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Corresponding Author:	Shohei Koyama Osaka University Suita, Osaka JAPAN
Corresponding Author's Institution:	Osaka University
Corresponding Author E-Mail:	koyama@imed3.med.osaka-u.ac.jp
Order of Authors:	Shohei Koyama
	Yujiro Naito
	Akio Osa
	Kentaro Masuhiro
	Takashi Hirai
	Atsushi Kuamnogoh
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TITLE:

Monitoring PD-1–Blocking Antibodies Bound to T Cells Derived from a Drop of Peripheral Blood

AUTHORS AND AFFILIATIONS:

Yujiro Naito[1], Akio Osa[1], Kentaro Masuhiro[1], Takashi Hirai[1], Shohei Koyama[1], Atsushi Kumanogoh[1]

[1]Department of Respiratory Medicine and Clinical Immunology, Osaka University Graduate School of Medicine, Osaka, Japan

Email addresses of co-authors:

Yujiro Naito (y.naito@imed3.med.osaka-u.ac.jp)
Akio Osa (akio_osa_20080622@yahoo.co.jp)
Kentaro Masuhiro (kmasuhiro0710@imed3.med.osaka-u.ac.jp)
Takashi Hirai (thirai@ent.med.osaka-u.ac.jp)
Atsushi Kumanogoh (kumanogo@imed3.med.osaka-u.ac.jp)

Corresponding author:

Shohei Koyama (koyama@imed3.med.osaka-u.ac.jp)

KEYWORDS:

PD-1–blocking antibodies, nivolumab, pembrolizumab, monitoring, binding status, blood drop, flow cytometry

SUMMARY:

We developed a simple flow cytometry assay for evaluating the binding of PD-1–blocking antibodies to T cells, requiring only a drop of peripheral blood from cancer patients.

ABSTRACT:

Immune checkpoint inhibitors, including PD-1–blocking antibodies, have significantly improved treatment outcomes in various types of cancer. The pharmacological efficacy of these immunotherapies is long lasting, extending even beyond the discontinuation of their injections, due to persistent blood concentrations. This means that immune-related adverse events (irAEs) caused by over-activation of the immune system can have extended durations as well. Since the clinical use of anti–PD-1 antibodies and the frequency of irAEs are increasing, it is becoming increasingly important to determine adequate strategies for immunosuppressive treatment to control irAEs, and to identify the optimal times to start subsequent therapies after PD-1 inhibitors. Here we developed a simple flow cytometry assay to evaluate the T cell binding status of the PD-1–blocking antibodies nivolumab and pembrolizumab. Like a glucose test, this assay requires just a single drop of peripheral blood. Visualizing antibody binding on T cells is more reliable than measuring antibody blood concentrations. In addition, if necessary, we can potentially analyze many distinctive immune-related markers on T cells bound to PD-1–blocking antibodies. Thus, this is a simple and minimally invasive strategy to analyze the pharmacological effect of PD-1–blocking antibodies in cancer patients.

INTRODUCTION:

PD-1–blocking antibodies have become the standard choice for treatment of various types of cancer, including non-small cell lung cancer (NSCLC)[1-4]. They show a remarkable therapeutic effect in a subset of cancer patients who have not responded to conventional cytotoxic chemotherapies. However, immune checkpoint inhibitors (ICIs), which include PD-1–blocking antibodies, can cause a unique and distinct spectrum of adverse events, termed immune-related adverse events (irAEs)[5]. Although irAEs can affect almost all tissues, they are most commonly observed in the gastrointestinal tract, endocrine glands, skin, and liver, and they can cause pruritus, rash, nausea, diarrhea, and thyroid disorders[6,7]. In general, most irAEs appear within 1 to 2 months after the initiation of ICIs. However, in some cases, they can occur later than 1 year after the beginning of treatment or even after treatment cessation[6,7]. They also cause various symptoms that may be difficult to discriminate from other pathologies. Thus, it can be challenging to promptly diagnose irAEs and treat them appropriately. irAEs can affect all tissues, and their onset is strongly influenced by circulating immune cells, especially T cells bound to PD-1–blocking antibodies. Therefore, a straightforward and minimally invasive method to monitor antibody-targeted T cells is important in clinical settings.

Here, we developed a simple method to assess the binding of PD-1–blocking antibodies to T cells using a drop of peripheral whole blood from cancer patients who received nivolumab or pembrolizumab. Using this approach, we were able to monitor each of the following: 1) the duration of antibody binding to T cells, 2) the occupancy of T cell PD-1 molecules by therapeutic antibodies, and 3) the activation status and immunological features of T cells. This method is a modification of a previously reported technique[8]. The amount of blood required is almost the same as that needed for a glucose test, and the approach does not require mononuclear cell enrichment or co-culturing with PD-1–blocking antibodies. We confirmed that this method can also be performed using frozen samples, including peripheral blood mononuclear cells (PBMCs) and cells from pleural effusion, pericardial effusion, bronchoalveolar lavage fluid, and cerebrospinal fluid, suggesting that this strategy may be useful in the context of a multicenter study. This method may facilitate the early diagnosis of irAEs, and also help to determine the appropriate immunosuppressive treatments to control their symptoms and to identify the optimal times to initiate subsequent therapies after PD-1 inhibitors.

