therapy (n = 18)          |
|                             | PEM-Mel                   |
|                             | melanoma patients         |
|                             | receiving pembrolizumab    |
|                             | therapy (n = 18)          |

| Sub-cohort                  | Group                      |
|-----------------------------|----------------------------|
|                             | 38.5 ± 10.7               |
|                             | 42/53/0                   |

| Sub-cohort                  | Group                      |
|-----------------------------|----------------------------|
|                             | 55.7 ± 8.4                |
|                             | 4/2/0                     |
|                             | 1 sample per person       |
|                             | available to researchers  |
|                             | (except for 3 patients     |
|                             | NSCLC1, NSCLC2, and NSCLC7|
|                             | who had 2 samples         |
|                             | available)                |
|                             |                          |
|                             | 67.6 ± 9.2                |
|                             | 2/3/0                     |

| Sub-cohort                  | Group                      |
|-----------------------------|----------------------------|
|                             | 65.3 ± 8.4                |
|                             | 5/5/0                     |
|                             |                          |
|                             | 5.8 × 10⁵ unique) per sample, with a combined total of ~4.2 × 10⁸ peptide sequences. SPEX2 exhaustive pattern search algorithm was used to group similar peptides and reveal enriched recognition patterns (epitopes) in the studied peptide sets (Supplementary Fig. 1a). Each sample was analyzed separately for identification of sample-specific epitopes that had ≥4 fixed amino acid positions. For data analysis of MelVac samples, the identification of epitopes was performed in a discriminative manner, where peptide sets from MelVac-CTRL and MelVac samples of the same patient were compared to each other. Epitopes that represented peptides that were at least 2-fold more enriched in the query sample (MelVac) as compared to paired sample peptide set (MelVac-CTRL) and with a hypergeometric p-value < 1 × 10⁻⁸ were selected for further analysis. For melanoma cohort (n = 5, PEM-Mel) the identification of epitopes was performed as non-discriminatory, where patient-specific epitopes were identified in comparison to a random-generated peptide set. Epitopes that represented peptides that were 10-fold more enriched in the query (PEM-Mel) than randomly generated reference peptide set and had a hypergeometric p-value < 1 × 10⁻⁸, were selected for further analysis. Altogether 54,055 core epitopes for melanoma and 18,021 epitopes for MelVac groups were selected, representing a dataset of melanoma-specific antibody immune response. In addition, pairwise comparison of MelVac-CTRL and MelVac sample datasets generated 17,690 pre-treatment-specific core epitopes.
Sequence alignment. The set of melanoma-associated antigens used in sequence alignment were chosen from Weinert et al., 2009 data describing genes expressed in the DDM-1.7 melanoma cells42 (Supplementary Fig. 1b). Sequences of the epitopes of the antigens were downloaded from Immune Epitope Database (IEDB)43, date accessed: 24.09.2020, www.iedb.org. Altogether, the IEDB database contained 2234 epitopes of 102 proteins expressed in the melanoma cell lysate DDM-1.742. All antigen alignments were conducted using custom Excel VBA scripts.

For sequence similarity analysis, 2234 linear IEDB epitopes were exactly aligned with 54,055 melanoma and 18,021 vaccination-specific epitopes generated with SPEXS2. Thirty-five database entries (altogether 34 unique proteins) with sequence identity to at least 1 epitope from both melanoma and vaccination-specific epitope sets were recruited for further antigen-specific analysis. Primary protein sequences were downloaded from UniProtKB database44 using accession codes matching IEDB epitope entry names (date accessed: 09.10.2020, www.uniprot.org). These 35 protein sequences were aligned with 54,055 melanoma, 18,021 vaccination-specific, and 17,690 pre-vaccination-specific epitopes, with the criteria that every fixed amino acid from SPEXS2-determined epitopes was to match with the protein sequence. Out of these, altogether 8562 epitopes aligned to sequences of 35 melanoma-associated antigens.

