ORIGINAL ARTICLE

Autoimmunity Against Surfactant Protein B Is Associated with Pneumonitis During Checkpoint Blockade

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Abstract

Rationale: Immune checkpoint inhibitor (ICI)–related pneumonitis is a serious autoimmune event affecting as many as 20% of patients with non–small-cell lung cancer (NSCLC), yet the factors underpinning its development in some patients and not others are poorly understood.

Objectives: To investigate the role of autoantibodies and autoreactive T cells against surfactant-related proteins in the development of pneumonitis.

Methods: The study cohort consisted of patients with NSCLC who provided blood samples before and during ICI treatment. Serum was used for proteomics analyses and to detect autoantibodies present during pneumonitis. T-cell stimulation assays and single-cell RNA sequencing were performed to investigate the specificity and functionality of peripheral autoreactive T cells. The findings were confirmed in a validation cohort comprising patients with NSCLC and patients with melanoma.

Measurements and Main Results: Across both cohorts, patients in whom pneumonitis developed had higher pretreatment levels of immunoglobulin G autoantibodies targeting surfactant protein (SP)-B. At the onset of pneumonitis, these patients also exhibited higher frequencies of $CD4^+$ IFN-γ–positive SP-B–specific T cells and expanding T-cell clonotypes recognizing this protein, accompanied by a proinflammatory serum proteomic profile.

Conclusions: Our data suggest that the cooccurrence of SP-B–specific immunoglobulin G autoantibodies and $CD4^+$ T cells is associated with the development of pneumonitis during ICI therapy. Pretreatment levels of these antibodies may represent a potential biomarker for an increased risk of developing pneumonitis, and on-treatment levels may provide a diagnostic aid.

Keywords: immune checkpoint inhibitor; non–small-cell lung cancer; autoimmunity; pneumonitis; surfactant protein B

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A data supplement for this article is available via the Supplements tab at the top of the online article.

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The development of immune checkpoint inhibitors (ICIs) has significantly advanced cancer treatment; however, ICI-induced immune activation often leads to immune-related adverse events (irAEs), which can affect various organs and may be treatment- or even life-limiting (1). Some irAEs are more frequent in certain types of cancer: for example, ICI-related pneumonitis (ICI-P) is much more common in patients with non–small-cell lung cancer (NSCLC) than in patients with melanoma or other cancers (2). Currently, we neither understand the immune processes underpinning the development of ICI-P in patients with NSCLC, nor can we predict which individuals it is most likely to affect (3). Some studies suggest that patients with preexisting lung disease, in particular interstitial lung disease, are at a higher risk of developing ICI-P (4, 5), although the data are not yet clear. These knowledge gaps prevent effective risk stratification and impede optimal clinical management.

This study aimed to identify the immune mechanisms underlying ICI-P to enable the development of strategies to predict, prevent, and treat this condition. Previous work proposed the recognition of shared tissue/tumor antigens by autoimmune T cells as a potential mechanism for irAEs (6–8). Recently, we showed that surfactant-related proteins, which are expressed by healthy and malignant lung tissue in patients with NSCLC, may act as immunogenic self-antigens, and that $CD8^+$ T cell responses to these antigens are associated with better responses to ICI treatment (6). Here, we asked whether and how autoantibodies and autoreactive T cells against surfactant-related proteins are linked with the risk or pathogenesis of ICI-P. Some of the results of these studies have been previously reported in the form of an abstract (9).

Methods

Study Design

This study was designed to define the immunological mechanisms mediating ICI-P in patients with NSCLC by comparison with appropriate control groups. This objective was addressed by analyzing: *1*) the clinical characteristics of patients with stage IV NSCLC receiving ICI treatment; *2*) serum samples from these patients to detect ICI-P–specific immune signatures; *3*) the T-cell responses of patients with NSCLC and ICI-P using approaches such as T-cell stimulation assays, T-cell receptor (TCR)-β chain sequencing, and single-cell RNA sequencing; *4*) the antibody-dependent cellular cytotoxicity of serum from patients with ICI-P; and *5*) samples of serum and BAL fluid from patients with NSCLC and ICI-P via ELISA to determine the role of autoantibodies and T cells in the development of ICI-P.

Collection of Clinical Samples

In the IMIT (Immunomonitoring of Immunotherapy) study, a prospective cohort of patients with stage IV NSCLC or melanoma who received ICI treatment (nivolumab, pembrolizumab [plus chemotherapy], ipilimumab [plus nivolumab], atezolizumab, avelumab) was established across four clinical centers in Switzerland (Kantonsspital St. Gallen, Spital Grabs, Spital Wil, and Spital Flawil) from July 1, 2016, to October 1, 2022. The study received ethical approval from the Ethikkommission Ostschweiz in 2016 (EKOS 16/079), and written informed consent in accordance with the Declaration of Helsinki guidelines was obtained from all patients.

ICI treatment was administered intravenously every 2–3 weeks (depending on the type of monoclonal antibody used). On-treatment peripheral blood mononuclear cell (PBMC) and serum samples from patients experiencing ICI-P were taken at the onset of ICI-P, and samples from control patients were taken to match the ICI-P samples as closely as possible. After isolation, PBMCs were cryopreserved at −150°C in fetal calf serum containing 10% DMSO, and serum was cryopreserved at −80°C. All samples were taken before the start of immunosuppressive treatment, and all ICI-P cases were graded according to Common Terminology Criteria for Adverse Events v.5.0 (as published on November 27, 2017).

A multicenter validation cohort was recruited from Kantonsspital St. Gallen (ethical approval, EKOS 16/079), Krankenhaus Nordwest Frankfurt (ethical approval, Biobank-Ethik Votum,

At a Glance Commentary

Scientific Knowledge on the Subject: Immune checkpoint inhibitor (ICI)–related pneumonitis is a serious adverse event that predominantly affects patients with non–small-cell lung cancer. The immune mechanisms mediating pneumonitis remain poorly understood. Recent evidence suggests that surfactant-related proteins may be involved in the development of pneumonitis during ICI therapy.

What This Study Adds to the Field: The results of this study suggest that the cooccurrence of surfactant protein B–specific IgG autoantibodies and surfactant protein B–specific $CD4^+$ T cells is associated with the risk of development of pneumonitis in patients with lung cancer treated with ICIs. These findings shed light on the immunological mechanisms involved in pneumonitis and may provide an aid in pneumonitis risk stratification and diagnosis in the clinical setting in the future.