PROTOCOL:

Sampling was performed during routine clinical procedures. All human samples were obtained after informed consent was provided by the subjects, in accordance with the Declaration of Helsinki and with the approval of the ethical review board of the Graduate School of Medicine, Osaka University, Japan (15383 and 752).

1. Whole blood sample preparation and staining

1.1. Collect whole blood samples into blood collection tubes containing ethylene diamine tetra-acetic acid (EDTA).

NOTE: Blood collection can be performed using either a regular needle or a blood lancet.

1.2. Transfer 20 µL of whole blood samples to 5 mL round-bottom polystyrene flow cytometry tubes.

NOTE: To reduce non-specific binding of cells to tubes, 1 mL of 2% fetal bovine serum (FBS) in phosphate buffered saline (PBS) is added into tubes and vortexed for 10 s before application to samples.

1.3. Add 20 µL of 2% FBS in PBS.

1.4. Add 10 µL of human-specific FcR blocking reagent. Mix well and incubate for 15 min at room temperature.

1.5. Add 500 µL of red blood cell lysis buffer. Mix well and incubate for 10 min at room temperature.

1.6. Add 4 mL of 2% FBS in PBS and spin down cells at 400 x *g* (1500 rpm) for 5 min at 4 °C. Remove supernatant by aspiration.

1.7. Repeat the wash and aspiration process described in step 1.6.

1.8. Re-suspend cells in 100 µL of 2% FBS in PBS and divide into two tubes of 50 µL each.

1.9. Add surface marker antibodies (**Table 1**). Mix well and incubate for 20 min at room temperature in the dark.

NOTE: When profiling the T cell immune status, the number of markers can be increased based on the flow cytometry machine quality.

1.10. Wash samples 2x as described in step 1.6.

1.11. Resuspend cells in 200 µL of 2% FBS in PBS.

2. Flow cytometric analysis

2.1. Insert tubes into the flow cytometer and acquire cells, basically following the recommended protocol[9].

2.2. Record 10,000 events as the lymphocyte gate (**Figure 1A**) and export flow data as .fcs files for analysis.

2.3. Open files in the analysis software. Visualize the cells on a forward scatter (FSC) (A) vs. side

scatter (SSC) (A) plot and gate lymphocytes (**Figure 1A**).

2.4. After selecting single cells using FSC (H) vs. FSC (W) and SSC (H) vs. SSC (W) (**Figure 1B**) and displaying them on a CD3 vs. CD8 or CD3 vs. CD4 plot, gate the CD8 T cells and CD4 T cells, respectively (**Figure 1C**).

2.5. After selecting the gated cells and displaying them on a PD-1 vs. human IgG4 plot, identify PD-1–blocking antibody–bound CD8 and CD4 T cells based on isotype control (**Figure 1D**).

REPRESENTATIVE RESULTS:

The gating strategy and flow cytometry analysis (**Figure 1**) can detect PD-1–blocking antibody binding to T cells obtained from a drop of NSCLC patient peripheral blood. Before PD-1–blocking antibody is administered, no human IgG4-positive CD8 or CD4 T cells are present, and PD-1 expression can be confirmed by a PD-1–detecting antibody (EH12.1) (**Figure 2A**). After nivolumab or pembrolizumab administration, IgG4 (nivolumab, pembrolizumab) can be detected on T cells by anti-IgG4 antibody (HP6025) whereas the PD-1–detecting antibody EH12.1 does not recognize any PD-1 on T cells because therapeutic PD-1 antibodies disturb EH12.1 binding. This means that we are indirectly measuring the therapeutic binding of PD-1–blocking antibody based on the lack of binding of PD-1–detecting antibody. Representative data show the different binding statuses of PD-1–blocking antibody (**Figure 2A**). Nivolumab and pembrolizumab binding and occupancy of PD-1 on T cells decrease over time[10], and there is partial binding (PB) and finally complete loss of binding (LB), the latter of which is shown in the double-positive area (**Figure 2B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Representative gating strategy to evaluate PD-1–blocking antibody binding to T cells from a drop of peripheral blood. (**A**) FSC (A) vs. SSC (A) plot and gating of lymphocytes. (**B**) Doublets are excluded by drawing gates around the main cell population on plots of FSC (H) vs. FSC (W) and SSC (H) vs. SSC (W). (**C**) CD3 vs. CD8 plot (upper) and CD3 vs. CD4 plot (lower) and gating of CD8 T cells and CD4 T cells, respectively. (**D**) PD-1 vs. human IgG4 plot and detection of the binding of nivolumab (a PD-1–blocking antibody) to CD8 and CD4 T cells. Orange dots and black dots indicate anti-IgG4 antibody and isotype control staining, respectively.