ELISA. Human cytomegalovirus (CMV) and Epstein-Barr virus (EBV) serostatases were measured from blood samples with ISO-17025 accredited methods. In brief, serological analyses were performed with anti-CMV ELISA (EUROIMMUN EI 2570–9601G) and with anti-EBV-CA ELISA (IgG) method (EUROIMMUN EI 2791–9601G) according to the manufacturer’s specifications. Absorbance was measured at 450 nm with SpectraMax Paradigm (Molecular Devices). For CMV serology, 41 samples tested positive, 13 negative and 2 samples were borderline and therefore excluded from further correlation analyses. For EBV serology, all measured samples were conclusive: 35 tested positive, 3 samples were negative.

Statistics and reproducibility. The study included 119 independent study subjects. Samples donated at different time points were considered as paired samples of the individual (n = 130). Technical replicates are defined as the same sample profiled in independent MVA experiments. No randomization or blinding to sample characteristics was conducted, samples were divided into groups based on clinically relevant diagnoses. Group-wise comparisons of median values were visualized using violin- or boxplots with individual data points, and statistical significance is shown where applicable. To evaluate the reproducibility of MVA data, the values of peptide abundance in two technical replicates were compared using Pearson’s correlation coefficient analysis (R package “ggpubr”) and the correlation value between replicates was established as R = 0.95 (P < 0.0001). Other samples were not measured repeatedly.

Statistical analysis. Statistical analyses were conducted with R statistical programming language v.4.0.4 and RStudio environment v.1.4.110615,46. Data were analyzed, graphs were produced and visualized using R packages “reshape2”, “tidyverse”, “precrec”, “ggpubr”, “ggsci”, “scales”, “patchwork”, “egg”, “ggalt” 2021 versions45–58.

Cosine similarity indices (CSIs) for sample comparisons based on top 2500 peptide abundance values and composition were calculated with the cosine function in R package “Isla”59.

Top 50 immunodominant characteristics were defined from group-specific epitopes generated in SPEXS2 analysis. For post-(Vac, n = 6) or pre- (Pre, n = 6) vaccination samples the abundance of group-specific epitopes (18,021 for Vac and 17,690 for Pre, respectively) were calculated as the number of IgG-bound peptides containing the epitope sequence in the sample. The 50 epitopes with the highest abundance values were selected for analysis. Z-scores for the comparison of antibody response to top 50 immunodominant characteristics were calculated individually for each patient. First, the mean of top epitope abundance values across both Pre and Vac samples was calculated, then the mean was subtracted from the value of each epitope (mean centered) and the result divided by the standard deviation (autoscaled). For graphical presentation the values are capped off at the 97.5th percentile value of each patient.

Boxplots were generated using the style of Tukey with R packages “ggpubr” or “ggplot2”47,48. In figures the upper, middle and lower boxplot lines represent the 75th, 50th, and 25th percentiles, while whiskers represent the largest or smallest value within 1.5 times interquartile range above the 75th percentile or below the 25th percentile, respectively. The p-values of two-sided Wilcoxon Rank Sum test were visualized with “ggpubr” or “ggplot2” packages47,48.

Wilcoxon Rank Sum test (with continuity correction, base R “stats” package46) was used to assess the group-differentiating features of 8562 unique epitopes aligning to melanoma-associated antigens, while custom Excel VBA script was used to determine the sensitivity and specificity while maximizing Youden’s index for each biomarker. MedCalc® Statistical Software (v.19.7.2, www.medcalc.org; 2021) was used to conduct logistic regression and ROC analysis of 15 epitopes as a combinatorial test.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Results

