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ORIGINAL RESEARCH

# Immune-related adverse events are clustered into distinct subtypes by T-cell profiling before and early after anti-PD-1 treatment

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## ABSTRACT

Although anti-programmed death-1 (PD-1) treatment has shown remarkable anti-tumor efficacy, immune-related adverse events (irAEs) develop with heterogeneous clinical manifestations. However, the immunological understanding of irAEs is currently limited. In the present study, we analyzed peripheral blood T cells obtained from cancer patients who received anti-PD-1 treatment to determine the immunological characteristics of irAEs. This study included 31 patients with refractory thymic epithelial tumor (TET) who were enrolled in a phase II trial of pembrolizumab (NCT02607631) and 60 patients with metastatic non-small cell lung cancer (NSCLC) who received pembrolizumab or nivolumab. T-cell profiling was performed by multi-color flow cytometry using peripheral blood obtained before treatment and 7 days after the first dose of anti-PD-1 antibodies. irAEs developed in 21 TET patients and 24 NSCLC patients. Severe (≥ grade 3) irAEs occurred in 7 TET patients (22.6%) and 6 NSCLC patients (10.0%). Patients with severe irAEs exhibited a significantly lower fold increase in the frequency of effector regulatory T (eTreg) cells after anti-PD-1 treatment, a higher ratio of T helper-17 (Th17) and T helper-1 cells at baseline, and a higher percentage of Ki-67$^+$ cells among PD-1$^+$CD8$^+$ T cells posttreatment. In clustering analysis using the T-cell parameters, patients with irAEs were grouped into four distinct subtypes: Th17-related, TNF-related, CD8-related Treg-compensated, and CD8-related Treg-uncompensated. The T-cell parameters showed a predictive value for the development of each subtype of severe irAEs. In conclusion, severe irAEs after anti-PD-1 treatment were clustered into four immunological subtypes, and potential biomarkers for early prediction of severe irAEs were proposed.

Immune checkpoint inhibitors (ICIs) have shown remarkable clinical benefit in patients with various types of cancer.[1,2] ICIs increase the activity of the immune system by blocking inhibitory signals of T-cell activation. Inhibitory immune checkpoint receptors, such as programmed death-1 (PD-1) and cytotoxic T lymphocyte antigen-4 (CTLA-4), serve important regulatory roles in self-tolerance. Therefore, ICIs can facilitate autoimmune and inflammatory responses.[3,4]

Anti-PD-1-treated patients may develop diverse forms of immune-related adverse events (irAEs). IrAEs commonly present as colitis, hepatitis, pneumonitis, endocrinopathies, arthritis, rash, pruritis, and fever, but may uncommonly involve the heart, kidney, and nervous system.[3,4] Autoantibodies,[5,6] tissue-infiltrating T cells,[7] cytokines,[8] early changes in B cells,[9] and the microbiome[10] have been proposed to be associated with irAEs, but the underlying pathophysiology of irAEs has not been fully elucidated. Such a broad spectrum of clinical presentation and associated factors suggests that irAEs are not caused by a single mechanism, but by heterogeneous mechanisms.

Most patients recover from irAEs with corticosteroids or immunosuppressive drugs, but severe cases can lead to treatment discontinuation, and even mortality.[4] For fatal cases, early detection and intervention are important for a proper management. However, clinically available biomarkers predicting severe irAEs have not been developed. A majority of severe irAEs occur as early as 1 or 2 months after starting anti-PD-1 treatment.[3,4] It indicates that irAE-related immunological changes may begin shortly after treatment, allowing the development of severe irAE-predictive biomarkers that are measured early after anti-PD-1 treatment.

In the present study, we performed a detailed assessment of the immunological parameters in patients treated with anti-PD-1 blocking antibodies to provide important insights into the underlying mechanisms that cause irAEs. Considering the crucial role of regulatory T (Treg) cells, T helper-17 (Th17) cells, and CD8$^+$ T cells in autoimmune and chronic inflammatory responses,[11–13] we

investigated the relative frequency and phenotype of different subsets of peripheral blood T cells in patients with non-small cell lung cancer (NSCLC) or thymic epithelial tumor (TET) who were treated with pembrolizumab or nivolumab. Early T-cell changes were analyzed 7 days posttreatment, when the proliferative response of CD8^{+} T cells has been shown to peak.[14,15] We clustered patients with irAEs into four distinct immunological subtypes according to peripheral blood T-cell parameters and suggest potential biomarkers for the prediction of severe irAEs.

## Results

### Patient characteristics

The baseline characteristics of the patients included in the study are summarized in Table 1. All patients with TET ($n$ = 31) received pembrolizumab, and 42 and 18 patients with NSCLC received pembrolizumab and nivolumab, respectively. None of the patients were treated previously with ICI agents. In addition, none of the patients had evidence of active autoimmune disease. One patient with thymoma had a prior history of myasthenia gravis but did not have any sign of active disease and did not receive immunosuppressive treatment for more than 1 year before administration of pembrolizumab.

### Peripheral blood T-cell profiling before and after anti-PD-1 treatment

We examined the phenotypes and relative frequency of subpopulations in peripheral blood CD8^{+} and CD4^{+} T cells at baseline and 7 days posttreatment. The gating strategies in flow cytometric analyses are presented in

Supplementary Figure S1. First, we examined PD-1^{+}CD8^{+} T cells for their expression of a proliferation marker (Ki-67) and activation markers (HLA-DR and CD38). The percentage of Ki-67^{+} cells (Figure 1A and B) and HLA-DR^{+}CD38^{+} cells (Figure 1C) among PD-1^{+}CD8^{+} T cells significantly increased after anti-PD-1 treatment in patients with TET and NSCLC. We also evaluated the relative frequency of CD4^{+}CD25^{+}CD127^{lo}FoxP3^{+} Treg cells and their subpopulations such as naïve Treg cells (CD45RA^{+}FoxP3^{lo}), effector Treg cells (eTreg or activated Treg cells; CD45RA^{−}FoxP3^{hi}), and non-suppressive cells (CD45RA^{−}FoxP3^{lo}) (Figure 1D).[16,17] Though the percentage of Treg cells among CD4^{+} T cells did not change with anti-PD-1 treatment (Figure 1E), the percentage of eTreg cells was significantly increased by anti-PD-1 treatment (figure 1F) in both TET and NSCLC patients. The percentage of naïve Treg cells or non-suppressive cells did not change (data not shown). We evaluated the production of effector cytokines by CD4^{+} T cells by intracellular cytokine staining following anti-CD3 stimulation (Figure 1G). In both TET and NSCLC patients, the percentage of IFN-γ^{+} (Figure 1H), IL-17A^{+} (Figure 1I), or TNF-α^{+} (Figure 1J) cells among CD4^{+} T cells did not significantly change with anti-PD-1 treatment. Similarly, the percentage of IFN-γ^{+} and TNF-α^{+} cells among CD8^{+} T cells did not significantly change (Figure 1K and L).