Landesärztekammer Hessen, MC 288/2015), Lungentumorenzentrum LMU Munich (ethical approval, EK Votum 476–16 UE, 12–16 and 376–11, Ludwig-Maximilians-Universität), University Hospital Basel (ethical approval, ENKZ2018–01990), and the University of Tübingen (ethical approval, 122/2022B02). The cohort consisted of patients with stage IV NSCLC or melanoma who received ICI treatment.

BAL fluid for ELISA experiments was provided by the Department of Pneumology of Kantonsspital St. Gallen and by the Department of Pneumology of Ludwig Maximillian University Hospital Munich. Immunopeptidome data generated from snap-frozen NSCLC tissue and benign adjacent lung tissue were provided by the University of Tübingen.

Olink Proteomics

Proteomic analysis of serum samples from patients was performed at the Swiss Institute of Allergy and Asthma Research (Davos, Switzerland) using the Olink platform "Inflammation panel" (https://olink.com/products-services/target/inflammation/) as previously described (10). *See* online supplement for a complete description of the approach.

Surfactant Proteins A2, B, C, and D and Napsin A IgG ELISA

To identify auto-IgG against pulmonary surfactant proteins (SPs), we performed ELISAs using recombinant proteins. *See* online supplement for a complete description of our approach.

T-Cell Stimulation Assay, Intracellular Cytokine Detection, and Flow Cytometry

Peptides used for stimulations were ordered from GenScript, JPT Peptide Technologies GmbH, or Intavis Peptide Services in lyophilized form. Standard T-cell stimulation assays and intracellular cytokine labeling of human PBMCs were performed as previously described (6). All samples used for the assays were collected before the start of immunosuppressive treatment. Sample data were acquired using an LSR Fortessa flow cytometer (BD) and analyzed using FlowJo software (version 10.6.2). IFN-γ, TNF-α, and IL-17A expression was used to identify antigen-specific T cells. The background signal determined in a medium-only negative control was subtracted from all measurements.

HLA Immunoaffinity Purification and Mass Spectrometric Data Acquisition and Processing

HLA-I and -II molecules were isolated from snap-frozen tissue by standard immunoaffinity chromatography using the monoclonal antibodies W6/32, TU39, and L243 (11). HLA ligand extracts were analyzed as described previously (12). Data processing was performed as previously described with Proteome Discoverer software (v1.4; Thermo Fisher Scientific) to integrate the search results of the SEQUEST HT search engine (University of Washington) against the human proteome (Swiss-Prot database) (13). The false discovery rate (estimated by Percolator algorithm 2.04) was limited to 1% for HLA class II.

Cell Sorting, Single-Cell RNA Sequencing, and TCR Sequencing

PBMCs from four patients with NSCLC that initially showed high frequencies of $CD4^+$ IFN-γ^+ T cells upon stimulation with SP-B were again stimulated over 10 days with SP-B–predicted epitopes (*see* T-Cell Stimulation Assay, Intracellular Cytokine Detection, and Flow Cytometry). After 10 days, cells were restimulated with peptides for 6 hours and stained for CD3, CD4, CD8, and CD137 as previously described (6). The marker CD137 (4-1BB) was used to sort activated T cells (14). Live $CD3^+CD4^+CD137^+$ T cells were isolated and processed for single-cell RNA (scRNA) sequencing as previously described (6). Libraries were sequenced using Illumina NextSeq 500 (TCR sequencing) at the Functional Genomic Center Zurich. *See* online supplement for a description of the analysis of scRNA-seq data.

Statistical Analysis

Statistical analyses and graphing were performed using Prism version 8.4.3 (GraphPad Software) or R (version 4.2.2, 2022; R Foundation for Statistical Computing).

Results

Our prospective study cohort comprised 144 patients with NSCLC, of whom 119 provided blood samples before and during ICI treatment. On-treatment samples from patients with ICI-P were provided at the onset of ICI-P, before receiving immunosuppressive therapy, and samples from patients without ICI-P in the cohort were taken to match the timing of the ICI-P samples as closely as possible. The study also included patients with melanoma and healthy volunteers who donated blood samples as controls. During the study, 15 of 144 patients with NSCLC (10.2%) experienced ICI-P, compared with only 2 of 64 patients with melanoma (3.2%; Figures 1A and E1A in the online supplement). The characteristics of patients with NSCLC and ICI-P are summarized in Table 1, the results of their diagnostic testing for ICI-P are shown in Table 2, and the characteristics of control patients are shown in Table E1. The development of irAEs in general is associated with improved outcomes in ICI-treated patients with NSCLC (15), and here we saw that skin irAEs correlated with longer