Highly individual patterns of top antibody response are elicited by immune therapy. To characterize immunotherapy-specific antibody repertoires and dissect the role of the immune system in biological therapies, our analysis included two different immunotherapy cohorts and controls comprising 119 individuals (Table 1 and Supplementary Table 1). First, sera samples of NSCLC patients from the phase II clinical trial receiving autologous DC vaccine MelCancerVac® (Supplementary Table 2) and second, sera samples of melanoma patients receiving monoclonal anti-PD-1 antibody pembrolizumab as part of their standard care. To characterize the most prevalent antibody immune response in our study cohort, we used mimotope variation analysis (Supplementary Fig. 1)40,41. Briefly, individual blood samples were incubated with M13 phage-displayed 12-mer peptide library to capture individual-specific IgG antibody repertoires and high-throughput sequencing was used to uncover captured peptides. Cosine similarity index (CSI), a measure of similarity between the samples, was calculated to compare the top antibody response by analysis of seroresponse to 2500 peptides with the highest antibody reactivity in cohort samples (n = 130, Table 1). Analysis showed that different individuals with the same disease and immunotherapy background presented remarkable differences in the composition and magnitude of the dominant antibody response to different peptide antigens (CSI < 0.3, Supplementary Fig. 2a, Supplementary Data 1). The dominant antibody response to peptide antigens within groups of MelVac and MelVac-CTRL, PEM-Mel and CTRL-Mel was highly dissimilar (CSI < 0.4, Fig. 1a). However, longitudinal samples from the same individual showed similar IgG response to the top antigens (Supplementary Fig. 2b). For example, clear similarity in the top immunoprofile features of a patient denoted as MelVac1, who received 35 different
Comparisons of the 4 longitudinal samples of one NSCLC patient, who received 35 doses of MelCancerVac
Pairwise comparisons of pre- and post-vaccination immunopro-
c(Supplementary Table 2), to the 4 samples themselves (vaccination
and their matched controls. $n=6$; MelVac - NSCLC patients who received MelCancerVac® vaccine ($n=6$); MelVac-CTRL - paired samples of MelVac group taken before vaccination ($n=6$); NSCLC - non-small cell lung cancer patients ($n=18$); CTRL-NSCLC - non-cancer controls for NSCLC group ($n=10$). a Pairwise comparisons of the 4 longitudinal samples of one NSCLC patient, who received 35 doses of MelCancerVac® and remained with stable disease (Supplementary Table 2), to the 4 samples themselves (MelVac1 vs MelVac6) and to the rest of the study cohort ($n=126$ samples, MelVac1 vs Cohort). b Pairwise comparisons of pre- and post-vaccination immunoprofiles of vaccinated NSCLC patients ($n=6$). MelVac Paired - comparison of pre- and post-vaccination samples of the same patient; MelVac Random - comparison of the pre-vaccination sample of one patient to the post-vaccination samples of all 5 other patients. Two-sided Wilcoxon Rank Sum test, **** $p<0.0001$, p-values not adjusted for multiple comparisons.

Fig. 2 The heterogeneity of antibody response converges on immunodominant epitopes. Heatmaps depict differential antibody response to the 50 most immunodominant epitopes detected in pre- (Pre, $n=6$) and post- (Vac, $n=6$) vaccination samples of patients who received MelCancerVac® treatment (MelVac1-MelVac6). Rows depict immunodominant epitopes with numbers on the left of each panel referring to the specific epitope sequences provided in Supplementary Data 2. The number of epitopes differs for each patient as some epitopes were in the top 50 for both Pre and Vac samples, while some were detected in only one sample of the patient. Z-scores depict the abundance of IgG-binding peptides containing the immunodominant epitope in each sample and are calculated separately for every patient by mean centering and autoscaling the abundance values across both Pre and Vac samples. Epitopes are ranked by highest-to-lowest abundance values as observed in the Vac sample. Z-score scale is cut-off at 97.5th percentile for better visualization of each panel.

vaccinations of MelCancerVac® and showed stable disease over a 2-year period, was evident irrespective of the vaccination stage (CSI > 0.5, Fig. 1b). Overall, the antibody response profiles from the paired MelVac and MelVac-CTRL samples shared more similar features with each other than with unpaired NSCLC samples (Fig. 1c, Wilcoxon Rank Sum test, $p<0.0001$, Supplementary Fig. 2b). Of note, the similarity to melanoma patients in the top 2500 peptide composition of NSCLC patients did not increase significantly upon vaccination with MelCancerVac® (Wilcoxon Rank Sum test, $p > 0.05$, Supplementary Fig. 2c). Next, to delineate epitopes characteristic to MelVac group we used SPEXS2 exhaustive pattern search algorithm (Supplementary Fig. 1a). Comparison of seroresponse values to the top 50 most targeted epitopes in both pre- and post-vaccination samples of the same patient revealed common antigenic features present in both conditions (Fig. 2 and Supplementary Data 2). However, we also observed that seroresponse to the top 50 antigens changed upon Mel-