### Immune-related adverse events

At a median follow-up of 16.3 months (range, 6.5–28.9 months), irAEs developed in 45 patients (Table 2). Grade 1/2 irAEs developed in 14 TET patients (32.3%) and 18 NSCLC patients (30.0%), and severe (grade ≥3) irAEs developed in 7 TET patients (22.6%) and 6 NSCLC patients (10.0%). Some irAEs such as myasthenia gravis, myocarditis, and nephritis were only observed in TET patients (Table 2). The severe irAEs are summarized in Table 3. The median time to severe irAEs was 4 weeks (range, 1–28 weeks) and 11 patients developed severe irAEs within 3 months (Table 3). Treatment was discontinued in 11 patients due to severe irAEs and a median of two cycles (range, 1–24 cycles) were administered, whereas patients that did not develop severe irAEs received a median of seven cycles of treatment (range, 1–53 cycles). Eleven patients with severe irAEs fully recovered from the events by discontinuation of anti-PD-1 agents and administration of immunosuppressive agents. One NSCLC patient experienced grade 5 pneumonitis and one patient with TET died from superimposed cytomegalovirus infection during immunosuppressive treatment.

Baseline clinicopahological characteristics of patients with TET were not significantly different except that those who developed severe irAEs had significantly higher proportion of patients with thymoma and PD-L1-positive tumors compared to patients with grade 1/2 irAEs or no irAEs (Supplementary Table S1). No significant differences in the baseline clinicopathological characteristics were observed between patients with grade 1/2 irAEs, severe irAEs, or no irAEs, in the NSCLC cohort (Supplementary Table S2).

**Table 1.** Baseline characteristics of patients with thymic epithelial tumor (TET) and non-small cell lung cancer (NSCLC).

| Characteristic | TET ($n$ = 31) | NSCLC ($n$ = 60) |
|---|---|---|
| Age, median (range), yrs | 58 (26–78) | 65 (35–88) |
| Sex, $n$ (%) | | |
| Male | 20 (64.5) | 47 (78.3) |
| Female | 11 (35.5) | 13 (21.7) |
| ECOG performance status | | |
| 1 | 31 (100) | 47 (78.3) |
| 2 | 0 | 13 (21.7) |
| Histology of TET, $n$ (%) | | |
| Thymoma | 6 (19.4) | - |
| Thymic carcinoma | 25 (80.6) | - |
| Histology of NSCLC, $n$ (%) | | |
| Squamous | - | 30 (50.0) |
| Non-squamous | - | 30 (50.0) |
| Tumor burden, median (range), cm | 12.4 (1.7–27.0) | 6.7 (1.5–15.7) |
| Prior autoimmune disease, $n$ (%) | | |
| Myasthenia gravis | 1 (3.2) | 0 (0) |
| None | 30 (96.8) | 60 (100) |
| Number of prior chemotherapy line, median (range) | 2 (1–5) | 2 (0–9) |
| Anti-PD-1 agent | | |
| Pembrolizumab | 31 (100) | 42 (70.0) |
| Nivolumab | 0 | 18 (30.0) |
| PD-L1 status, $n$ (%) | | |
| ≥ 1% | 18 (58.0) | 38 (63.3) |
| < 1% | 7 (22.6) | 11 (18.3) |
| not available | 6 (19.4) | 11 (18.3) |

ECOG, Eastern Cooperative Oncology Group; PD-L1, programmed cell death ligand 1.

**Figure 1.** Changes in peripheral blood T cells following anti-PD-1 treatment.
Flow cytometric analysis was performed with PBMCs obtained before and 7 days after anti-PD-1 treatment from patients with TET ($n$ = 31) or NSCLC ($n$ = 60). (A) Representative plots of Ki-67 expression after gating for PD-1$^+$CD8$^+$ T cells in patients with TET (left panel) and NSCLC (right panel). (B) The percentage of Ki-67$^+$ and (C) HLA-DR$^+$CD38$^+$ cells among PD-1$^+$CD8$^+$ T cells pre- and posttreatment. (D) Representative plots of the subsets of Treg cells after gating for CD4$^+$ T cells in patients with TET (left panel) and NSCLC (right panel). (E) The percentage of total Treg (CD25$^+$CD127$^{lo}$FoxP3$^+$) and (F) eTreg (CD25$^+$CD127$^{lo}$CD45RA$^-$FoxP3$^{hi}$) cells among CD4$^+$ T cells pre- and posttreatment. (G) Representative plots of anti-CD3-stimulated production of IFN-γ and IL-17A after gating for CD4$^+$ T cells from patients with TET (left panel) and NSCLC (right panel). (H-L) The percentage of IFN-γ$^+$ (H and K), IL-17A$^+$ (I), and TNF-α$^+$ (J and L) cells among CD4$^+$ (H-J) or CD8$^+$ (K and L) T cells pre- and posttreatment. Statistical analyses were performed using paired t-test. ns, not significant. *$P$ < .05, **$P$ < .01, ****$P$ < .0001.

In the combined cohort of TET and NSCLC patients, the development of grade 1/2 or severe irAEs, was not significantly associated with objective response rate (ORR; Supplementary Table S3), PFS (Supplementary Figure S2A), and OS (Supplementary Figure S2B). Among patients with TET, the development of grade 1/2 or severe irAEs was not predictive of superior ORR (Supplementary Table S3), PFS (Supplementary Figure S2C), or OS (Supplementary Figure S2D). However, in NSCLC patients, the development of grade 1/2 irAEs was associated with significantly higher ORR (Supplementary Table S3) and PFS (Supplementary Figure S2E), but not OS (Supplementary Figure S2F) compared to patients who did not develop irAEs. However, patients with severe irAEs did not exhibit a significant difference in ORR, PFS, and OS with patients who did not experience irAEs (Supplementary Table S3 and Supplementary Figures S2A-F).

Table 2. Immune-related adverse events in patients with TET and NSCLC.

| Immune-related adverse events | TET ($n$ = 31) | | NSCLC ($n$ = 60) | |
|---|---|---|---|---|
| | Grade 1/2 | Grade ≥ 3 | Grade 1/2 | Grade ≥ 3 |
| Hepatitis | 0 (0) | 3 (9.7) | 2 (3.2) | 1 (1.7) |
| Myasthenia gravis | 1 (3.2) | 1 (3.2) | 0 (0) | 0 (0) |
| Myocarditis | 0 (0) | 2 (6.5) | 0 (0) | 0 (0) |
| Hypothyroidism | 3 (9.7) | 1 (3.2) | 3 (5.0) | 2 (3.2) |
| Colitis | 0 (0) | 1 (3.2) | 1 (1.7) | 0 (0) |
| Nephritis | 1 (3.2) | 1 (3.2) | 0 (0) | 0 (0) |
| Subacute myoclonus | 0 (0) | 1 (3.2) | 0 (0) | 0 (0) |
| Pneumonitis | 6 (19.4) | 0 (0) | 1 (1.7) | 3 (5.0) |
| Pruritis | 4 (12.9) | 0 (0) | 11 (18.3) | 0 (0) |
| Rash | 4 (12.9) | 1 (3.2) | 8 (13.3) | 0 (0) |
| Arthritis | 1 (3.2) | 0 (0) | 0 (0) | 0 (0) |
| Sensory neuropathy | 2 (6.5) | 0 (0) | 0 (0) | 0 (0) |
| Fever | 1 (3.2) | 0 (0) | 0 (0) | 0 (0) |