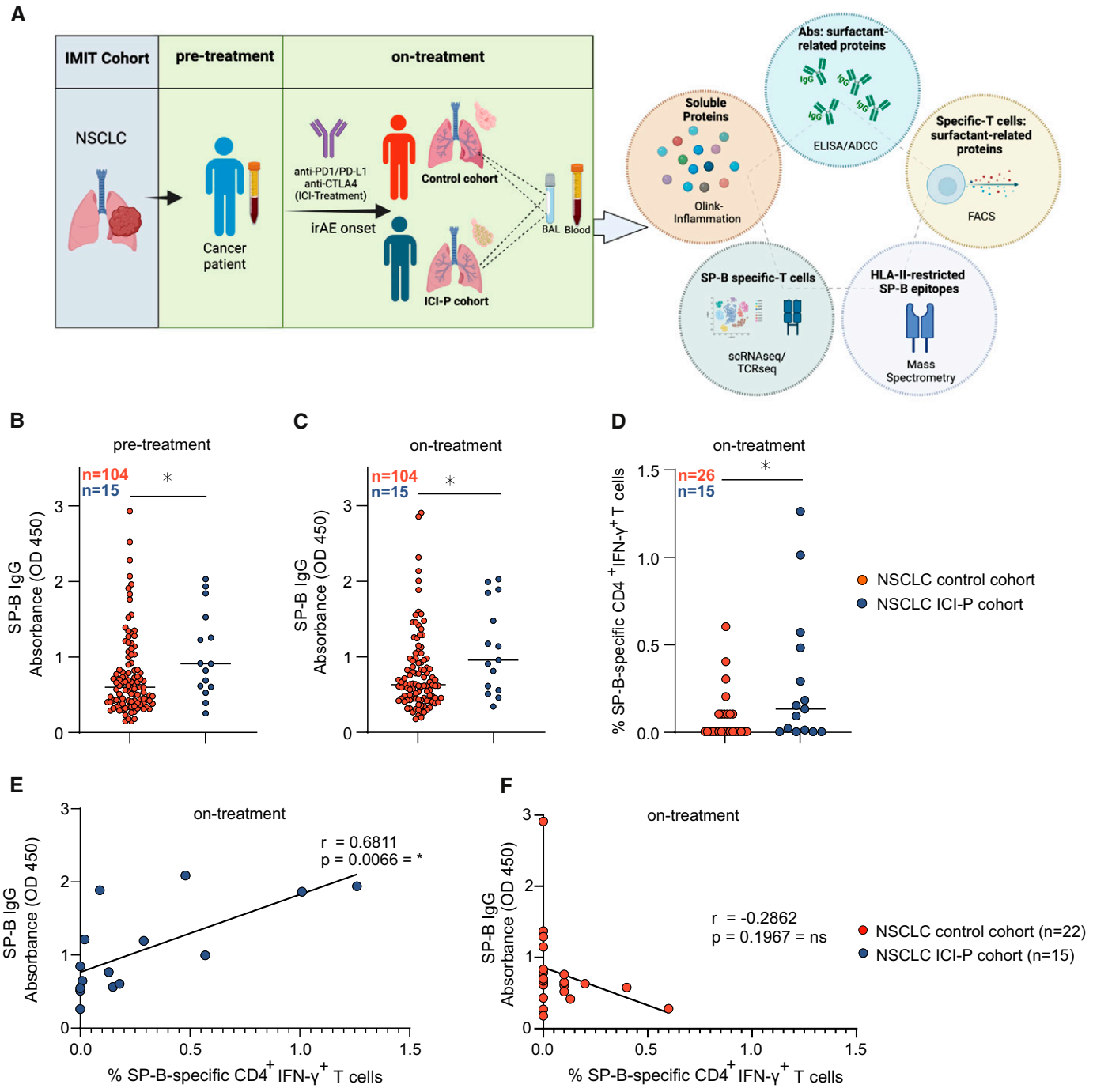

Figure 1. Patients with non–small-cell lung cancer (NSCLC) and immune checkpoint inhibitor–related pneumonitis (ICI-P) have increased levels of surfactant protein (SP)-B–specific IgG and $CD4^+IFN\text{-}\gamma^+$ T cells in their blood. (*A*) Graphical representation of the experimental approach (generated with BioRender). (*B* and *C*) Pretreatment and on-treatment levels of IgG targeting SP-B in serum from patients with NSCLC and ICI-P ($n = 15$) compared with those without ICI-P ("control patients"; $n = 104$) (Mann-Whitney test, $P = 0.0370$ before treatment, $P = 0.0366$ during treatment). (*D*) On-treatment peripheral blood mononuclear cells from patients with NSCLC and ICI-P ($n = 15$) and NSCLC control patients ($n = 26$) were stimulated with peptide pools of SP-B. IFN-γ production by $CD4^+$ T cells was measured to determine the frequency of activated T cells in the cell cultures (Mann-Whitney test, $P = 0.0278$). (*E* and *F*) Correlation of serum levels of IgG targeting SP-B (*y*-axis) and the frequency of SP-B–specific $CD4^+$ $IFN\text{-}\gamma^+$ T cells (*x*-axis) for patients with ICI-P ($n = 15$) and control patients ($n = 22$) (linear regression and Spearman correlation). * = $P < 0.05$; IMIT = Immunomonitoring of Immunotherapy (study); irAE = immune-related adverse event; ns = not significant; OD 450 = optical density of the ELISA.

Table 1. Clinical Characteristics of Patients with Non–Small-Cell Lung Cancer and Immune Checkpoint Inhibitor–Related Pneumonitis ($n = 15$)

Patient ID	Age at Start of Immunotherapy, y	Sex	Histology	Type of Immunotherapy	Therapy Line	Tumor Driver Mutations	Preexisting Lung Disease	PD-L1 Expression Status, %	Response to Immunotherapy	Pack-Years	Lung RT before Pneumonitis Onset	Other irAEs during Treatment*	CTCAE Pneumonitis Grade
IMIT15	62	F	AC	Nivolumab	Second	KRAS	COPD	0	Yes	40	No	Arthritis	2
IMIT34	76	M	AC	Pembrolizumab	First	Unknown	TBC as child	60	No	10	No	None	2
IMIT37	66	M	AC	Nivolumab	Third	KRAS	COPD	NA	No	45	No	None	2
IMIT53	74	M	AC	Nivolumab	Second	Unknown	COPD	0	Yes	99	Yes, >12 mo	Rash, thyroiditis	2
IMIT84	71	M	AC	Pembrolizumab	Second	KRAS	COPD	80	No	60	No	Nephritis	2
IMIT89	61	F	AC	Pembrolizumab	First	BRAF	None	80	Yes	45	No	Colitis, hepatitis, rash	2
IMIT104	68	M	SCC	Pembrolizumab	Second	ALK transl.	None	5	No	65	No	Arthritis, Colitis	2
IMIT109	53	F	AC	Atezolizumab	Second	None	None	0	No	30	No	None	3
IMIT122	67	M	SCC	Pembrolizumab	First	TP53	COPD	60	Yes	50	Yes, >12 mo	Colitis, Rash	3
IMIT124	72	M	SCC	Pembrolizumab	First	Unknown	COPD	90	Yes	50	Yes, >12 mo	Arthritis	2
IMIT130	69	F	AC	Atezolizumab	Second	KRAS	None	NA	No	25	Yes, >12 mo	None	2
IMIT137	57	F	AC	Pembrolizumab	Third	TP53	COPD	>1	Yes	35	Yes, >12 mo	Arthritis, rash	2
IMIT165	59	M	AC	Pembrolizumab	First	KRAS	COPD	0	Yes	40	No	None	2
IMIT173	67	M	AC	Pembrolizumab (+chemotherapy)	First	Unknown	COPD, asthma	0	No	40	Yes, >12 mo	Rash	3
IMIT199	78	M	AC	Pembrolizumab (+chemotherapy)	First	KRAS	Emphysema	0	No	30	No	Nephritis	3

Definition of abbreviations: AC = adenocarcinoma; ALK transl. = translocation in the ALK (anaplastic lymphoma kinase) gene; BRAF = B-Raf proto-oncogene; COPD = chronic obstructive pulmonary disease; CTCAE = Common Terminology Criteria for Adverse Events; IMIT = Immunomonitoring of Immunotherapy (study); irAE = immune-related adverse event; KRAS = KRAS proto-oncogene; NA = not available; PD-L1 = programmed death-ligand 1 protein; RT = radiation therapy; TBC = tuberculosis; TP53 = tumor protein p53.
*Not treatment-limiting.

survival, whereas ICI-P did not (Figures E1B and E1C), in line with previous studies (16, 17).