Immune reactivity targets epitopes of melanoma antigens. We hypothesized that dendritic cell vaccine therapy based on mela-

noma cell lysate could elicit melanoma-antigen-specific antibody
response in NSCLC patients. In particular, considering that out of all protein antigens known to be expressed by the DDM-1.7 melanoma cells, 102 proteins were reported to have epitopes showing serologically positive findings in Immune Epitope Database (IEDB). To characterize protein-specific immune responses in immunotherapy patients, we used SPEXS2 exhaustive pattern search algorithm to group individual peptides of MelVac, MelVac-CTRL, and PEM-Mel groups into representative epitopes and compared the delineated peptides with known antigenic sequences. Altogether, antibody response to IEDB epitopes with sequence similarity to 35 proteins was detected in both MelVac and PEM-Mel groups (Supplementary Table 3). To characterize potential new antibody targets, we aligned the cancer-group-specific epitopes with the primary sequence of these 35 proteins. With an average of 340 epitope alignments per protein, 8562 unique epitopes from either MelVac, MelVac-CTRL or PEM-Mel group matched exactly with 100% identity to the specified proteins (shown above each graph, Supplementary Data 4) from panel a across study sub-cohorts. Two-sided Wilcoxon Rank Sum test, ns p > 0.5, ** p < 0.01, *** p < 0.001, **** p < 0.0001, p-values not adjusted for multiple comparisons. CTRL-Mel – healthy controls for melanoma group (n = 21, all individuals older than 45 years); PEM-Mel – melanoma patients receiving pembrolizumab treatment (n = 5); MelVac-CTRL – paired samples of MelVac group taken before vaccination (n = 6); MelVac – NSCLC patients who received MelCancerVac® vaccine (n = 6); NSCLC – non-small cell lung cancer patients (n = 18).

Fifteen group-discriminating epitopes converge on antigens associated with modulation of extracellular matrix and tumor cell survival pathways. To identify specific changes in immune response upon immunotherapy, we divided the study cohort into two subsets: controls (n = 90) and cancer samples with melanoma-associated attributes (n = 11, comprising of both MelVac and PEM-Mel groups). ROC analysis of 8562 antigen-associated epitopes resulted in 15 most group-discriminating antigenic determinants (markers M1 to M15) with sensitivity > 0.72 and specificity > 0.67 (Supplementary Fig. 3 and Supplementary Table 4). Notably, several of the resolved 15 biomarkers (M1-M15) mapped to the same antigens, but to different epitopes (M1-M15) mapped to the same antigens, but to different epitopes (M2 and M7 to MAGD2; M2 and M5 to MAGE1; M2 and M6 to PMEL; M3 and M11 to MORC4; M4 and M10 to MAGEMG50; M8 and M14 to CSPG4; M9 and M13 to CRGB1, Supplementary Table 5). Analysis of protein structure and biological relevance data from UniProtKB database indicated that majority of these 15 epitopes aligned to antigenic regions that were enriched in polar amino acids and located preferentially in regions with no known structural domains or in disordered segments. For example, M2 and M7 to MAGD2, M2, and M5 to MAGE1, M7 to MAGEMG50; M9 and M13 to CRGB1 and M12 to MAGE6. Some epitopes encompassed well-conformed domains like coiled-coil repeat of G3V599 for M1, leucine rich repeat of PRA22 for M3, cadherin-like CSPG repeats for M8 and M14. Biologically, these antigens are associated with extracellular-matrix formation (MAGEMG50) and modulation collagen protein turnover pathways (G3V599, CSPG4, and TGO1), with p53-associated apoptosis (MAGEMG50) and/or via ubiquitin ligase activity (MAGE1, MAGD1, MAGA3), but also with melanosome...
biogenesis (PMEL), ciliary signaling (ARMC) and lipoprotein signaling (G3V599). We also analyzed whether antibody response to common human herpesviruses, including cytomegalovirus (CMV) and Epstein-Barr virus (EBV) contributed to the treatment-elicited anti-cancer immunity given that EBV and CMV are the most prevalent infection types in tumors60 and can act as independent biomarkers for cancer immunotherapy61. For that, we analyzed EBV and CMV serology and showed that seropositivity to these common herpesviruses (Supplementary Fig. 4) was not correlated with treatment-elicited antibody response to the resolved top 15 melanoma-associated epitopes (Supplementary Data 5, 6). Based on both clinical serology and MVA data, we concluded that melanoma-associated antibody response linked to immunotherapy pointed onto apoptotic signaling and extracellular matrix-remodeling pathways conveyed by tumor-antigens but was not correlated with the common herpesviral antigens.