### Peripheral blood T-cell parameters that correlate with severe irAEs

Next, we attempted to find T-cell parameters correlating with grade 1/2 irAEs or severe irAEs in each cohort of patients with TET and NSCLC. Parameters exhibiting both a significant difference among patients with and without irAEs and a significant association with irAEs in the univariate logistic regression analysis were selected. None of the T-cell parameters were significantly associated with the development of grade 1/2 irAEs in the total cohort (Supplementary Table S4 and S7), TET cohort (Supplementary Table S5 and S7), or NSCLC cohort (Supplementary Table S6 and S7). Next, we compared the patients with or without severe irAEs. In the total cohort, percentage of Ki-67$^+$ among PD-1$^+$CD8$^+$ T cells posttreatment (Ki-67$_{post}$; odds ratio [OR] 1.06, 95% confidence interval [CI] 1.01–1.11, $P$ = .027), fold change in eTreg frequency posttreatment (eTreg$_{post/pre}$; OR 0.14, 95% CI 0.03–0.75, $P$ = .021), percentage of posttreatment Treg cells (OR 0.71, 95% CI 0.52–0.97, $P$ = .032) and eTreg cells (OR 0.45, 95% CI 0.23–0.89, $P$ = .022) among CD4$^+$ T cells were significantly associated with development of severe irAEs (Supplementary Table S8 and S11). eTreg$_{post/pre}$, percentage of posttreatment Treg cells, and eTreg cells are correlated and therefore, we used eTreg$_{post/pre}$ for further analysis due to its strongest association with the development of severe irAEs (Supplementary Table S11). In TET patients, eTreg$_{post/pre}$ (OR 0.06, 95% CI 0.00–0.95, $P$ = .046; Supplementary Table S9 and S11, Figure 2A and B) and baseline ratio of IL-17A$^+$ cells and IFN-γ$^+$ cells among CD4$^+$ T cells (Th17/Th1$_{pre}$; OR 6.52, 95% CI 1.09–39.22, $P$ = .040; Supplementary Table S9 and S11, Figure 2C and D) were significantly associated with the development of severe irAEs. In NSCLC patients, Ki-67$_{post}$ (OR 1.12, 95% CI 1.03–1.22, $P$ = .008; Supplementary Table S10 and S11, Figure 2E and F) was significantly associated with the development of severe irAEs.

### Clustering of severe irAEs into distinct subtypes

Because eTreg$_{post/pre}$, Th17/Th1$_{pre}$, and Ki-67$_{post}$ were identified as T-cell parameters correlating with severe irAEs, we performed unsupervised clustering analysis with these parameters in the 13 patients with severe irAEs. In this first-round

clustering analysis, we found that the patients with severe irAEs were clustered into four distinct subgroups: Th17-related, CD8-related, Treg-related, and not otherwise specified (NOS) (Figure 3A). The Th17-related subgroup had a high Th17/Th1$_{pre}$ value (Figure 3B), the CD8-related subgroup had a high Ki-67$_{post}$ value (Figure 3C), the Treg-related subgroup had a low eTreg$_{post/pre}$ ratio (Figure 3D), and the NOS subgroup had none of the three risk parameters. We further analyzed the T-cell parameters listed in Supplementary Table S2 and S3 to find risk parameters associated with the NOS subgroup. The posttreatment percentage of TNF-α$^+$ cells among CD4$^+$ (CD4TNF$_{post}$; Figure 3E) and CD8$^+$ (CD8TNF$_{post}$; Figure 3F) T cells were significantly higher in the NOS subgroup than in all other patients.

Finally, we performed clustering analysis again with the five significant parameters, including eTreg$_{post/pre}$, Th17/Th1$_{pre}$, Ki-67$_{post}$, CD4TNF$_{post}$, and CD8TNF$_{post}$, in the 13 patients with severe irAEs. Re-clustering analysis revealed four distinct subgroups: Th17-related (high Th17/Th1$_{pre}$), TNF-related (high CD4TNF$_{post}$ and CD8TNF$_{post}$), Treg-related (low eTreg$_{post/pre}$), and CD8-related (high Ki-67$_{post}$) (Figure 3G and Table 3). Each risk parameter was specific to each subgroup, and none of the subgroups had multiple features of high Th17/Th1$_{pre}$, high CD4TNF$_{post}$ and CD8TNF$_{post}$, low eTreg$_{post/pre}$, or high Ki-67$_{post}$. Of note, the Th17-related and Treg-related subgroups were restricted to patients with TET, and the TNF-related subgroup mostly consisted of NSCLC patients (Figure 3G). PFS and OS of the patients of the different subgroups of severe irAEs did not significantly differ (Supplementary Figure S3).

### Clustering analysis of patients with all-grade irAEs

Considering the distinct properties of different subgroups of severe irAEs, we reasoned that this may be also the case with grade 1/2 irAEs, and further performed clustering analysis using the five T-cell parameters, eTreg$_{post/pre}$, Th17/Th1$_{pre}$, Ki-67$_{post}$, CD4TNF$_{post}$, and CD8TNF$_{post}$, in the 45 patients with all-grade irAEs (Figure 4A). Including all-grade irAEs showed a similar subgroup pattern (Figure 4A) compared to the analysis that included only the severe cases (Figure 3G). The irAEs included in each subgroup are summarized in Supplementary Table S12. The TNF-related subgroup exhibited a higher value of CD4TNF$_{post}$ and CD8TNF$_{post}$ compared to other subgroups and patients without irAEs (Figure 4A and B). The Th17-related subgroup exhibited a higher value of Th17/Th1$_{pre}$ compared to other subgroups and patients without irAEs (Figure 4A and C). Patients with irAEs not included neither in the TNF-related nor the Th17-related cluster exhibited a higher value of Ki-67$_{post}$ (Figure 4A and D), which is similar to the CD8-related subgroup found in the clustering analysis with severe irAEs (Figure 3G). In addition, we found two subclusters in the CD8-related subgroup which are the Treg-compensated subgroup and Treg-uncompensated subgroup (Figure 4A). The Treg-compensated subgroup had a significantly higher eTreg$_{post/pre}$ value compared to the Treg-uncompensated subgroup and other subgroups (Figure 4E). The ratio of Ki-67$_{post}$ and eTreg$_{post/pre}$ (Ki-67$_{post}$/eTreg$_{post/pre}$) was significantly higher in the Treg-uncompensated subgroup than the Treg-compensated

