Immune checkpoint inhibitors (ICI) have shown efficacy for treatment of metastatic melanoma, lung cancer, and renal cell carcinoma. Immune checkpoint inhibitors are expected to be applicable to a wide range of cancers. However, ICI have a relatively high incidence of severe and sometimes life-threatening immune-related adverse events (irAEs). At present, no good biomarker is available to predict such irAEs. It is imperative to identify the biomarkers that can predict the risk of severe irAEs. Here we report a melanoma patient who developed severe myocarditis, myositis, and myasthenia gravis (MG) after only one dose of nivolumab. By comprehensive analysis of T cell receptor (TCR) repertoires of T lymphocytes in skeletal muscle tissue, melanoma tumor tissue, and PBMC samples, we identified oligoclonal expansion of T cells in the muscle tissues, implying a strong T cell response in the tissue, as well as drastic systemic immunological changes that were evoked by the nivolumab treatment.

Materials and Methods

Patient. An 80-year-old man developed multiple lymph node and skin metastasis of malignant melanoma, received nivolumab monotherapy. Two weeks after the first dose, he experienced anorexia and fatigue, and suffered from progressive, severe dyspnea and muscle weakness. We diagnosed him with myocarditis, myositis, and myasthenic crisis induced by nivolumab. We commenced steroid therapy, immune absorption therapy, plasma exchange therapy, and i.v. immunoglobulin therapy, and succeeded in saving his life. Because his serum level of anti-acetylcholine receptor antibodies in a sample collected before nivolumab treatment were positive and were elevated significantly after nivolumab, we suspected that nivolumab triggered a severe autoimmune response, which progressed subclinical myasthenia gravis to myasthenic crisis. We carried out T cell receptor repertoire analysis using next-generation sequencing technologies and identified infiltration of clonally expanded T cell populations in the skeletal muscle after nivolumab treatment, implying a very strong T cell immune response against muscular cells. To avoid severe immune-related adverse events, the exclusion of patients with subclinical autoimmune disease is very important for treatment with immune checkpoint inhibitors.

Key words
Melanoma, myasthenia gravis, myositis, nivolumab, tumor-infiltrating lymphocyte

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An 80-year-old man, who developed multiple lymph node and skin metastasis of malignant melanoma, received nivolumab monotherapy. Two weeks after the first dose, he experienced anorexia and fatigue, and suffered from progressive, severe dyspnea and muscle weakness. We diagnosed him with myocarditis, myositis, and myasthenic crisis induced by nivolumab. We commenced steroid therapy, immune absorption therapy, plasma exchange therapy, and i.v. immunoglobulin therapy, and succeeded in saving his life. Because his serum level of anti-acetylcholine receptor antibodies in a sample collected before nivolumab treatment were positive and were elevated significantly after nivolumab, we suspected that nivolumab triggered a severe autoimmune response, which progressed subclinical myasthenia gravis to myasthenic crisis. We carried out T cell receptor repertoire analysis using next-generation sequencing technologies and identified infiltration of clonally expanded T cell populations in the skeletal muscle after nivolumab treatment, implying a very strong T cell immune response against muscular cells. To avoid severe immune-related adverse events, the exclusion of patients with subclinical autoimmune disease is very important for treatment with immune checkpoint inhibitors.
considered to be induced by nivolumab. We commenced 3 days of steroid pulse therapy (1000 mg/day) followed by oral prednisolone at a dose of 1 mg/kg, which was tapered to 20 mg/day after the serum CK level decreased to the normal level. For myasthenic crisis, we carried out immune absorption therapy, plasma exchange therapy, and i.v. Ig therapy (400 mg/kg/day). After these treatments, symptoms in his respiratory muscles, peripheral limbs, and eye opening improved, and the serum level of anti-AChR-Ab decreased to 3.3 nmol/L. After 4 months of treatment in our intensive care unit, the patient is currently receiving maintenance therapy of 20 mg/day prednisolone and low-dose pyridostigmine and continuing rehabilitation in the general ward. The condition of his skin metastasis and left inguinal lymph node metastasis is currently stable, and no new metastatic lesion has appeared. We investigated the serum level of anti-AChR-Ab in a sample collected before nivolumab treatment and confirmed that it were 10.2 nmol/L. The anti-AChR-Ab were thought to exist subclinically and increased following nivolumab treatment, resulting in myasthenic crisis.