Figure 2: Representative flow cytometry analysis indicating the change in binding status of PD-1–blocking antibodies. (**A**) Staining of PD-1 and human IgG4 in CD8 T cells from ICI-pretreated patient blood was evaluated by flow cytometry (left). Ten microliters of pretreated blood was treated with serial dilution of nivolumab for 15 min, and steps 1.4 to 1.10 of the protocol were completed. Complete binding (CB) (red), partial binding (PB) (blue), and loss of binding (LB) (green) are defined by the indicated gates. (**B**) The status of PD-1–blocking antibody binding to CD8 T cells was analyzed at the follow-up time points, as indicated, in NSCLC patients who discontinued nivolumab and pembrolizumab.

Table 1: Antibodies used in flow cytometric analysis.

DISCUSSION:

In this article, we report a method using a flow cytometer to detect PD-1–blocking antibodies bound to T cells derived from a drop of peripheral blood, which we originally developed for nivolumab detection[10]. Although this technique is very simple and easy to perform, two important points should be noted in order to obtain accurate results. One is that to detect PD-1 molecules, an appropriate antibody that competes with nivolumab and pembrolizumab should be used. This issue was evaluated in a previous study[11]. The other is that RBC lysis should be performed thoroughly before surface staining. As long as an isotype control is established for each assay to determine the gate of the IgG4-positive cluster, the frequency is unaffected. However, with this protocol, when RBC lysis is insufficient there may be reductions of both the T cell count in the lymphocyte gate and of the intensity of each surface marker. In addition, the RBC lysis step must be performed before surface staining, otherwise the anti-IgG4 antibody (HP6025) does not function properly.

The limitation of this method is that the population of T cells bound to PD-1–blocking antibodies include specific T cell clones responsible for therapeutic effects and irAEs; however, the frequencies of these specific clones among the antibody-bound population are quite low. Therefore, we still need to enrich the specific target using certain markers, for instance CD39[12]. Another limitation is that the fluorescence intensity of IgG4 is not high in some cases, which makes it difficult to determine binding status (i.e., PB and LB).

Other studies have monitored therapeutic PD-1 antibodies in the blood to evaluate pharmacokinetics. Measuring the plasma concentration of nivolumab or pembrolizumab is essential to determine how the residual amount of these antibodies in blood correlates with time. However, we previously reported that the concentration of nivolumab does not completely correlate with residual binding to T cells[10]. Compared to the measurement of plasma antibody concentration[13], our method may be more appropriate for evaluating the residual binding of anti–PD-1 antibodies to T cells. The original method of monitoring nivolumab binding to T cells in blood required incubation with fluorescence-labeled nivolumab[8]. We simplified this approach and succeeded in substantially reducing the assay time and the amount of blood needed for analysis.

Frozen cells can also be analyzed using this assay, suggesting that this method is a good fit for multicenter studies. The usefulness of this approach will be further enhanced by combining monitoring binding status with other immunological markers, such as Ki-67, of T cells bound to PD-1–blocking antibodies[10]. Future studies should use our method in conjunction with single-cell RNA sequencing and T cell receptor sequencing to try to identify and characterize the specific subsets of T cells that are bound to PD-1–blocking antibodies and are responsible for irAEs.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