We previously showed that changes in the levels of chemokines and cytokines associated with the IFN-γ signaling pathway are associated with increased risk of future irAEs (10). We therefore asked whether a specific immune signature was related to the risk of developing ICI-P in our cohort and measured the relative concentrations of 92 inflammation-related proteins in the serum (Olink inflammation panel; *see* METHODS) before and during treatment. During treatment, patients with ICI-P showed significantly altered expression levels of a variety of immune mediators compared with controls (Figures E2A and E2B). Proteins that were present at higher concentrations in the ICI-P group during treatment were associated with "antiinflammatory signaling," "control of immune tolerance," and "[severe acute respiratory syndrome coronavirus 2] SARS-CoV-2 innate immunity evasion and cell-specific immune response" pathways, whereas the proteins expressed at a lower level in the ICI-P group were significantly correlated with "inflammatory response" and "allograft rejection" pathways (Tables 3 and 4). Our receiver operating characteristic analysis revealed that patients with high levels of FGF5, βNGF, and IL-4 were more likely to have ICI-P (*see* Figure E2C), indicating the potential of this proteomic signature as a noninvasive diagnostic biomarker. Interestingly, pretreatment protein expression profiles between the two groups were comparable (see Figure E2D), suggesting that the inflammatory process of pneumonitis is induced *de novo* by treatment with ICIs, and that these proteomic changes could form the basis for improved noninvasive diagnosis.

In the context of the immunological changes described above, we then asked about the potential role of autoantibodies. High levels of autoantibodies against surfactant-related proteins underpin the pathology of severe acute respiratory distress syndrome in patients with coronavirus disease (COVID-19) (18). In addition, preexisting autoantibodies against shared tumor–tissue antigens have been linked to the development of irAEs in ICI-treated patients (19). We therefore measured the serum levels of IgG targeting surfactant-related proteins (SP-A2, SP-B, SP-C, SP-D, and napsin A) in pretreatment and

Table 2. Summary of Invasive Diagnostic Procedure Results in Patients with Non–Small-Cell Lung Cancer and Immune Checkpoint Inhibitor–Related Pneumonitis ($n = 15$)

Procedure and Test Result	Total ($n = 15$)	IMIT15	IMIT34	IMIT37	IMIT53	IMIT84	IMIT89	IMIT104	IMIT109	IMIT122	IMIT124	IMIT130	IMIT137	IMIT165	IMIT173	IMIT199
BAL	9 (60%)	Yes	Yes	No	Yes	Yes	Yes	No	No	No	Yes	No	No	Yes	Yes	Yes
Total cell count, M/L	—	647	633	—	171	32	399	—	—	—	322	—	—	Very low	12.71	Very low
(<300 M/L for smoker)																
Malignant cells	—	NA	None	—	None	Few	None	—	—	—	None	—	—	None	None	None
Macrophages, % (>81%)	—	NA	51.8	—	51	81.5	42.5	—	—	—	68	—	—	Few	11	NA
Lymphocytes, % (<13%)	—	18.8	45.8	—	42	10.5	47	—	—	—	14	—	—	NA	0.92	NA
CD4/CD8 quotient	—	0.9	0.9	—	0.3	NA	4.5	—	—	—	0.3	—	—	NA	NA	NA
(1.1–1.5 for smoker)																
$CD4^+$ T cells, %	—	NA	46.89	—	20.5	NA	78.4	—	—	—	22	—	—	NA	NA	NA
$CD8^+$ T cells, %	—	NA	51.2	—	66	NA	17.6	—	—	—	72	—	—	NA	NA	NA
Neutrophils, % (<3%)	—	24.7	2.5	—	5	6.5	9.7	—	—	—	17	—	—	NA	0.83	Few
Eosinophils, % (<1%)	—	NA	0	—	2	1.5	0.8	—	—	—	0.3	—	—	NA	0	NA
Mastocytes, % (<1%)	—	NA	0	—	0	0	0	—	—	—	0	—	—	NA	0	NA
Plasma cells, % (<1%)	—	NA	0	—	0	0	0	—	—	—	0	—	—	NA	0	NA
Lung biopsy	3 (20%)	No	Yes, lym↑	No	No	No	Yes, unsp	No	No	No	No	No	Yes, unsp	No	No	No
Pleural effusion analysis	3 (20%)															
Reactive lymphocyte proliferation	—	Yes, lym↑	No	No	No	Yes, lym↑	No	No	No	No	No	No	No	No	No	Yes, lym↑

Definition of abbreviations: IMIT = Immunomonitoring of Immunotherapy (study); M/L = total cell count/liter; NA = not available; unsp = unspecific; lym↑ = increased lymphocyte count.

on-treatment samples from a pilot cohort consisting of patients with NSCLC with ICI-P, patients with NSCLC without ICI-P, control patients with melanoma, and healthy volunteers. Interestingly, we found significant differences in the levels of SP-B IgG among the different groups but not in the levels of the other proteins (Figure E3). We therefore focused on SP-B IgG levels and repeated the experiments with our entire prospective NSCLC cohort (termed the discovery cohort). We found that patients with NSCLC showed significantly higher levels of pretreatment IgG targeting SP-B compared with patients with melanoma and healthy volunteers (Figure E4A) and patients with melanoma during treatment (*see* Figure E4B). In addition, among patients with NSCLC, we found significantly higher levels of IgG targeting SP-B in patients with ICI-P than in the rest of the NSCLC cohort before and during treatment (Figures 1B and 1C). We also found that patients with ICI-P had significantly higher levels of IgG targeting SP-B in their BAL fluid compared with control patients (*see* Figure E4C). We next determined whether high IgG levels affected surfactant function. Patients with NSCLC and high IgG levels against SP exhibited increased surface tension, indicating impaired surfactant function, which was associated with an increased risk of alveolar collapse (*see* Figure E4D). To further evaluate the functional role of IgG targeting SP-B, we performed antibody-dependent cellular cytotoxicity assays using serum from patients with ICI-P that contained polyclonal IgG antibodies. We coincubated A549 NSCLC cells overexpressing SP-B with patient serum and PBMCs. Despite the polyclonal nature of the serum (and hence the strong dilution of SP-B–specific antibodies), effective target cell killing was achieved with an antibody concentration of 100 μg/ml (*see* Figure E4E). The frequency of cell killing was significantly higher with NSCLC target cells compared with melanoma target cells (*see* Figure E4F). These findings show that patients experiencing ICI-P have increased levels of IgG targeting SP-B in their blood and BAL fluid compared with control patients and that SP-B IgG antibodies exhibit antibody-dependent cellular cytotoxicity functions.