**Table 3.** Characteristics of patients who experienced severe immune-related adverse events (irAEs).

| Category | Age/Sex | Histology | Immune-related adverse events | Th17/Th1$_{pre}$ | eTreg$_{post/pre}$ | Ki-67$_{pre}$ | CD8TNF$_{post}$ | CD4TNF$_{post}$ | Preexisting autoimmune syndrome | Anti-PD-1 agent | Time to irAEs (wks) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Th17-related | 60/F | Thymoma (B3) | G4 Glomerulonephritis | 2.0 | 1.7 | 18.0 | 0.2 | 7.4 | - | Pembrolizumab | 13 |
| | 64/F | Thymoma (B2) | G4 Myocarditis, G3 hypothyroidism, G3 hepatitis | 1.0 | 1.2 | 8.7 | 6.9 | 6.5 | - | Pembrolizumab | 4 |
| TNF-related | 51/F | Thymoma (B2) | G4 Myocarditis | 1.4 | 1.1 | 14.4 | 3.0 | 3.7 | - | Pembrolizumab | 4 |
| | 65/M | Squamous cell ca. | G3 Pneumonitis | 0.04 | 1.1 | 11.3 | 27.1 | 7.8 | - | Pembrolizumab | 4 |
| | 72/M | Squamous cell ca. | G3 Hypothyroidism | 0.08 | 1.2 | 23.3 | 22.3 | 18.4 | - | Nivolumab | 25 |
| | 57/M | Squamous cell ca. | G5 Pneumonitis | 0.05 | 1.0 | 20.7 | 13.8 | 11.2 | - | Pembrolizumab | 4 |
| | 43/M | Thymic carcinoma | G3 Hepatitis | 0.2 | 0.7 | 10.3 | 22.5 | 21.0 | - | Pembrolizumab | 4 |
| | 88/M | Squamous cell ca. | G3 Hypothyroidism | 0.003 | 1.2 | 13.1 | 18.6 | 28.4 | - | Nivolumab | 5 |
| Treg-related | 26/M | Thymic carcinoma | G4 Myasthenia gravis | 0.5 | 0.4 | 22.6 | 2.2 | 8.2 | - | Pembrolizumab | 4 |
| (or CD8-related Treg-uncompensated) | 66/F | Thymoma (B2) | G4 Hepatitis, G3 colitis | 0.003 | 0.7 | 24.6 | 0.2 | 7.4 | Myasthenia gravis | Pembrolizumab | 1 |
| CD8-related | 52/M | Adenocarcinoma | G3 Pneumonitis | 0.3 | 0.9 | 38.9 | 3.5 | 8.6 | - | Pembrolizumab | 4 |
| (or CD8-related Treg-uncompensated) | 59/M | Thymic carcinoma | G3 Subacute myoclonus | 0.3 | 1.4 | 34.0 | 3.4 | 5.6 | - | Pembrolizumab | 28 |
| | 66/M | Squamous cell ca. | G3 Hepatitis | 0.4 | 1.2 | 40.9 | 4.0 | 4.4 | - | Nivolumab | 1 |

Th17/Th1$_{pre}$, ratio of Th17 and Th1 cells pre-treatment; eTreg$_{post/pre}$, fold change of the frequency of eTreg cells posttreatment; Ki-67$_{post}$, percentage of Ki-67$^+$ cells among PD-1$^+$CD8$^+$ T cells posttreatment; CD8TNF$_{post}$, percentage of TNF-α$^+$ cells among CD8$^+$ T cells posttreatment; CD4TNF$_{post}$, percentage of TNF-α$^+$ cells among CD4$^+$ T cells posttreatment.
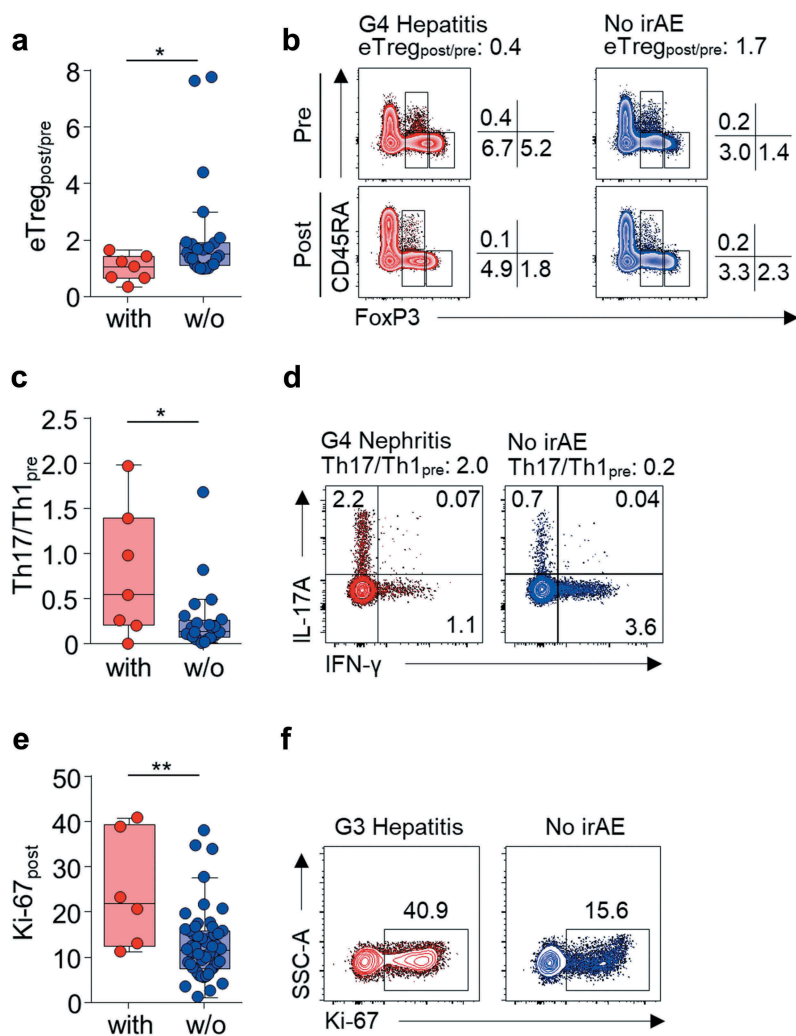
**Figure 2.** Severe irAE-associated peripheral blood T-cell parameters.

(A) Fold change in the percentage of eTreg cells among CD4$^+$ T cells after anti-PD-1 treatment (eTreg$_{post/pre}$) in TET patients with ($n = 7$; red) and without ($n = 24$; blue) severe irAEs. (B) Representative plots of the subsets of Treg cells after gating for CD4$^+$ T cells in a TET patient that experienced G4 hepatitis and a representative patient who did not experience irAEs. (C) Th17 (IL-17A$^+$CD4$^+$ T cells) to Th1 (IFN-γ$^+$CD4$^+$ T cells) ratio pre-treatment (Th17/Th1$_{pre}$) in TET patients with ($n = 7$; red) and without ($n = 24$; blue) severe irAEs. (D) Representative plots of IFN-γ and IL-17A production after gating for CD4$^+$ T cells in a patient who developed G4 nephritis and a representative patient who did not experience irAEs. (E) The percentage of Ki-67$^+$ cells among PD-1$^+$CD8$^+$ T cells posttreatment (Ki-67$_{post}$) in NSCLC patients with ($n = 6$; red) and without ($n = 54$; blue) severe irAEs. (F) Representative plots of Ki-67$^+$ expression after gating for PD-1$^+$CD8$^+$ T cells in a patient who developed G3 hepatitis and a representative patient who did not experience irAEs. The box plot represents the IQR with the horizontal line indicating the median. Whiskers extend to a maximum of 1.5 × IQR beyond the box. Statistical analyses were performed using Mann–Whitney U test. *$P < .05$, **$P < .01$.

subgroup, other subgroups, and patients without irAEs (Figure 4f). However, Ki-67$_{post}$/eTreg$_{post/pre}$ was not significantly different between the Treg-compensated subgroup and other subgroups or patients without irAEs (Figure 4F). No severe cases were included in the Treg-compensated subgroup, while five of the seven patients in the Treg-uncompensated subgroup experienced severe irAEs. PFS and OS were not significantly different among the patients of the different subgroups of irAEs (Supplementary Figure S4).

