PD-L1 Expression Affects Neoantigen Presentation

Masahiro Okada, Kanako Shimizu, Tomonori Iyoda, ..., Takashi Watanabe, Osamu Ohara, Shin-ichiro Fujii

HIGHLIGHTS

- Poor prognosis of PD-L1-expressing tumors in some cancers
- Identification of TCRVα and Vβ repertoire responsive for H2-Kb-restricted neoantigens
- Neoantigen-epitope-pulsed DC therapy demonstrates antitumor effect in vaccine

Okada et al., iScience 23, 101238
June 26, 2020 © 2020
https://doi.org/10.1016/j.isci.2020.101238
PD-L1 Expression Affects Neoantigen Presentation

Masahiro Okada,1 Kanako Shimizu,1 Tomonori Iyoda,1 Shogo Ueda,1 Jun Shinga,1 Yoshiki Mochizuki,2 Takashi Watanabe,2 Osamu Ohara,2 and Shin-ichiro Fujii1,3,4,*

SUMMARY
Although PD-L1 expression on tumor is related to the prognosis of immune checkpoint blockade (ICB) therapy, a recent study also demonstrated clinical benefits even in patients without PD-L1 expression. To understand the relationship between innate resistance and antitumor cytotoxic T lymphocyte (CTL) responses especially against neoantigens, the interaction between PD-L1+ or genetically PD-L1-deleted colorectal tumors and CTLs was assessed under an ICB therapy, finding the robust CTL activation in PD-L1-deleted tumor-bearing mice. Using antigen libraries based on immunogenomics, we identified three H2-Kb-restricted, somatic-mutated immunogenic neoantigens by utilizing enhanced CTLs responses due to PD-L1 deficiency. Furthermore, we identified three T cell receptor (TCR) repertoires relevant to the neoantigens, confirming the response of TCR-gene-transduced CTLs to parental tumor cells. Notably, neoantigen-pulsed dendritic cell (DC) therapy reversed the tumor tolerance. Thus, innate resistance of tumors determines their responsiveness to neoantigens and mixed neoantigen peptides may be useful in DC therapy against innate resistance type tumor.

INTRODUCTION
Cancer immunotherapy has recently evolved into one of the most promising cancer treatment modalities. Although tumor cells hijack the immune system, in particular causing T cell exhaustion, several reports have demonstrated its reversal through immune checkpoint blockade (ICB), i.e., antibodies targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) (Hodi et al., 2016; Shimizu et al., 2018; Wei et al., 2018). Currently, these have been used as effective immunotherapeutic drugs. Specifically, ICB therapy has demonstrated improved overall survival (OS) and progression-free survival (PFS) in the treatment of many different tumor types, such as melanoma (Robert et al., 2015), non-small-cell lung cancer (NSCLC) (Brahmer et al., 2015), head and neck cancer (Ferris et al., 2016), and other cancers, due to the durable antitumor CD8+ cytotoxic T lymphocytes (CTLs). In addition, ICB-therapy-induced recruitment of T cells in the tumor increases in hot tumors, but not in cold tumors (Tumeh et al., 2014; Topalian et al., 2016). Despite its successful clinical activity, still limited patients received the clinical benefits, for which many studies have addressed the factors related to ICB sensitivity and resistance.

The interplay between PD-1 and PD-L1 regulates T cells in the tumor microenvironment (TME). PD-1 expression is upregulated in activated T cells and remains high in exhausted T cells from tumor-infiltrating lymphocytes (Ahmadzadeh et al., 2009). Constitutive PD-L1 expression caused by aberrant transactivation due to genetic or signaling alteration in an intrinsic manner defines the innate resistance. After exposure to IFN-γ released by effector T cells, PD-L1 and other inhibitory molecules were induced at the transcription level and triggers the adaptive resistance (Topalian et al., 2015). Clinically, PD-L1 expression on tumors or immune cells in the tumor microenvironment is associated with durable clinical responses to anti-PD-1/PD-L1 therapies in many tumor types (Topalian et al., 2012; Herbst et al., 2014). However, the significance of PD-L1 expression in each cell type is controversial. PD-L1 expression on tumors or immune cells can independently attenuate antitumor T cell immunity. PD-1+ T cells in the tumor are impaired due to interactions with PD-L1 expressed on either tumor cells (Juneja et al., 2017) or other tumor-infiltrating immune cells (e.g., myeloid cells or TAM) (Lin et al., 2018; Tang et al., 2018) in the TME. Furthermore, immune checkpoint knockout knockout mice or wild-type (WT) mice treated with anti-PD-1 antibodies (Abs) suppressed tumor cells, indicating that the anti-PD-1 Ab has an antitumor effect on both PD-L1-expressing host tumor-associated...
Regarding PD-L1 expression in ICB treatment, PD-L1-mediated immune suppression by IFN-γ-induced immunity. Therefore, DCs vaccination has potential application for resistant tumors. In contrast, the mechanism of PD-L1-mediated immune suppression by the tumor cells (Kleeff et al., 2015; Lin et al., 2018). Recently, the functional importance of immune cells relative to the tumor has been reported in the regulation of the antitumor T cell response in NSCLC patients (Kowanetz et al., 2018), but its importance on the tumor still has to be evaluated. Furthermore, despite low PD-L1 expression on tumor, recent several reports demonstrated that some patients respond well to PD-1 pathway blockade (Brahmer et al., 2015; Zou et al., 2016; Rittmeyer et al., 2017; Eggermont et al., 2018), indicating that the relation of PD-L1 expression and ICB sensitivity partly depends on pathological conditions, thus ascribing PD-L1 expression to innate or acquired resistance. To elucidate the underlying mechanisms of these phenomena, CTL responses in the presence or absence of ICB treatment, with focus on PD-L1 expression on tumor cells should be compared.