We next asked whether surfactant-related proteins were also recognized by T cells from these patients. PBMCs taken during treatment from 15 patients with NSCLC and ICI-P and from 26 control patients with NSCLC and no ICI-P were

Table 3. Pathways, Names, and Functions of Immune-related Proteins Expressed at Significantly Different Levels in Serum from Patients with and without Immune Checkpoint Inhibitor–Related Pneumonitis during Treatment

Abbreviation	Name	Function
βNGF	β-nerve growth factor	Autocrine hormone that has a role in inflammatory responses and tissue repair
IL10RB	Interleukin 10 receptor β subunit	Participates in IL10-mediated antiinflammatory functions
FGF5	Fibroblast growth factor 5	Regulatory effect on inflammation-related disease
TGFβ1	Transforming growth factor β 1	Pleiotropic effects on adaptive immunity, regulation of effector and regulatory $CD4^+$ T cell responses
NT3	Neurotrophin 3	Antiinflammatory capacity
IL10RA	Interleukin 10 receptor α subunit	Participates in IL10-mediated antiinflammatory functions, limiting excessive tissue disruption caused by inflammation
IL24	Interleukin 24	Cytokine predominantly released by activated monocytes, macrophages, and Th2 cells; acts on skin, lung, and reproductive tissues
AXIN1	Axin 1	Modulation of inflammatory cytokine product
MCP1	Monocyte chemoattractant protein 1	Regulates migration and infiltration of monocytes to the site of inflammation
CD5	Cluster of Differentiation 5	Expressed on T and B-1a lymphocytes, immunomodulator
CASP8	Caspase 8	Plays cell-intrinsic role in inflammatory cytokine production
IL4	Interleukin 4	Cytokine, induces differentiation of naive helper T cells (Th0 cells) to Th2 cells
NRTN	Neurturin	Neurotrophin plays critical role in bidirectional signaling between immune cells and neurosensory network structures in airways and skin
IL15RA	Interleukin 15 receptro α subunit	Potentially proinflammatory roles in autoimmune disease
IL10	Interleukin 10	Cytokine, antiinflammatory, plays central role in limiting host immune response
IL1α	Interleukin 1 α	Major alarmin cytokine, initiation and propagation of sterile inflammation
IL2	Interleukin 2	Key growth and death factor for antigen-activated T lymphocytes
ARTN	Artemin	Supports survival of peripheral neurons
IL22RA1	Interleukin 22 receptor α subunit 1	Modulates tissue responses during inflammation
IL13	Interleukin 13	Immunoregulatory cytokine
CXCL5	C-X-C motif chemokine 5	Chemokine, crucial inflammatory mediator
MCP4	Monocyte chemoattractant protein 4	Potent chemoattractant for monocytes and T lymphocytes
CD6	Cluster of differentiation 6	Important for continuation of T cell activation
TWEAK	Tumor necrosis factor superfamily member 12	Controls several cellular responses, including induction of inflammatory cytokines
CST5	Cystatin-D	Cysteine proteinase inhibitor
CCL28	CC-chemokine ligand 28	Drives mucosal homing of T and B lymphocytes that express CCR3 and CCR10
FGF23	Fibroblast growth factor 23	Circulating proinflammatory hormone

Definition of abbreviation: Th = T helper.

stimulated with peptide pools representing SP-A2, SP-B, SP-C, SP-D, and napsin A. We detected SP-specific $CD4^+$ and $CD8^+$ T cells in the blood of patients with NSCLC and ICI-P (Figure E5A) and in the NSCLC control group (*see* Figure E5B). Interestingly, the frequency of SP-B–specific $CD4^+IFN\text{-}\gamma^+$ T cells was significantly higher in the ICI-P group compared with the control group of patients without ICI-P (Figures 1D and E5C), whereas the frequency of SP-B–specific $CD8^+IFN\text{-}\gamma^+$ T cells was comparable between patients with ICI-P and controls (*see* Figure E5D). To further investigate the relationship between T-cell responses and the presence of autoantibodies, we performed a correlation analysis between the percentage of SP-B–specific $CD4^+IFN\text{-}\gamma^+$ T cells and SP-B–specific IgG levels. Notably, a significant positive correlation was observed in patients with NSCLC and ICI-P (Figure 1E), but not for the rest of the NSCLC cohort (Figure 1F), indicating a potential interplay of T-cell reactivity and production of SP-B–specific antibodies that may be specific to patients with ICI-P. We also conducted a second correlation analysis between the concentrations of the 92 previously measured inflammatory proteins and the levels of SP-B–specific IgG or the frequency of $CD4^+IFN\text{-}\gamma^+$ T cells in patients with NSCLC and ICI-P. This analysis revealed significant positive correlations between the levels of SP-B–specific IgG and βNGF and IL4 and a significant negative correlation with levels of MCP4 (*see* Figure E5E). Taken together, patients with NSCLC and ICI-P have a significantly higher frequency of SP-B–specific $CD4^+$ T cells in their blood during treatment compared with control patients. Furthermore, we found that the cooccurrence of SP-B–specific IgGs, SP-B–specific $CD4^+$ T cells, and inflammatory proteins is associated with the pathogenesis of ICI-P. These findings suggest that monitoring the levels of SP-B–specific T cells and IgGs could potentially aid in the identification and management of ICI-P.