### Predictive values of T-cell parameters for each subgroup of irAEs

Since high CD4TNF$_{post}$ and CD8TNF$_{post}$, high Th17/Th1$_{pre}$, high Ki-67$_{post}$ features, and Ki-67$_{post}$/eTreg$_{post/pre}$, were only present in the corresponding Th17-related, TNF-related, CD8-related, and CD8-related Treg-uncompensated

subgroups, respectively, we evaluated predictive values of each parameter in predicting each subgroup of irAEs.

CD4TNF$_{post}$ and CD8TNF$_{post}$ significantly predicted all-grade or severe TNF-related irAEs (Figure 5A and B). In addition, Th17/Th1$_{pre}$ significantly predicted all-grade or severe Th17-related irAEs (Figure 5C). Ki-67$_{post}$ was predictive of both CD8-related Treg-compensated and CD8-related Treg-uncompensated irAEs (Figure 5D), but Ki-67$_{post}$/eTreg$_{post/pre}$ only significantly predicted CD8-related Treg-uncompensated irAEs (Figure 5E).

### Discussion

In this study, we demonstrated that the development of irAEs is associated with the baseline T-cell profile or early T-cell response after anti-PD-1 therapy. In addition, we showed the heterogeneity of irAEs based on the immune phenotype of the
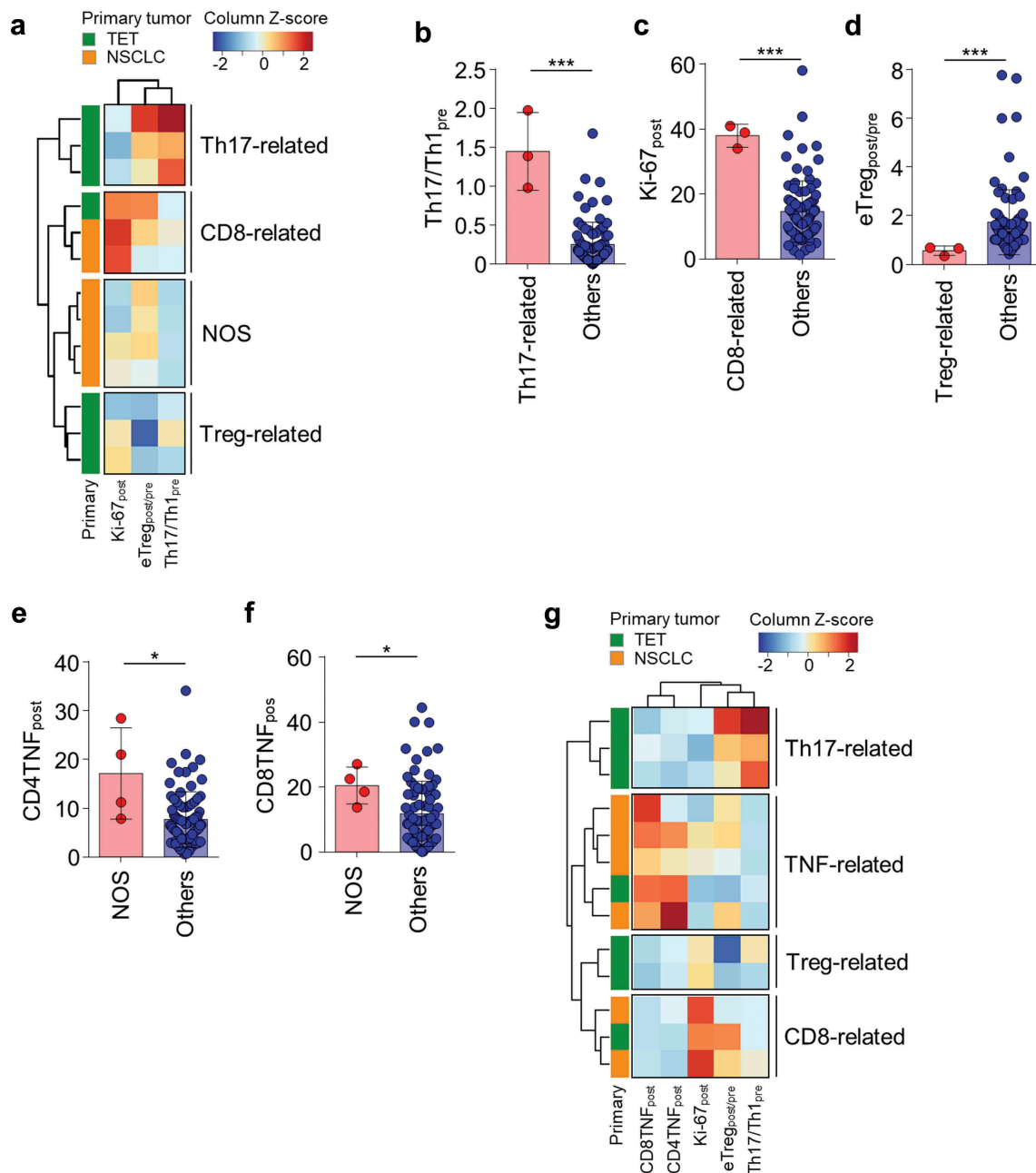
**Figure 3.** Clustering of patients with severe irAEs based on peripheral blood T-cell parameters.

(A) Unsupervised hierarchical clustering using three T-cell parameters (Th17/Th1$_{pre}$, Ki-67$_{post}$, and eTreg$_{post/pre}$) in 13 patients with severe irAEs (1 patient/row). The z-scores are column normalized. (B) Th17/Th1$_{pre}$ in the Th17-related subgroup ($n = 3$) and all patients other than the Th17-related subgroup ($n = 88$), (C) Ki-67$_{post}$ in the CD8-related subgroup ($n = 3$) and all patients other than the CD8-related subgroup ($n = 88$), and (D) eTreg$_{post/pre}$ in the Treg-related subgroup (n = 3) and all patients other than the Treg-related subgroup ($n = 88$). (E) The percentage of TNF-α$^+$ cells among CD4$^+$ (CD4TNF$_{post}$) or (F) CD8$^+$ (CD8TNF$_{post}$) T cells in the NOS subgroup ($n = 4$) and all patients other than the NOS subgroup ($n = 87$). (G) Unsupervised hierarchical clustering using five T-cell parameters (eTreg$_{post/pre}$, Th17/Th1$_{pre}$, Ki-67$_{post}$, CD4TNF$_{post}$, and CD8TNF$_{post}$) in the 13 patients with severe irAEs (1 patient/row). The z-scores are column normalized. Error bars indicate standard deviation of the mean. Statistical analyses were performed using Mann–Whitney U test (B-F). *$P < .05$, ***$P < .001$.

patients. Four clusters of patients with irAEs were identified with distinct features of peripheral blood T-cell phenotypes.