MelCancerVac® boosts prior antibody response against a subset of melanoma-associated antigens. Analysis of longitudinal samples revealed that antibody response to the majority of 15 melanoma-specific epitopes was pre-existing before MelCancerVac® vaccination and boosted upon vaccine stimulation. (Fig. 4a) Comparison of antibody response to 15 epitopes in samples taken before (n = 6, MelVac-CTRL) and after vaccination (n = 6, MelVac) in six MelCancerVac® receiving patients (MelVac1-MelVac6). x-axis denotes 15 epitopes as biomarkers (M1-M15), y-axis (Abundance ratio) shows the ratio of abundance values of IgG-bound peptides between paired MelVac and MelVac-CTRL samples of the patient (MelVac[Mabundance + 1]/MelVac-CTRL[Mabundance + 1], i - number of patient, M - biomarker) in base 10 logarithmic scale. Dashed line indicates ratio value 0 (1 in linear scale), i.e., where antibody reactivity to peptides containing the specific epitopes remained unchanged in MelCancerVac® post-vaccination cohort. Values > 0 indicate rise in seroreactivity after vaccination while <0 indicates decrease. Source abundance values for each epitope are presented in Supplementary Data 5. (b) Vaccine-dependent antibody response enhancement to the resolved epitopes was common. Data are shown for epitopes M4 and M5 by comparing abundances of IgG-bound peptides from the vaccinated patients, before (MelVac-CTRL) and after vaccination (MelVac). Abundance = number of IgG-bound peptides containing the specified epitope sequence detected in the sample. Two-tailed paired Wilcoxon Rank Sum test, *p < 0.05, p-values not adjusted for multiple comparisons. (c) Box plots show the abundance of IgG-bound peptides containing the specified epitopes (M1, M3, M9, M13, and M14) upon MVA competition analysis. MelVacComp - data from competition with DDM-1.7 melanoma cell lysate is shown. Relative abundance - the abundance of IgG-bound peptides containing the specified epitopes normalized to values of the paired vaccination-specific sample (MelVac) for each patient. (d) Box plots show the abundance of IgG-bound peptides containing sequences of the viral capsid antigen p18 (EBV VCA p18 epitope (161 GGQPHD TA PRGARKK 175) and the epitope of glycoprotein B (CMV gB;70 ETIYNTTLKY 80)40 from MVA competition analysis. MelVacComp - data from competition with DDM-1.7 melanoma cell lysate is shown. Abundance - the abundance of IgG-bound peptides in base 10 logarithmic scale.
of the 15 melanoma-differentiating epitopes was detectable in MelVac-CTRL samples with levels boosted by MelCancerVac® administration in paired MelVac samples (Fig. 4a and Supplementary Fig. 5). Although the seroresponse changes to the majority of the 15 epitopes were patient-specific, reactivity to the epitope markers M4 (MAGEM50) and M5 (MAGE1) was similarly boosted by vaccine in all patients (Wilcoxon Rank Sum test, p < 0.05, Fig. 4b). Furthermore, MVA with melanoma cell lysate competition confirmed that the high antibody reactivity observed in MelVac samples was specific to melanoma proteins as blocking with cell lysate interfered with IgG binding to most of the aforementioned epitopes, with significant effects for M1, M3, M9, M13, and M14 (Fig. 4c and Supplementary Fig. 6). The specificity of blocking with anti-melanoma-lysate was further confirmed by analyzing independent EBV and CMV-associated epitopes. Namely, the antibody response to the viral capsid antigen p18 (EBV VCA p18; 1GPGHDTAPRGARKK 179) and glycoprotein B (CMV gB-70 ETVYNTLKY 80-87) did not change upon competition (Fig. 4d). Therefore, we conclude that antibody response boosted upon MelCancerVac® vaccination is specific to epitopes related to melanoma-associated antigens and is frequently correlated with pre-existing immune response to these antigens.