Table 4. Significantly Different Expression of Immune-related Proteins in Serum from Patients with and Without Immune Checkpoint Inhibitor–Related Pneumonitis during Treatment

Term in Wikipathways	Overlap	*P* Value	Adjusted *P* Value	Genes
Up in NSCLC pneumonitis vs control cohort				
IL-10 antiinflammatory signaling pathway WP4495	3/12	7.48×10^{-8}	6.46×10^{-6}	IL10, IL10RB, IL10RA
Control of immune tolerance by vasoactive intestinal peptide WP4484	3/13	9.72×10^{-8}	6.46×10^{-6}	IL10, IL4, TGFB1
SARS-CoV-2 innate immunity evasion and cell-specific immune response WP5039	4/66	1.44×10^{-7}	6.46×10^{-6}	IL10, TGFB1, CASP8, CCL2
Down in NSCLC pneumonitis vs. control cohort				
Cytokines and inflammatory response WP530	2/26	1.06×10^{-4}	0.004149455	IL13, IL2
Allograft rejection WP2328	2/89	0.00125537	0.024479803	IL13, IL2

Definition of abbreviations: NSCLC = non–small-cell lung cancer; SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2.

To investigate the role of SP-B as a target antigen for $CD4^+$ T cells in patients with NSCLC and ICI-P, we next searched in the HLA Ligand Atlas for HLA-II–restricted SP-B–specific lung tissue–exclusive epitopes for the 15 patients in the ICI-P group (Tables 5 and 6) (12). Using PBMC restimulation assays with the identified SP-B epitopes, we found that several patients with ICI-P harbored epitope-specific $CD4^+IFN\text{-}\gamma^+$ T cells, which ranged between 0.2% and 5.5% of their total $CD4^+$ T cells at ICI-P onset (Figures 2A and 2B). To determine whether SP-B peptides are naturally presented in the HLA class II immunopeptidome of patients with NSCLC, we applied mass spectrometry–based immunopeptidomics. We analyzed HLA peptide presentation in snap-frozen tumor and adjacent normal lung tissue samples from 17 patients with NSCLC from a separate cohort (Table E2) and found that, in almost one fourth, SP-B peptides were presented by NSCLC tissue and by the adjacent benign lung tissue. In an additional three patients, SP-B–derived peptides were exclusively presented by the adjacent benign lung tissue (*see* Figure E5F). Importantly, 3 of the 15 identified SP-B peptide sequences present in the lungs of patients were identical to the previously described SP-B–specific peptide sequences identified with the HLA Ligand Atlas (Tables 6 and 7). These findings confirmed that patients with NSCLC have circulating SP-B–specific $CD4^+$ T cells at the time of ICI-P onset. Furthermore, these data showed that SP-B–derived peptides are naturally presented on HLA-II alleles by NSCLC tissue and benign lung tissue and therefore might serve as targets in ICI-P.

To gain further insight into SP-B–specific $CD4^+$ T cells, we chose four patients whose T cells were activated after stimulation with individual SP-B epitopes. We repeated the stimulation experiments for these patients and subsequently isolated activated $CD137^+CD4^+$ T cells. We then performed paired scRNA and single-cell TCR sequencing. The clustering analysis revealed that cluster 3 consisted of T cells highly expressing activation markers such as TNF and CD69. We therefore considered the TCR clonotypes within this cluster to be of the highest importance and focused our subsequent analysis on them (Figures 2C and 2D). We next performed bulk TCR CDR3β sequencing on unsorted PBMCs

Table 5. HLA Class II Alleles of IMIT Patients with Non–Small-Cell Lung Cancer and Immune Checkpoint Inhibitor–Related Pneumonitis

Sample ID	HLA-DRB1	HLA-DRB1-1	HLA-DQB1	HLA-DQB1-1	HLA-DPB1	HLA-DPB1-1
IMIT15	04:04:01	14:54:01	03:02:01:02	05:03:01:01	02:01	04:01
IMIT34	11:04:01	13:01:01	03:01:01:03	06:03:01	03:01:01G	04:02:01G
IMIT37	04:04:01	11:01:01	03:01:01:03	03:02:01:02	02:01	04:01
IMIT53	01:01:01	13:02:01	05:01:01:03	06:09:01	02:01	04:01
IMIT84	04:04:01	15:01:01	03:02:01	06:02:01	04:01:01	04:02:01
IMIT89	03:01:01	04:01:01	02:01:01	03:02:01	03:01:01	04:01:01
IMIT104	07:01:01	11:04:01	02:02:01	03:01:01	04:02:01	04:01:01
IMIT109	13:03:01	15:01:01	03:01:01	06:02:01	03:01:01:01	04:01:01
IMIT122	01:01:01	—	05:01:00	—	04:02:01	10:01:01
IMIT124	13:01:01	15:01:01	06:03:01	06:02:01	01:01:01	02:01:02
IMIT130	03:01:01	—	02:01:01	—	04:01:01	—
IMIT137	07:01:01	11:01:01	02:02	03:BJJBW	03:01:01	11:01:01
IMIT165	13:02:01	14:54:01	05:03:01	06:04:01	02:01:02	10:01:01
IMIT173	01:01:01	04:01:01	03:01:01	05:01:01	03:01:01	04:01:01
IMIT199	07:01:01	15:01:01	02:02:01	06:02:01	03:01:01	04:01:01

Definition of abbreviation: IMIT = Immunomonitoring of Immunotherapy (study).

Table 6. SP-B–Specific Peptide Sequences Identified with the HLA Ligand Atlas

Peptide ID	SP-B Peptide Sequence	Strong Binder HLA-II Allele	Weak Binder HLA-II Allele
1	EDIVHILNKMAKE*	DRB1*15:01	DQB1*05:01
2	GPEFWCQSLEQALQ	DPB1*02:01 DRB1*10:01	—
3	IPKGALAVAVAQVCR	—	DQB1*03:01 DRB1*01:01
4	IPKGALAVAVAQVCRVVP	—	DQB1*03:01 DRB1*01:01
5	IPKGALAVAVAQVCRVVPL	—	DQB1*03:01
6	IQAMIPKGALAVAVA*	DQB1*03:01	DPB1*02:01 DRB1*01:01
7	TPQLLTLVPRGWDAH*	—	DRB1*08:01 DRB1*13:03
8	YSVILLDTLLGRMLPQ	DRB1*03:01	DPB1*03:01 DRB1*11:01

Definition of abbreviation: SP-B = surfactant protein-B.
*Peptide sequences also found to be naturally presented on human lung tissue (Table 7).

from the same patients before treatment, during treatment, and at the onset of ICI-P. This method allowed for a more precise estimation of individual clonotype frequency. By matching the TCR clonotypes in the peripheral T-cell repertoire with those present in cluster 3 T cells, we identified T cells within the unsorted PBMCs that were highly likely to be SP-B–specific. Notably, these identified TCR clonotypes exhibited a greater proportion of expanded cells compared with non–SP-B–specific clonotypes at the onset of ICI-P. This suggests previous activation and proliferation following exposure to their cognate antigen, SP-B (Figures 2E and 2F). Moreover, when monitoring the abundance of SP-B–specific TCR clonotypes over time in the peripheral blood, we observed an increase in the total frequency of SP-B–specific T cells in three of the four patients leading up to the onset of ICI-P (Figure 2G). These findings suggest that SP-B–specific T cells may potentially contribute to the development of ICI-P during treatment.