The Th17-related subgroup was characterized by a high baseline Th17 to Th1 ratio. The role of Th17 cells has been demonstrated in multiple autoimmune diseases.[12,18] Th17 cells secrete a highly inflammatory cytokine, IL-17A, which plays protective roles in host defense against bacterial and fungal pathogens at epithelial and mucosal barriers. Dysregulated production of IL-17A can result in

autoimmunity and tissue damage. In addition, previous studies have suggested that the balance between Th17 and Th1 is associated with autoimmune disease.[19,20] Recently, an IL-17A blocking antibody was developed and introduced for the treatment of psoriasis,[21] psoriatic arthritis,[22] and ankylosing spondylitis.[23] Intriguingly, IL-17A blocking antibody was also tried in the management anti-PD-1-induced irAEs.[24,25]

In the TNF-related subgroup, the percentage of TNF-α$^+$ cells among the CD4$^+$ and CD8$^+$ T cell populations was typically high
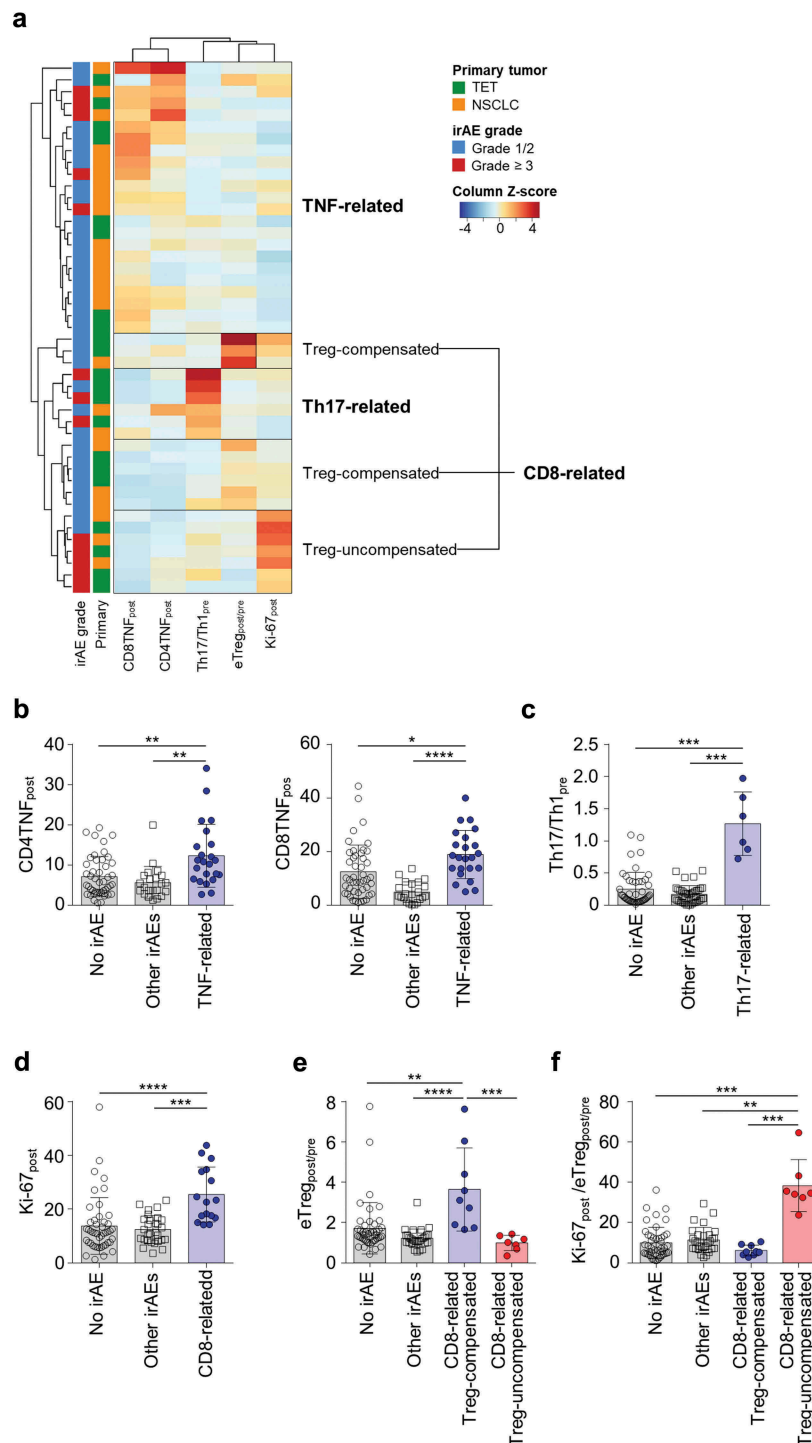
**Figure 4.** Clustering of patients with any grade irAEs based on peripheral blood T-cell parameters.

(A) Unsupervised hierarchical clustering using five T-cell parameters (eTreg$_{post/pre}$, Th17/Th1$_{pre}$, Ki-67$_{post}$, CD4TNF$_{post}$, and CD8TNF$_{post}$) in the 45 patients with any grade irAEs (1 patient/row). The z-scores are column normalized. Patients with grade 1/2 irAEs are indicated in blue and patients with grade ≥3 irAEs are indicated in red. Patients were clustered into four subgroups; TNF-related, Th17-related, CD8-related Treg compensated, and CD8-related Treg non-compensated. (B) CD4TNF$_{post}$ or CD8TNF$_{post}$ in patients with no irAE (n = 46), other irAEs (Th17-related and CD8-related; n = 22), and TNF-related irAEs (n = 23). (C) Th17/Th1$_{pre}$ in patients with no irAE (n = 46), other irAEs (TNF-related and CD8-related; n = 39), and Th17-related irAEs (n = 6). (D-F) Ki-67$_{post}$ (D), eTreg$_{post/pre}$ (E), ratio of Ki-67$_{post}$ and eTreg$_{post/pre}$ (Ki-67$_{post}$/eTreg$_{post/pre}$) (F), in patients with no irAE (n = 46), other irAEs (TNF-related and Th17-related; n = 29), CD8-related Treg-compensated (n = 11), and CD8-related Treg-uncompensated irAEs (n = 7). Error bars indicate standard deviation of the mean. Statistical analyses were performed using Kruskal–Wallis test and post-hoc analysis by Dunn's test (B-F). *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$.