Three-epitope signature as a biomarker of immunotherapy-elicited melanoma-specific response. As high melanoma-specific response appears to be a feature of tumor immunogenicity and its sensitivity to targeted treatments, we were interested to examine the prognostic utility of the resolved epitopes in therapy-elicited response. We used logistic regression and ROC analysis and found that 3 epitope biomarkers: M3 (ARMC9/PRA22/MORCA4) (73% sensitivity, 97% specificity), M9 (CRBG1) (Sens 82% sensitivity, 81% specificity) and M11 (MORCA4) (73% sensitivity, 90% specificity) in combination differentiated cancer patients based on melanoma-specific response elicited by treatments from controls with area under curve (AUC) of 0.991, ~91% sensitivity and 100% specificity (Fig. 5a, b and Supplementary Fig. 7). Here we show that antibody reactivity to a small subset of epitopes of known melanoma-associated antigenic determinants serves as a biomarker associated with melanoma-specific immunity elicited by cancer immunotherapies.

Discussion

Here, we note unique and divergent changes in melanoma-antigen immune profiles in cancer patients receiving immunotherapy treatments that implicate distinct humoral immune functions connected to the therapy. The IgG response to specific epitopes of a subset of melanoma-antigens was associated with dendritic cell vaccine treatments in lung cancer. Patients receiving MelCancerVac® showed prior response to some epitopes which was enhanced upon treatments, concluding these as potential biomarkers associated with anti-melanoma immunity already at the pre-treatment stage. The same epitopes were targeted by antibodies in melanoma patients receiving anti-PD-1 therapy. The resolved antigenic determinants were in proteins involved in the formation and modulation of extracellular matrix, and also tumor cell survival. Resolved antibody response targeting melanoma or melanoma-like features in cancer elicited by different types of immunotherapies sets the stage for future investigations of the epitopes and their clinical relevance as biomarkers for predicting therapy efficacy in larger studies.

Herein, we used MVA to discover epitope biomarkers associated with anti-cancer antibody response. Our data establish that individuals with cancer show highly heterogeneous immune response to peptide antigens that is neither clinical group, cancer type nor immunotherapy specific. This finding was expected and could be related to the genetic variation and history of previously encountered pathogens. However, we found clear similarities in antibody response to melanoma-associated proteins, including CTAs, a group of antigens now exceeding more than 200 proteins in patients upon different immunotherapy treatments. The resolved epitopes were mapped to extra- and intracellular proteins associated with the formation and modulation of extracellular matrix, but also with tumor cell survival, ciliary functions and lipoprotein signaling. Despite the fact that CTAs are mostly internal tumor antigens, the restricted expression of CTAs in tissues and their antigenicity has promoted utilizing them as targets for immunotherapies. Interestingly, among other epitopes targeted by treatment-elicited antibody response in melanoma and NSCLC cases was of MAGE-A3 protein. MAGE-A3 has been detected in up to 76% of melanomas and in 30–50% of NSCLCs and is thus currently trialed as a target for immunotherapy. Antibody response to epitopes coalescing on other MAGE group of proteins could be related to the poor prognostic features of metastases and melanoma progression. On the other hand, given that the resolved group of antigens included widely expressed proteins (including MAGE family of antigens like MAGE-D), the humoral response towards these could mark excessive immune-attack and damage to self-tissues as concluded by others from studies of ICI-based therapies of melanoma and NSCLC. This suggests that MVA-defined epitopes from intracellular antigens could be indicators of immunotherapy-associated tumor cell death. Our data on heterogenic immune response to melanoma-antigens are in good harmony with the findings on the heterogeneous expression of...
CTAs. Therefore, characterization of the antibody response towards to canonical tumor antigens, including CTAs at pre- and post-treatment stage at epitope resolution could provide new strategies to detect and tackle cancer.