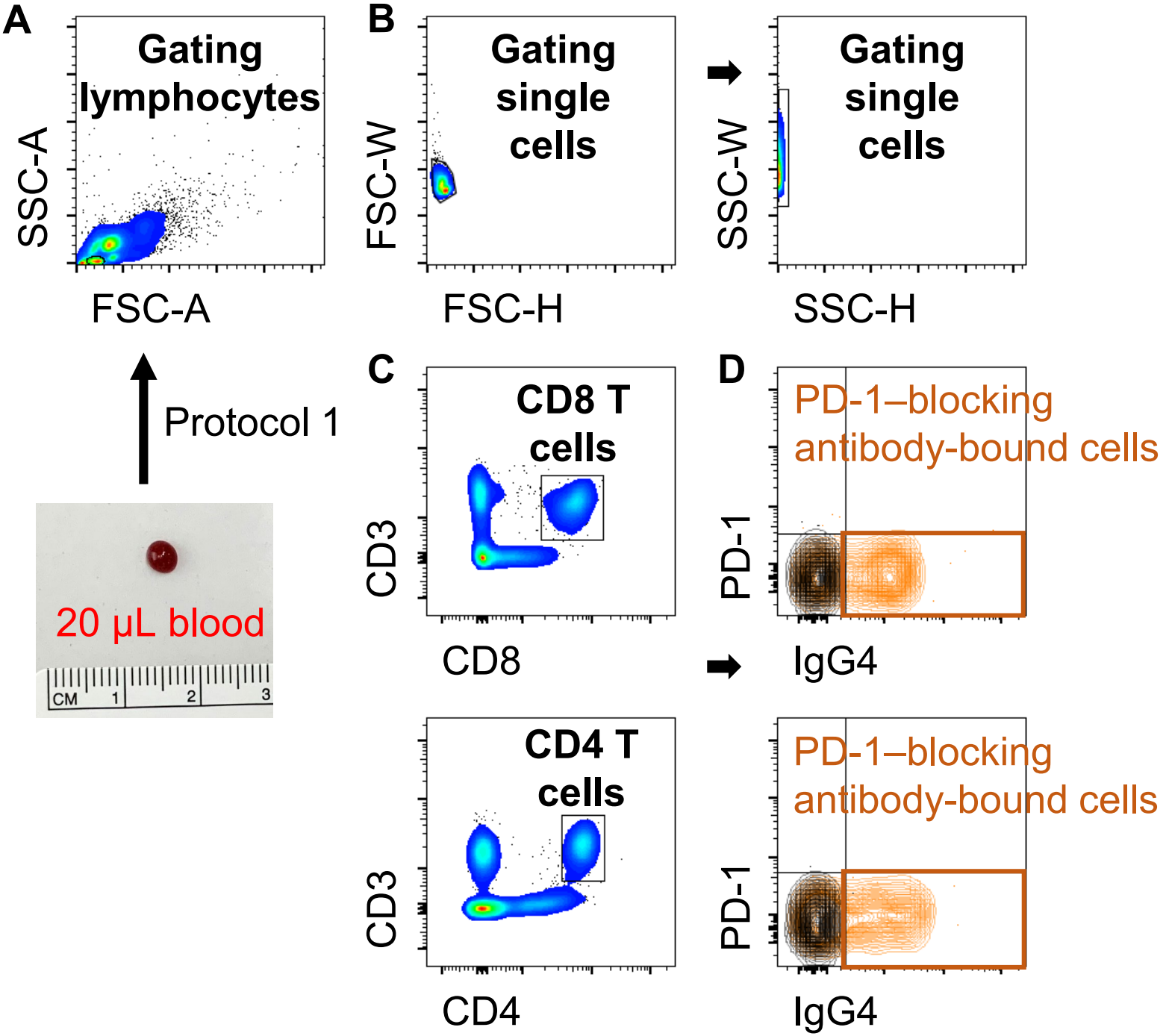
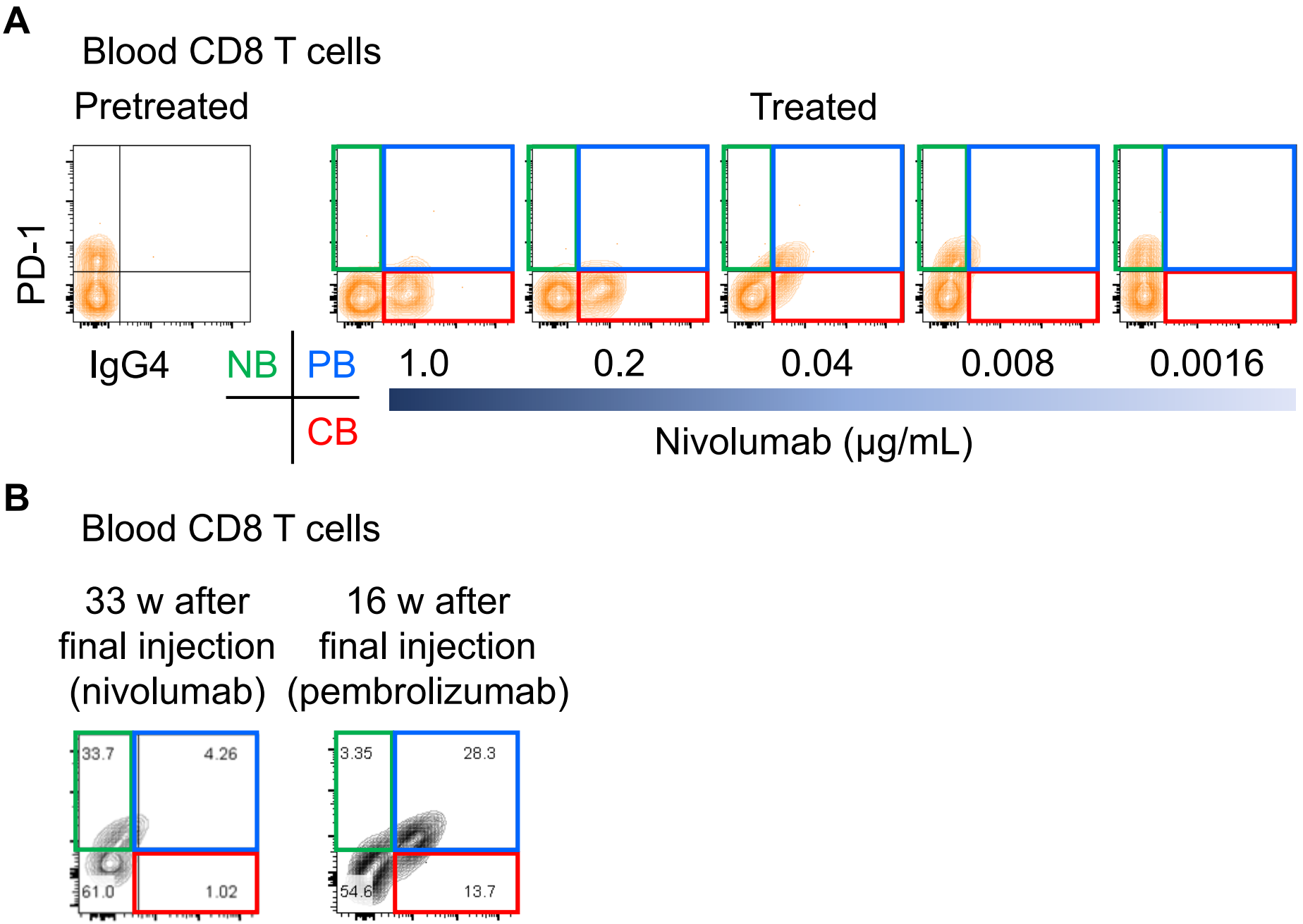