posttreatment. The role of TNF-α in autoimmune disease has been well described, and TNF-α blockers are currently used in clinics.[26] Infliximab, a TNF-α blocking antibody drug, is also recommended for the management of severe irAE patients who do not respond to corticosteroids.[27] Clinical identification of TNF-related patients with severe irAEs will be important for the appropriate management of patients using TNF-α blockers, such as infliximab.
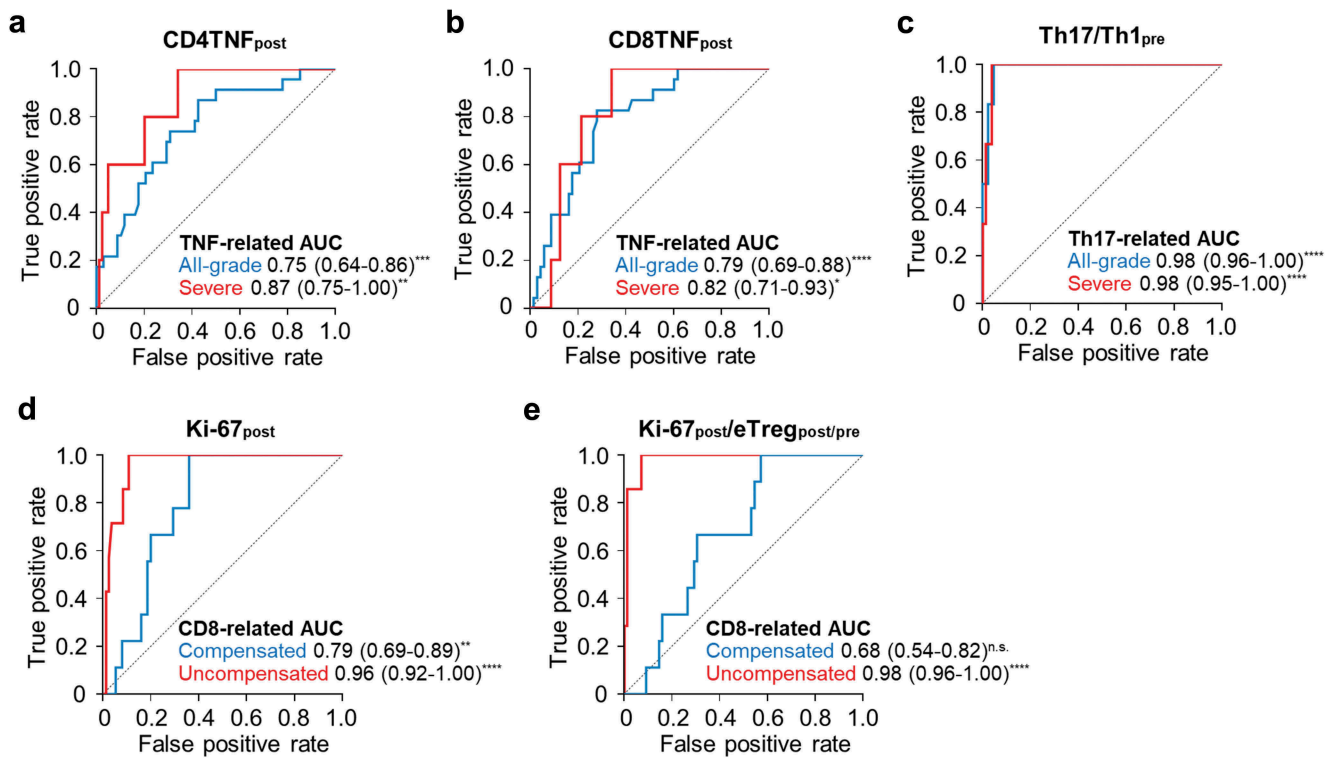
**Figure 5.** Predictive values of T-cell parameters for each subgroup of irAEs.
Receiver operating characteristic curve of T-cell parameters predicting each subgroup of irAEs. (A) Th17/Th1$_{pre}$ predicting Th17-related any grade (blue) or severe (red) irAEs. (B,C) CD4TNF$_{post}$ (B) and CD8TNF$_{post}$ (C) predicting TNF-related any grade (blue) or severe (red) irAEs. (D,E) Ki-67$_{post}$ (D) and Ki-67$_{post}$/eTreg$_{post/pre}$ (E) predicting CD8-related Treg-compensated (blue) or Treg-uncompensated (red) irAEs. Area under the curve (AUC) values are presented on the right bottom of each panel and 95% confidence interval values are described in the parenthesis. ns not significant, *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$.

In the CD8-related subgroup, the percentage of Ki-67$^+$ cells among PD-1$^+$CD8$^+$ T cells was typically high posttreatment. PD-1 blockade may enhance the activation of not only tumor antigen-specific CD8$^+$ T cells, but also autoreactive CD8$^+$ T cells, resulting in breaking self-tolerance. Recent reports have shown abundant CD8$^+$ T cell infiltration in the affected organs of patients who experienced irAEs after anti-PD-1 treatment or combined immune checkpoint blockade.[7,28] Future studies evaluating the infiltration of CD8$^+$ T cells into the affected organs of patients in the CD8-related subgroup may further confirm the role of CD8$^+$ T cells in these patients.

Wtihin the CD8-related subgroup, we found two distinct subtypes according to the fold increase of eTreg frequency (eTreg$_{post/pre}$). The first subtype, Treg-compensated group, exhibited a high eTreg$_{post/pre}$ value and consisted of patients with only grade 1 or 2 irAEs. However, the second subtype, Treg-uncompensated group, did not show an increase in eTreg frequency following treatment and exhibited a significantly higher Ki-67$_{post}$ to eTreg$_{post/pre}$ ratio (Ki-67$_{post}$/eTreg$_{post/pre}$) compared to other subgroups. Most of the patients in the Treg-uncompensated subgroup experienced severe irAEs. eTreg cells are highly suppressive activated Treg cells,[29] and their frequency was significantly increased 1-week after anti-PD-1 treatment. Although the precise mechanism of the early increase in the frequency of eTreg cells is unknown, we hypothesize that it may be a counter-mechanism to balance the increased activity of

effector T cells after PD-1 blockade. Considering the suppressive role of eTreg cells, the decrease or poor increase in this population can be assumed to break immune homeostasis and facilitate autoimmune responses.

Some of the irAEs such as myocarditis were only present in a specific subgroup, while hepatitis, pneumonitis, and hypothyroidism were present in multiple subgroups. Due to the small number of events we were not able to conclude whether a specific irAE is associated with a particular immunological subgroup. However, irAEs present in multiple subgroups implies that the underlying immunological mechanisms may be different even in patients with similar clinical manifestations. If this is the case, a personalized approach in managing severe irAEs may be needed.

The development of irAEs after anti-PD-1/PD-L1 treatment has been proposed to be positive prognostic factors.[6,30–32] In our study, we also found that NSCLC patients with grade 1/2 irAEs had significantly higher ORR and PFS. The occurrence of irAEs may be a sign of more robust immune response after PD-1 blockade which may lead to better treatment outcome. Therefore, it should be taken into account that anti-inflammatory strategies to treat irAEs may have protumoral effects as shown from a previous report.[24] Thus, the benefit by controlling the symptoms of irAEs by anti-inflammatory strategies and their risk of protumoral effects should be carefully weighed for each patient when managing irAEs.