Most of the anti-melanoma-associated antigen immune response that we described for the NSCLC MelCancerVac cohort was also detected in patients with unresectable metastatic melanoma who received anti-PD-1 antibody (pembrolizumab) immunotherapy. We found that the resolved epitopes of MAGE-A antigens were similarly targeted by antibodies in both cases, suggesting the redundant cellular functions of the underlying antigens in lung cancer and melanoma. We expected to detect the anti-melanoma immunogenicity mainly in post-vaccination cases, but interestingly we determined antitumor immunity already at the pre-vaccine stage. Data from IFNγ analysis of MelCancerVac trial demonstrated T cell-specific response correlating with vaccine-specific immunity and sustained stable disease. These findings on the ICI-associated restoration of T cell activity by MelCancerVac are in good agreement with the observed anti-melanoma-specific humoral response patterns detected by our analysis. This once again highlights the importance to determine the elicited antibody response to specific tumor antigens as a measure of the anti-tumor activity associated with immune treatments for assessing the clinical utility of the treatment. However, to confirm the relationship between the melanoma-antigen associated epitopes and clinical efficacy, the number of patients needs to be substantially increased in future studies.

Autoantibodies have the potential to provide unique fingerprints that reflect the nature of the malignant process in the affected organ. Studies of B cell immunity on melanoma have demonstrated its important role in anti-tumor response, but also affected organ. Studies of B cell immunity on melanoma have needs to be substantially increased in future studies.

CancerVac targeted by our analysis. This once again highlights the importance to exclude neoepitopes, cryptic epitopes, and metagenome-associated epitopes for which we do not have data. Finally, although this study hessesh the use of samples from a phase II clinical trial for the discovery of blood biomarkers, due to the limited number of samples further clinical studies are warranted.

Data availability
Source data underlying the main figures are provided as Supplementary Data files 1–6. The whole sequencing datasets generated and/or analyzed in this study are not publicly available due to containing sensitive clinical information but are available from the corresponding author upon reasonable request via a material transfer agreement.

Code availability
The R packages used to generate and analyze data presented in this study are described in the Methods section and available to all. The SPEXS2 algorithm for delineating epitopes from peptide sets can be found at https://github.com/epodelbre/spxs2. The custom Excel VBA used to find sequence similarities between generated epitopes and protein sequences are available from the corresponding author upon request.

Received: 20 July 2021; Accepted: 25 April 2022;
Published online: 11 May 2022

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**Acknowledgements**

Previous members of DanDrit Biotech A/S team Eric Leire (Genflow Biosciences, Immunethep), Mai-Britt Zocca (IO Biotech ApS) are thanked for assistance on study execution. We thank A.-H. Pool from the Department of Neuroscience, University of Texas Southwestern Medical Center, for the expertise that supported this work. The members of Protiobios team are thanked for their excellent technical support. This study was supported by research funding grants of Protiobios (5.1–4/20/170, and PRG573) from the Estonian Ministry of Education and Estonian Research Council, respectively, and H2020-MSCA-RISE-2016 (EU734791) and H2020 PANBioRA (EU760921) projects from the European Union. T.T. and J.T. were partially supported by Estonian Research Council (grant PRG805) and European Union through the European Regional Development Fund (Project No. 2014–2020.4.01.15–0012). J.T. was partially supported by the grant from Estonian Ministry of Education and Research (2014–2020.4.01.21–0315).

**Author contributions**

A.R., M.J., H.S., N.P., A.P., M.A. and K.P. conceived and designed the study. M.A. and A.M. consulted the clinical data. A.R., M.J., H.S., N.P., A.P., J.T., and K.P. analyzed and interpreted the data. M.A., T.T., and K.P. supervised the study. A.R., A.G., and K.P. drafted the manuscript. All authors revised and approved the final manuscript for submission.

**Competing interests**

A.P. and K.P. are inventors of the patent application (PCT Application No. US/14079626) filed by Protiobios that covers the use of phage display method for manipulating and monitoring humoral immunity. All other authors declare no competing interests.

**Additional information**

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1038/s43856-022-00114-7.

**Correspondence** and requests for materials should be addressed to Kaia Palm.

**Peer review information** *Communications Medicine* thanks the anonymous reviewers for their contribution to the peer review of this work.

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