**T cell receptor repertoire analysis.** High numbers of infiltrated T cells were found in the skeletal muscle biopsy. Immunohistochemical staining revealed that CD4+ and CD8+ T lymphocytes infiltrated into the muscle fibers (Fig. 1b,c). To characterize these T cells, we used a TCR repertoire analysis. As shown in Figure 2, the read frequencies of five TCR-α and two TCR-β clonotypes were as high as >5% (5.7–16.2%) in the inflamed skeletal muscle tissue. Although most of these clonotypes were also detected in the PBMC samples (P1 and P2) as well as in a melanoma tissue (T1), the read frequencies in those samples were very low (<0.08%; Fig. 3). In addition, we identified strong enrichment (>1.0% frequency) of four TCR-β clonotypes (as the read depth of TCR-α was not high enough, we focused on TCR-β) which were different from the clonotypes observed in the muscle tissue, in PBMC (P2) after nivolumab treatment. The frequencies of these four TCR-β clonotypes in the P1 sample were 0.00027–0.0013%, indicating more than 1000-fold expansion triggered by inhibition of the interaction between programmed cell death protein-1 (PD-1) and its ligand (PD-L1), although it is not clear whether their enrichment was associated with any pathological processes.

**Gene expression assays.** To further characterize the systemic immune signature before and after nivolumab treatment, we also carried out gene expression analysis for several immune-

### Results

**T cell receptor repertoire analysis.** Total RNA from inflamed skeletal muscle tissue after nivolumab treatment, melanoma tumor tissue (T1) before nivolumab treatment, and PBMC samples before (P1) and after (P2) nivolumab treatment as controls were isolated, and then a TCR repertoire analysis was carried out using a next-generation sequencer MiSeq (Illumina, San Diego, CA, USA). The read frequencies of five TCR-α and two TCR-β clonotypes were as high as >5% (5.7–16.2%) in the inflamed skeletal muscle tissue. Although most of these clonotypes were also detected in the PBMC samples (P1 and P2) as well as in a melanoma tissue (T1), the read frequencies in those samples were very low (<0.08%; Fig. 3). In addition, we identified strong enrichment (>1.0% frequency) of four TCR-β clonotypes which were different from the clonotypes observed in the muscle tissue, in PBMC (P2) after nivolumab treatment. The frequencies of these four TCR-β clonotypes in the P1 sample were 0.00027–0.0013%, indicating more than 1000-fold expansion triggered by inhibition of the interaction between programmed cell death protein-1 (PD-1) and its ligand (PD-L1), although it is not clear whether their enrichment was associated with any pathological processes.
related genes. Gene expression levels of CD8 and cytolytic activity markers, as well as ratios of CD8/FOXP3, CD8/CD4, TBX21/GATA3, GZMA/CD3, and PRF1/CD3, were significantly increased at 1.3- to 120-fold after severe MG compared with those before nivolumab treatment. Interestingly, expression levels of CD4 and FOXP3 were robustly decreased to 1.6% and 9.4%, respectively (Fig. 4). These results indicate that, although the patient was considered to be in the immunotolerant phase while receiving 100 mg oral prednisolone after steroid pulse therapy, nivolumab treatment shifted the Th1/Th2 balance towards Th1 polarization and decreased the Treg subpopulation, leading to the systemic activation of autoreactive CD8⁺ cytotoxic T lymphocyte (CTL) effectors with a higher cytolytic activity that could extravasate and infiltrate into the peripheral tissues, such as muscle, to cause a severe adverse reaction. As the CD4⁺ T cell population was extremely low in the peripheral blood in the autoimmune condition, we assume that pre-existing autoreactive B cells producing anti-AChR-Ab may also be activated by the PD-1/PD-L1 inhibition and resulted in a high titer of anti-AChR-Ab during his treatment period.

Discussion

It is notable that only a single dose of nivolumab induced such a large change in the T cell population. We speculate that several CTL clonotypes, increased by nivolumab treatment, are involved in myositis, myocarditis, and MG. A case of MG who had anti-AChR-Ab subclinically before nivolumab therapy has been reported. Our patient was complicated with myocardiitis and myositis, not only MG. Another case of myositis induced by atorvastatin and nivolumab has been reported, but our patient has not taken any statins. Infiltrations of T cells into muscle tissue in rhabdomyolysis is unusual, therefore we thought that autoimmune myositis was induced by nivolumab in our patient. Suzuki et al. reported that 0.8% of MG patients have complicated myositis and/or...
myocarditis. Although the detailed mechanism of the complication of myositis/myocarditis and MG is not clear, a similar phenomenon might have been evoked in our patient.

The reason why nivolumab treatment drastically decreased CD4 and FoxP3 expression levels in peripheral blood remains unclear, but one possibility is that the predominant expansion of autoreactive CTLs could have competitively suppressed the generation or recruitment of CD4+ T or Treg cells in circulating blood.

To avoid the severe irAEs, the exclusion of patients with subclinical autoimmune disease could be a very important consideration for the treatment of ICI. Monitoring of anti-AChR-Ab before nivolumab treatment could be a prediction tool to avoid MG. More importantly, development of predictive immune biomarkers to avoid the various kinds of irAEs is urgently needed.

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Disclosure Statement

The authors have no conflict of interest.

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