In our study, severe irAEs occurred at a median time of 4 weeks after treatment initiation and mostly within 3

months. A previous study also reported that severe irAEs after anti-PD-1/PD-L1 treatment occurred at median 40 days after treatment onset,[4] indicating that irAE-related immunological changes may begin early after anti-PD-1 treatment. In our study, we obtained peripheral blood samples at baseline and 7 days after treatment. Each T-cell parameter used for clustering analysis had a predictive value for each subgroup of severe irAEs, suggesting potential biomarkers that may be used to identify patients at higher risk of developing severe irAEs. However, the immunological change early after treatment may not entirely reflect the relevant immunological changes associated with irAEs that develop months later. Blood samples drawn at the time of irAEs may be helpful to further investigate the immunological changes of these late-occurring irAEs.

The current study has some limitations. First, we included patients with TET in the present study. TET is well-known to accompany autoimmune diseases, such as myasthenia gravis, even without anti-PD-1 treatment. Moreover, recent studies have reported that severe irAEs are more common after anti-PD-1 treatment in TET patients than patients with other types of cancer.[33,34] In addition, the Th17-related and Treg-related subgroups were restricted to patients with TET in the current study. Due to the unique features of TET, further investigation is required to validate our findings in other types of cancer. Second, although we focused on T cells, recent reports have claimed the role of B cells, autoantibodies, and serum cytokines.[5,6,9] Analysis of all peripheral blood components will be required to comprehensively understand the pathophysiology of irAEs. Third, some late-onset irAEs were observed and further investigation is required to explain the long latent period between the immunological change and clinical manifestation. Fourth, although 91 patients were recruited in the current study, only 13 patients with severe irAEs could be analyzed for the clustering of subtypes. Therefore, the current findings need to be validated in larger cohorts with various types of cancer. Furthermore, patient recruitment periods were different between the TET and NSCLC patients, and more experience with the detection and management of adverse events may have had an impact on the severity of the irAEs.

In this preliminary, hypothesis-generating study, we found peripheral blood T-cell parameters associated with the development of irAEs after anti-PD-1 therapy and describe four distinct subgroups of patients: Th17-related, TNF-related, CD8-related Treg-compensated, and CD8-related Treg-uncompensated. Our findings suggest there may be distinct underlying immunological mechanisms for different subtypes of irAEs. In addition, early evaluation of the immunological responses may have clinical implications in the prediction of irAEs. Further prospective validation in a more homogeneous population with a larger number of patients is essential to better guide prediction and management of irAEs after anti-PD-1 treatment.

## Materials and methods

### Patients and sample collection

This study included 31 patients with TET who were enrolled in a phase II trial (NCT02607631)[33] that evaluated the safety and efficacy of pembrolizumab (200 mg every 3 weeks). In addition, we prospectively recruited 60 patients with metastatic NSCLC treated with pembrolizumab (200 mg every 3 weeks; $n = 42$) or nivolumab (2 mg/kg every 2 weeks; $n = 18$). TET patients were enrolled from March 2016 through June 2016, and NSCLC patients were enrolled between April 2016 and February 2018. Patients with active autoimmune disease who required systemic treatment within 1 year, interstitial lung disease, active infection requiring systemic therapy, known history of human immunodeficiency virus infection, active hepatitis B or hepatitis C virus infection, or received systemic chemotherapy or radiotherapy within 2 weeks were not included in this study. Peripheral blood was collected immediately before treatment and 7 days after the first dose of anti-PD-1 agents to detect the peak immune response after treatment and investigate for early biomarkers to predict severe irAEs. Previous reports have shown that the proliferative response of T cells peaks at 7 days posttreatment and gradually decrease during the following treatment period.[14,15] Furthermore, irAEs can develop before the second dose of treatment, and therefore early evaluation is needed. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by standard Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. This study was approved by the Institutional Review Board of Samsung Medical Center and was conducted in accordance with the Helsinki Declaration. All patients provided informed consent before inclusion in the study.

### Clinical assessment

Physical examination and laboratory tests were performed every 2 or 3 weeks. IrAEs were defined as adverse events of clinical interest with potentially drug-related immunological causes. All adverse events were graded in accordance with the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0. Grade 3 or higher irAEs were defined as severe irAEs. Anti-PD-1 treatment was withheld if patients presented evidence of severe irAEs, and treatment was discontinued if the adverse events did not resolve within 12 weeks from the last dose or if corticosteroid could not be reduced to ≤10 mg per day (or equivalent dose of prednisone per day) within 12 weeks.

The tumor response was assessed according to the Response Evaluation Criteria In Solid Tumors (RECIST), version 1.1. An objective response was defined as a radiologically confirmed complete or partial response. Patients were suspicious of pseudoprogression at first response evaluation that were clinically stable continued treatment and were reevaluated with a new CT scan 4 weeks later to confirm progression. Progression-free survival (PFS) was defined as the time from the start of anti-PD-1 treatment to either disease progression (according to RECIST v1.1) or death from any cause. Overall survival (OS) was defined as the time from the start of anti-PD-1 treatment to death from any cause.

## Multi-color flow cytometry

The following fluorochrome-conjugated monoclonal antibodies were used in multicolor flow cytometry: anti-CD8 (SK1 and RPA-T8), anti-CD3 (UCTH1 or SK7), anti-CD45RA (HI100), anti-CD4 (SK3), anti-CD25 (M-A251), anti-granzyme B (GB11) (all from BD Biosciences, San Jose, CA); anti-PD-1 (EH.12.2H7), anti-Ki-67 (Ki-67), anti-CD38 (HB7), anti-CD127 (A019D5) (all from Biolegend, San Diego, CA); anti-HLA-DR (LN3), anti-FoxP3 (PCH101), anti-CD14 (61D3), anti-CD19 (HIB19) (all from eBioscience, San Diego, CA); and anti-human IgG4 Fc (HP6025, Southern Biotech). Dead cells were stained using the LIVE/DEAD Fixable Red Dead Cell Stain Kit (Invitrogen, Carlsbad, CA) and excluded from the analysis. Intracellular Ki-67, granzyme B, and FoxP3 were stained using a FoxP3 transcription factor staining buffer set (eBioscience, San Diego, CA) and specific antibodies. All stained samples were counted on an LSR II flow cytometer (BD Biosciences, San Jose, CA) and the data analyzed using FlowJo software version 10.4.0 (Treestar, San Carlos, CA).

## Intracellular cytokine staining

For intracellular cytokine staining, PBMCs were stimulated with an anti-CD3 antibody (1 μg/mL; OKT3, eBioscience) for 6 h. Brefeldin A (GolgiPlug, BD Biosciences) was added 1 h after anti-CD3 stimulation. Cytokine production was analyzed by intracellular staining using anti-interferon (IFN)-γ (B27), anti-tumor necrosis factor (TNF)-α (MAb11), and anti-interleukin (IL)-17A (N49-653) (all from BD Biosciences, San Jose, CA) antibodies.

## Statistical methods

Continuous and categorical variables were compared as indicated in the figure legends. Univariate logistic regression was used to evaluate the correlation of severe irAEs and T-cell parameters. Unsupervised hierarchical clustering was performed and a heatmap was generated using pheatmap package version 1.0.12. Two-sided $P$-values <0.05 were considered significant. All statistical analyses were performed with Prism software version 6.0 (GraphPad, La Jolla, CA) and R statistical software version 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria).

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Funding

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