Taken together, these data suggest that the cooccurrence of SP-B–specific IgG autoantibodies and SP-B–specific $CD4^+$ T cells is associated with the development of ICI-P in patients with NSCLC. Because serum is easier to obtain, store, and analyze than PBMCs, we asked whether screening for SP-B–specific autoantibodies could serve as a candidate biomarker for ICI-P in ICI-treated patients with NSCLC. We collected serum samples from a multicenter validation cohort consisting of patients with NSCLC or melanoma and ICI-P, as well as control patients, and measured levels of IgG targeting SP-B before and during treatment (Table S3). We found significantly higher levels of SP-B IgG before and during treatment in the serum of patients with NSCLC and ICI-P compared with those without ICI-P, confirming our initial findings (Figures E6A and E6B).

Interestingly, we also found that patients with melanoma with ICI-P had significantly higher levels of SP-B IgG compared with patients with melanoma without ICI-P (*see* Figure E6C). A visible trend was observed for higher levels of SP-B IgG in pretreatment serum of patients with melanoma and ICI-P, but this was not significant (*see* Figure E6D). The results of the validation cohort confirm that patients with NSCLC and ICI-P have higher pretreatment and on-treatment levels of SP-B IgG and suggest these findings may apply to other cancer types as well.

Discussion

In the present study, we analyzed the cellular and humoral immune response against surfactant-related proteins in patients with NSCLC in whom ICI-P develops. Building on our previous work (6, 18), we uncovered an autoantibody- and $CD4^+$ T cell–driven

Table 7. Naturally Presented Surfactant Protein-B Epitopes on Malignant Non–Small-Cell Lung Cancer and Adjacent Benign Lung Tissue

Malignant Exclusive Peptide Sequence	Benign Exclusive Peptide Sequence	Malignant/Benign Peptide Sequence
DTLLGRMLP	EDIVHILNKMAKEA	DIVHILNKMAKEA
AKEAIFQDTMRKFLEQ	DIVHILNKMAKE	EDIVHILNKMAKE*
DIVHILNKMAK	LPQLVCRLVLR	TPQLLTLVPRGWDAH*
DPLPDPLLDKLVL	DPLPKPLRDPLPDPLLDK	
TPQLLTLVPRGWDA	IQAMIPKGALAVAVA*	
LPDPLLDKLVLPVLPG		
DDYFPLVID		

*Peptide sequences also identified using the HLA ligand atlas (Table 6).