Figure 2



Table

	PE	BV421	PE-Cy7	APC-Cy7	BV510
Binding evaluation	IgG4	CD3	PD-1	CD4	CD8
Isotype control	Isotype control	CD3	Isotype control	CD4	CD8

Name of Material/Equipment	Company	Catalog Number	Comments/Description
10X RBC Lysis Buffer (Multi-species)	Thermo Fisher Scientific	00-4300-54	50 mL
APC/Cyanine7 anti-human CD4 Antibody	BioLegend	300518	Clone RPA-T4
BD FACS Canto II Flow Cytometer	BD		
Brilliant Violet 510 anti-human CD8a Antibody	BioLegend	301048	Clone RPA-T8
Dulbecco's Phosphate Buffered Saline	nacalai tesque	14249-95	500 mL
Falcon Round-Bottom Polystyrene Tubes	STEMCELL Technologies	352058	5 mL
FcR Blocking Reagent, human	Miltenyi Biotec	130-059-901	2 mL
FLOWJO	BD		
Gibco Fetal Bovine Serum	Thermo Fisher Scientific	12676029	500 mL
Mouse IgG1 monoclonal - Isotype control	abcam	ab81200	
Mouse monoclonal Anti-Human IgG4 Fc	abcam	ab99825	Clone HP6025
Pacific Blue Mouse Anti-Human CD3	BD	558117	Clone UCHT1
PE-Cy7 Mouse anti-Human CD279 (PD-1)	BD	561272	Clone EH12.1
PE-Cy7 Mouse IgG1 κ Isotype Control	BD	557646	

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CORRESPONDING AUTHOR

Name:	Shohei Koyama
Department:	Department of Respiratory Medicine and Clinical Immunology
Institution:	Osaka University Graduate School of Medicine
Title:	M.D., Ph.D.
Signature:	Shohei Koyama
Date:	July 27th, 2019

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Based on the editorial`s and reviewers' helpful comments, we revised the manuscript and figure and addressed the reviewers' concerns.

Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. Please use 'mL' and 'µL' instead of 'ml' and 'µl', including in figures. Please ensure there is a space between numbers and corresponding units (except %).

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Falcon, Miltenyi Biotec, Thermo Fisher Scientific, eBioscience, BD®, FACS Canto II, abcam®, Flowjo BD

We appreciate these helpful comments. We edited and corrected all relevant units and symbols.

Protocol:

1. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

2. For each protocol step, please ensure you answer the "how" question, i.e., how

is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Thank you for these comments. We added an ethics statement and revised protocols.

Figures and Tables:

1. Please remove the embedded figures and tables from the manuscript.

2. Please upload Table 1 as an .xls/.xlsx file in your Editorial Manager account.

3. Please upload one file per figure (2 in total). Please remove 'Figure 1' and 'Figure 2' from the figures themselves.

4. Figure 2B: Please use 'weeks' instead of 'w'.

In accordance with your comment, we edited the figures and manuscripts.