The underlying genomic features of tumor cells contribute to ICB responses, and increased tumor mutation burden has been shown to be associated with survival benefits from both anti-CTLA-4 and anti-PD-1 therapy in multiple malignancies (Snyder et al., 2014; Le et al., 2015; Rizvi et al., 2015; Hugo et al., 2016). High mutation burden was frequently diagnosed in mutagen-associated cancers, such as melanoma and NSCLC as well as cancers associated with DNA-mismatch repair gene defects, such as microsatellite instability in colorectal cancers (Schumacher and Schreiber, 2015; Riaz et al., 2016). These high mutation burden tumors potentially generate immunogenic neoantigens. In fact, tumors with clonal neoantigens may significantly elicit effective immune responses (McGranahan et al., 2016), and peptides containing these mutations presented on MHC class I (MHC-I) can be recognized as “non-self” by T cells. For this purpose, neoantigens in high mutation burden tumors can be identified by whole-exome sequence and RNA sequence (RNA-Seq), and accompanying bioinformatics approaches can predict specific neoepitopes in individual cancers using MHC-I-binding algorithms and immunogenomics methods (Castle et al., 2012; Karasaki et al., 2017). Furthermore, ICB immunotherapy has sometimes been found to be effective even in cancer with a low number of mutations but containing some indels, such as renal cell carcinoma, indicating that the number of mutations present in tumors does not solely determine ICB responsiveness (Turajlic et al., 2017; Motzer et al., 2018; Yang et al., 2019). Based on these reports, there are many possible implications regarding ICB sensitive and resistant cases, particularly their PD-L1 expressions and potential neoantigen responses under ICB therapy. Nonetheless, comprehensive analyses are yet to be performed.

Development of a therapeutic strategy for ICB-resistant cancers is an important issue. Of which, dendritic cell (DC) vaccine was identified as a candidate, which plays a central role in linking innate and adaptive immune system responses (Fuji et al., 2004, 2007; Steinman, 2012; Bottcher and Reis, 2018; Fuji and Shimizu, 2019). Ex vivo-expanded DCs pulsed with MHC-I-restricted cancer testis antigen peptides (Bezu et al., 2018) and DCs electroporated with transfected mRNA coding tumor antigen (Bo et al., 2015) and tumor lysates or irradiated tumor cells (Fuji et al., 1999; Rojas-Sepulveda et al., 2018; Tanyi et al., 2018) have been demonstrated in many basic and clinical studies (Steinman and Banchereau, 2007; Saxena et al., 2018). It is noteworthy that utilizing neoantigens, several reports applied DC vaccines in clinical study and showed that neoantigen-pulsed DC can induce tumor regression through neoantigen-specific T cell responses in refractory solid tumors such as melanoma (Carreno et al., 2015; Chen et al., 2019).

Regarding PD-L1 expression in ICB treatment, PD-L1-mediated immune suppression by IFN-γ-induced adaptive resistance in T cell infiltrating hot tumors has been described (Spranger et al., 2013; Tumeh et al., 2014; Wilky, 2019). In contrast, the mechanism of PD-L1-mediated immune suppression by the tumor in a cell intrinsic manner, i.e., innate resistance, and its reversal by ICB therapy remain to be fully resolved. Furthermore, little is known about whether PD-L1 expression on tumors controls T cell immunity against neoantigens, which can partly determine the ICB response. Therefore, in the current study, we used several models—PD-L1-expressing or PD-L1-deleted tumor cells, with or without ICB (anti-PD-1 Ab and anti-CTLA-4 Ab) therapy—to explore how PD-L1 expression on tumor cells leads to innate resistance to ICB therapy and also elucidated potential mechanism of PD-L1-deleted tumor responses in ICB therapy. Further, we demonstrated the availability of our identified neoantigens-based DC therapy to compensate for ICB resistance, resulting in the generation of neoantigens responding T cell immunity as well as enhanced pre-existing immunity. Therefore, DCs vaccination has potential application for resistant tumors.