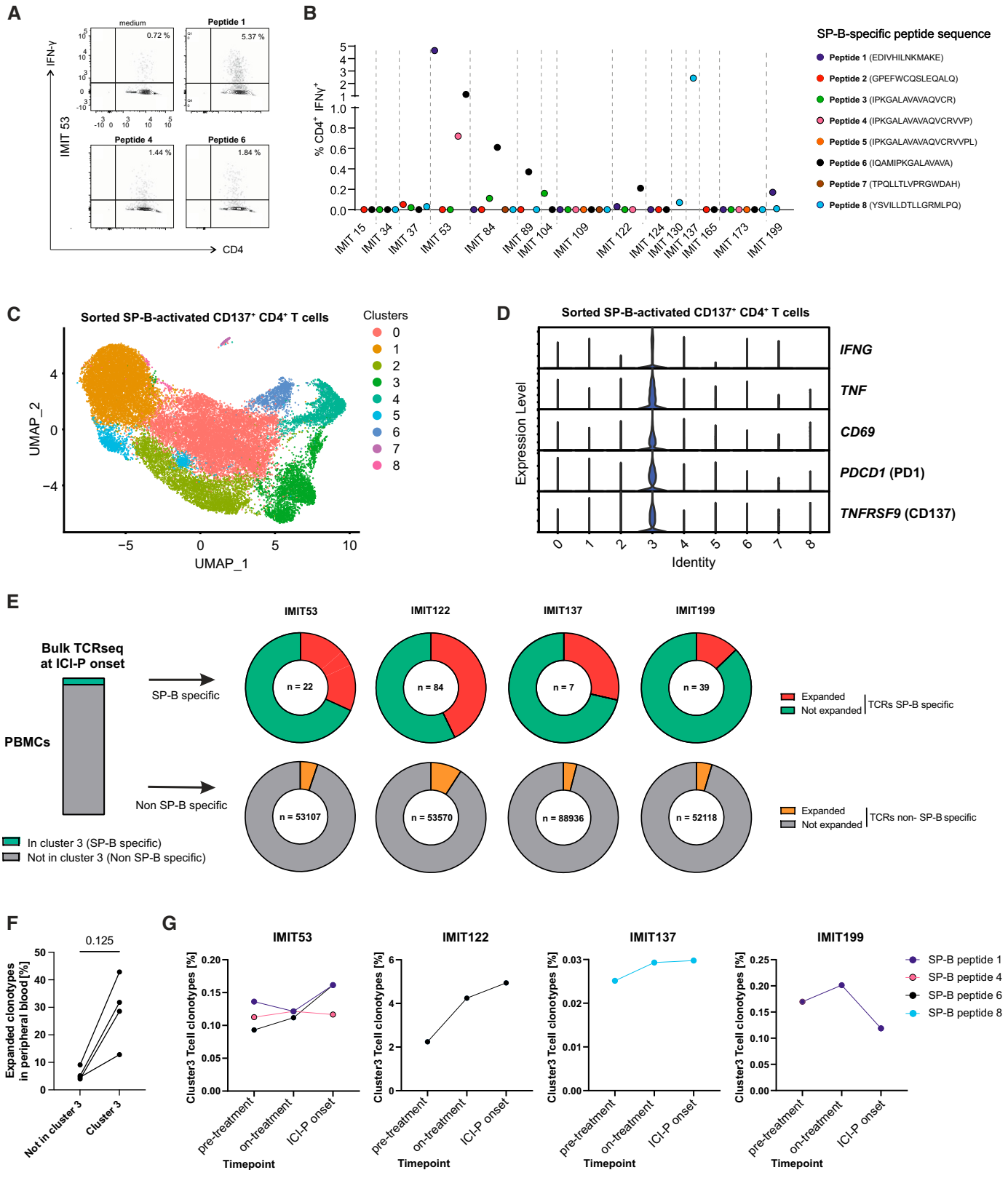

Figure 2. The T-cell repertoire of patients with non–small-cell lung cancer and immune checkpoint inhibitor–related pneumonitis (ICI-P) contains proinflammatory surfactant protein (SP)-B–specific T cells. (*A*) Representative flow cytometry plots depicting the frequency of $CD4^+IFN\text{-}\gamma^+$ T cells after incubation with SP-B–specific peptides. Peptides are summarized in Table 6 (*B*) Overview of $CD4^+IFN\text{-}\gamma^+$ T-cell responses in

Figure 2. (*Continued*). patients with ICI-P ($n = 15$) whose peripheral blood mononuclear cells (PBMCs) were stimulated with HLA-matched SP-B–specific peptides. (*C*) UMAP plot showing the identified clusters after sorting for SP-B–specific activated CD137$^+$ (4-1BB$^+$) CD4$^+$ T cells followed by single-cell RNA and single-cell T-cell receptor sequencing. SP-B–specific single peptides used for the T-cell stimulations are summarized in Table 6. (*D*) Violin plots depicting expression of activation markers in the identified clusters. (*E*) Donut plots showing the fraction of expanded clonotypes (red) in PBMCs at ICI-P onset, divided into SP-B–specific clonotypes (upper row) and non–SP-B–specific clonotypes (lower row). The clonotypes were characterized by matching the CDR3β amino acid sequences from the PBMCs with those in cluster 3, as shown in *C*. PBMCs from one patient (IMIT53) were incubated separately with three SP-B–specific epitopes and those from three patients (IMIT122, IMIT137, and IMIT199) with one SP-B–specific epitope (*C*). (*F*) Comparison of the fraction of expanded clonotypes among non–SP-B–specific and SP-B–specific clonotypes in PBMCs at ICI-P onset (Wilcoxon test). (*G*) Frequency of SP-B–specific T cells before treatment, during treatment, and at ICI-P onset. SP-B–specific clonotypes were identified as in *E*. SP-B–specific peptide sequences are summarized in Table 6. IMIT = Immunomonitoring of Immunotherapy (study); TCRseq = T-cell receptor sequencing; UMAP = Uniform Manifold Approximation and Projection.

immune response against SP-B that is a likely pathophysiological mechanism of ICI-P. We found that patients with NSCLC and ICI-P had higher serum levels of IgG autoantibodies targeting SP-B before treatment and at the onset of pneumonitis compared with patients in whom ICI-P did not develop. We confirmed our findings in a multicenter validation cohort of patients with NSCLC or melanoma undergoing ICI treatment. A wide-ranging proteomics analysis also revealed the importance of FGF5, IL-4, and bNGF in patients with NSCLC and ICI-P. In addition, we found that blood samples from patients with NSCLC and ICI-P contain significantly higher frequencies of CD4$^+$ T cells that recognize SP-B epitopes compared with samples from other patients with NSCLC, and that SP-B is part of the HLA class II immunopeptidome of patients with NSCLC. Using scRNA, single-cell TCR, and bulk TCR sequencing, we confirmed that patients with ICI-P showed expanded SP-B–specific clonotypes in their blood, which also increased in frequency over time. Last, we identified a positive correlation between the levels of SP-B–specific IgG and the frequency of SP-B–specific CD4$^+$ T cells in patients with NSCLC and ICI-P. We hypothesize that, during ICI therapy, there is reinvigoration of not only CD8$^+$ T cells, but also CD4$^+$ T cells that have cytotoxic characteristics, as also documented in other cancer types (20). In patients with increased autoantibody levels, this combination may lead to the development of irAEs such as ICI-P.

These data show in two independent cohorts that measuring SP-B IgG levels before treatment may be a useful marker for predicting ICI-P risk in patients with NSCLC, which may also be applicable to other cancer types. Although SP-B IgG levels were higher in serum and BAL of patients with ICI-P, given the less invasive nature of blood tests and the higher concentration of these antibodies in serum compared with BAL, we believe that measurement of serum SP-B IgG levels represents a more optimal profile as a biomarker for predictive screening. Future studies will be required to establish optimal cutoffs points for SP-B IgG. Combining measurement of SP-B IgG with other immune immunological mediators such as those identified here in our proteomics analysis would likely further improve the specificity and the sensitivity of such an assay. Our data show that the cooccurrence of SP-B IgG and SP-B–specific CD4$^+$ T cells is clearly associated with ICI-P. However, at present, identifying and quantifying antigen-specific T cells is not practical in a typical clinical setting; on the contrary, measuring antibody levels is rapid, noninvasive, and relatively inexpensive. The identification of an ICI-P risk biomarker would better equip clinicians to personalize upfront decision-making and would allow timely interventions as a result of the closer monitoring of the patient. Alternative treatment options could also be considered. Ultimately, such a biomarker could help to improve safety and tolerability for patients undergoing ICI treatment. In addition to providing a potential predictive marker for ICI-P, measuring SP-B IgG levels during treatment, when a suspicion of pneumonitis arises, could represent a useful diagnostic aid for ICI-P alongside conventional clinical tests for pneumonitis. Diagnosing ICI-P is currently a formidable challenge, and the detection of SP-B IgG autoantibodies during treatment may help in this difficult process. Future studies in larger cohorts will be required to validate the findings so far and determine the utility of SP-B as a biomarker for ICI-P risk stratification and ICI-P diagnosis. Nevertheless, the findings of this study shed light on immunological mechanisms and antigenic targets that likely mediate ICI-P, of which very little is known thus far, and provide a well-validated basis for the development of SP-B–related assays that may bring important clinical benefit to clinicians and patients. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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