References:

1. Please do not abbreviate journal titles.

Thank you for this comment. We applied the JoVE EndNote style for references.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

2. Please remove trademark (™) and registered (®) symbols from the Table of Materials.

We edited the Table of Materials according to the editor's comment.

Reviewers' comments:

Reviewer #1:

JoVE60608

Summary:

In this manuscript the authors describe a simple method to identify immune related adverse events after anti-PD-1 therapy. This assay identifies the binding of PD-1 antibody to T cells and can performed using peripheral blood and as simple as glucose test. This assay is advantageous than measuring antibody concentrations as once can visualize the binding of the antibody to T cells and also can further measure other immune-related markers using flow cytometry. Overall this manuscript is a very interesting and timely. However, the following comments need to be addressed to improve the overall quality of the manuscript.

Specific comments:

1. A Lancet and lancet device for blood collection would be simpler than using a needle. 100-200 ul of blood can be obtained using a lancet.

We appreciate this helpful comment. We replaced the glucose test needle with a blood lancet.

2. Why does the authors perform FCR blocking prior to the RBC lysis?

Thank you for this helpful comment. We used eBioscience 10XRBC Lysis Buffer (Catalog Number: 00-4300). According to manufacturer's protocol, we originally performed Fc blocking and cell surface marker staining before RBC lysis. However, we found that this RBC lysis step quenched PE-conjugated anti-IgG4 antibody HP6025 from Abcam (ab99825). Therefore, we performed Fc blocking before RBC lysis, and surface staining after RBC lysis. We describe this in the discussion section.

3. The authors need to cite a study or perform experiments to confirm that therapeutic PD-1 antibodies block the EH12.1 PD-1 antibody binding

We cited "Zelba H et al Cancer Immunol Immunother. 2018 Dec;67(12):1845-1851" as reference 11.

4. The authors need to describe adequately that the they are measuring anti-PD-1 antibody binding based on no binding of PD-1 antibody. While this is acceptable, this is indirect way.

We appreciate this helpful comment. We added the following sentence to the representative results section: "This means that we are indirectly measuring the therapeutic binding of PD-1–blocking antibody based on the lack of binding of PD-1–detecting antibody."

5. Does this method work with all annti-PD-1 antibodies in the market.

We appreciate this helpful comment. We confirmed that PD-1 on T cells showed competitive binding with EH12.1 and both nivolumab and pembrolizumab, the latter of which are clinically available for lung cancer patients in Japan. Another study (reference 11) demonstrated that other commercially available PD-1–detecting antibodies also bind PD-1 competitively with nivolumab and pembrolizumab.

6. The authors need to cite studies which describe multicolor flowcytometry in detail (Bommareddy PK et al J Biol Methods. 2019;6(2)

We cited “Bommareddy PK et al J Biol Methods. 2019;6(2)” in the protocol section as reference 9.

Overall the manuscript is well written and interesting, these comments need to be addressed to improve the quality of the manuscript.

Reviewer #2:

Manuscript Summary:

Immune checkpoint inhibitors, including PD-1 blocking antibodies, are now widely used as immunotheraputics for multiple types of cancer because they have been show to be highly effective at improving outcomes. Blocking the PD-1 pathway

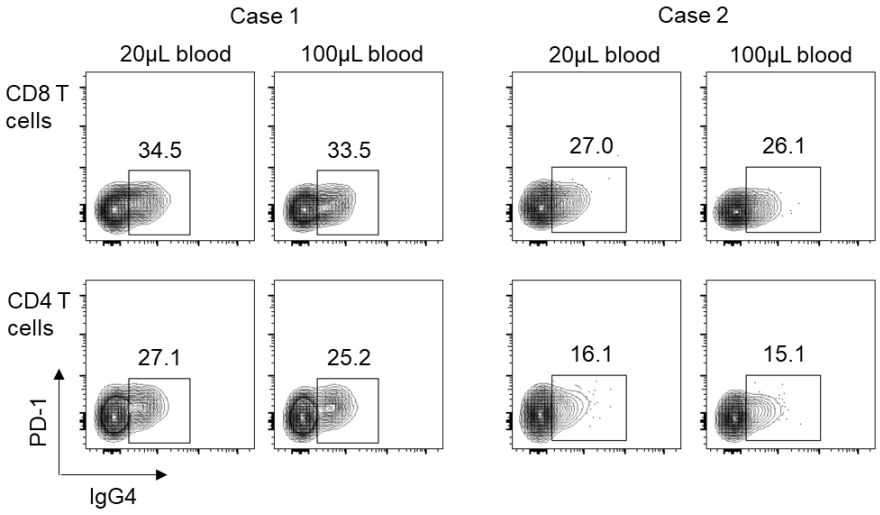
has been shown to increase the function and killing capacity of tumor specific T cells leading to enhanced clearance of cancer cells. However, the use of checkpoint inhibitors, including anti PD-1, are also associated with immune related adverse events and it has been shown that T cells bound to PD-1 blocking antibodies play an important role in the onset of these immune-related adverse events so determining the level of PD-1 on cell after blocking antibodies are given is potentially a way to facilitate the early diagnosis of irAEs and help determine the appropriate dosing of of anti PD-1 antibodies. This manuscript details a method to assess the level of anti PD-1 on T cell in the blood. It was designed as a screening test to determine the level of anti PD-1 binding in subject receiving PD-1 blocking antibodies as part of immunotherapy for various types of cancer in hopes that it will help predict irAEs. A rapid assay like this could be useful in may clinical setting.

Major Concerns:

Why use only 20 uls of blood? Is this representative and are the numbers of T cell analyzed sufficient to accurately determine the level of PD-1 binding. Using more blood would potentially make the assay more rigorous. Conversely shown data that larger volumes of blood provide the same information that 20uls does would be helpful. Only 10,000 events are collected which is fairly low for a flow cytometry-based assay.

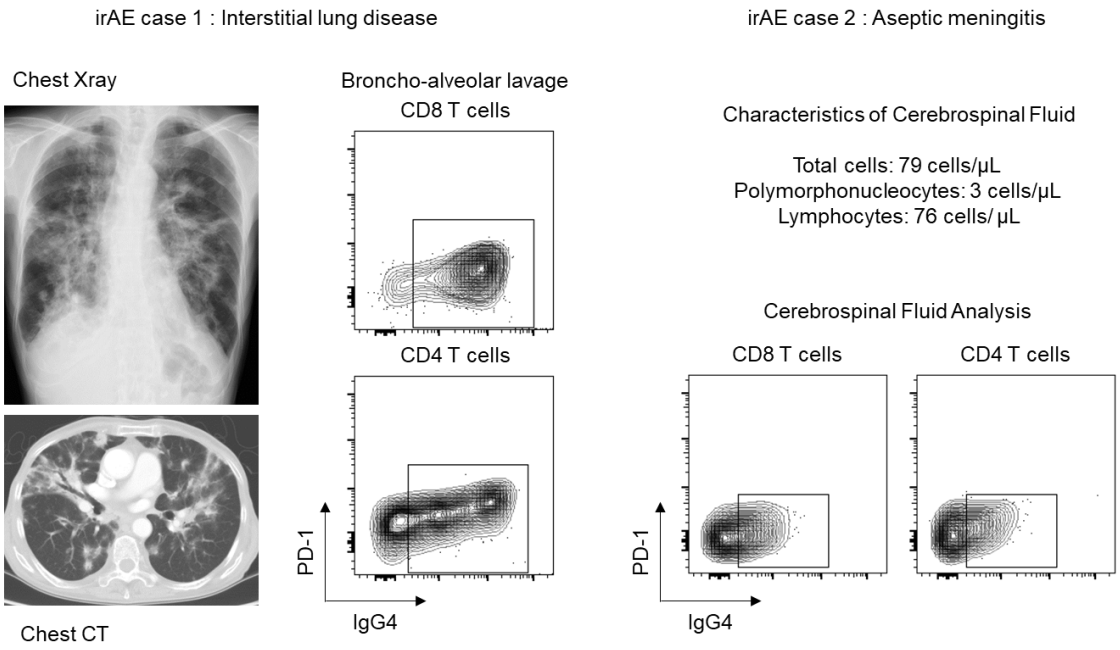
We appreciate this very helpful comment. It is true that larger volumes and cell counts can potentially make the assay more rigorous. Originally we evaluated 100 µL blood

samples according to the protocol of eBioscience 10XRBC Lysis Buffer (Catalog Number: 00-4300). However, titration assays confirmed that the frequency of antibody binding was no different between 20 µL and 100 µL. We also focused on reasonable antibody volumes and RBC lysis efficiency to simplify the assay while still retaining good accuracy.



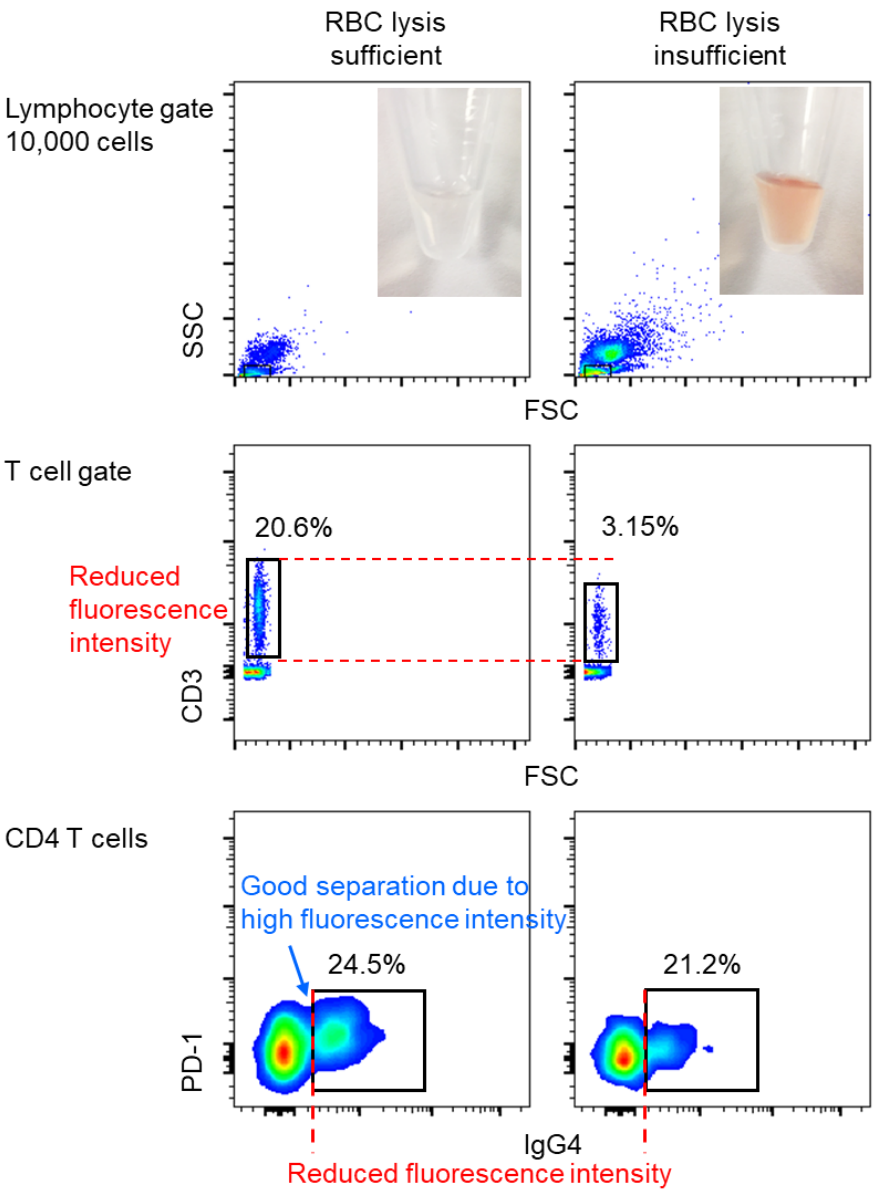
How well does PD-1 binding in the blood reflect PD-1 of T cells in tissues. All the irAEs occur in tissues and thus it is the most important site to assay.

Thank you for this comment. In cases of irAEs, specific types of tissues can be collected if biopsies can be performed. Although we do not have data from isolated biopsy samples, we were able to confirm antibody binding to T cells in bronchoalveolar lavage and cerebrospinal fluid from patients who developed interstitial lung disease and aseptic meningitis, respectively (data shown below). Because it is very important to explore this issue, future experiments will focus on biopsy samples.



The authors mention that the RBC lysis set is critical and that if not done properly the result are suspect. Including a control to ensure that the RBC lysis work appropriately would strengthen the test.

We appreciate this helpful comment. Basically, it is important to check if the macroscopic appearance of the solution is clear before staining. As long as an isotype control is established for each assay to determine the gate of the IgG4-positive cluster, the frequency is not affected. However, using this protocol, if RBC lysis is insufficient there may be reductions both in the T cell count in the lymphocyte gate and in the intensity of each surface marker. We included this explanation in the manuscript.



Some data that links this test with clinical outcomes would strengthen the manuscript. The authors report a similar test had been developed but it appears none of the authors on this paper were part of that study. It seems to me that they need to show this assay does indeed correlate with irAEs.

We appreciate this helpful comment. The authors of this manuscript mainly work on developing novel and useful analysis methods for clinical specimens. We also work together with physicians in our department to collect specimens. As described in our previous report (Osa et al JCI insight 2018, reference 10), we would like to apply this strategy to further evaluate the efficacy of PD-1–blocking antibodies and clarify the mechanism of irAE. irAE is a systemic disease, and collecting biopsy samples from affected tissues is sometimes difficult. At the same time, a subset of circulating T cells bound to PD-1–blocking antibody can recognize autoantigen because treatment triggers the onset of irAE. We confirmed that our method was applicable to liquid samples from a limited number of irAE cases. Future research will focus on applying this strategy to analyzing biopsy samples, combining with TCR sequences of antibody-bound T cells, and antigen screening using collected tissue samples.