RESULTS

TCGA Analysis Indicates the Association between PD-L1 and Survival in Cancer Patients

PD-L1 expression in tumor sites is generally known to be associated with overall survival but differs in patients and their contexts. To clarify the variable association of PD-L1 expression and overall survival, we first
analyzed all study cohorts in publicly available TCGA database (The Cancer Genome Atlas; Cancer Genome Atlas Research Network, 2014). PD-L1 expression in some cancers were related to better prognosis but varied among study (Figure 1A). Because the cytolytic score (CYT), that is, the level of granzyme A and perforin, was previously reported to be mainly associated with antitumor immunological effects (Rooney et al., 2015; Roufas et al., 2018), we sub-grouped based on PD-L1 expression, CYT score, and estimated survival. We found that PD-L1 low expression on hot tumors (CYT high) remarkably correlated with better prognosis, than the other three groups—PD-L1 low expression on the cold tumor (CYT low) or PD-L1 high expression—in colorectal adenocarcinoma (COAD) and uterine corpus endometrial carcinoma (UCEC) (Figure 1B). Some other cancers (breast invasive carcinoma [BRCA], cervical squamous cell carcinoma [CESC], diffuse large B cell lymphoma [DLBC], acute myeloid leukemia [LAML], liver hepatocellular carcinoma [LIHC], and sarcoma [SARC]), but not all, also show similar tendencies (Figure S1). In addition to previous observations of survival in patients with high microsatellite instability (MSI) (Gryfe et al., 2000; Le et al., 2015), a group of cancer patients with low PD-L1 expression and high MSI were also associated with better prognosis in these COAD and UCEC studies (Figure 1C). Thus, our results indicated that low PD-L1 expression with strong antitumor immunity or with potential mutation load is related to survival benefits in patients with certain tumors.

**Anti-tumor CTL Responses in PD-L1+ or PD-L1-KO Tumors**

In accordance with clinical observation, several murine tumors have been characterized, including B16 melanoma showing low PD-L1 expression and low immunogenicity with low T cell infiltration (known as a cold tumor type) and a murine colorectal MC38 expressing high PD-L1 and high immunogenicity with high T cell infiltration (known as a hot tumor type). In this study, MC38, which is known to be highly immunogenic and relatively responds to ICB treatment, was used to compare PD-L1 expression and T cell responses in ICB therapy (Yadav et al., 2014; Tanegashima et al., 2019). To examine an effect of PD-L1 of tumor cells on the antitumor immunity, we first generated genetically PD-L1-deleted tumors (hereafter MC38-PD-L1-KO cells).
by plasmid transfection-based transient CRISPR-Cas9 expression in the MC38 cell line. MC38-PD-L1-KO cells expressed MHC-I, but not PD-L1 (Figure S2A). We then examined the tumor proliferation activity in vitro and in vivo. There was no difference in the proliferation of tumor cells between MC38 and MC38-PD-L1-KO in an in vitro culture (Figure 2A). Subsequently, we compared tumor growth after a subcutaneous injection of MC38 and MC38-PD-L1-KO and found that tumor growth of MC38-PD-L1-KO was more slowly progressive than MC38 in WT mice, but not in Rag1−/− mice (Figure 2B), indicating T cell dependence. We also established the PD-L1-deficient murine breast cancer cell line, E0771, and confirmed similar phenomena (Figure S2B), but were not found with B16F10, probably due to the cold tumor type (Figure S2C). These imply that PD-L1 expression and tumor antigen presentation in tumor cells directly dampened the T-cell-dependent tumor suppression. Further, anti-PD-1 Ab treatment alone showed an antitumor effect by reactivating T cells in the tumor, although somewhat insufficient (Figure S2D). The combination of anti-PD-1 Ab plus anti-CTLA-4 Ab has been shown to be more promising for effective CTL generation (Hodi et al., 2016; Shimizu et al., 2018; Wei et al., 2018). To understand the relationship between CTL

Figure 2. Enhanced Immune Responses of CTLs from PD-L1-KO Tumor

(A) Proliferating activity of MC38 or MC38-PD-L1-KO cells in vitro by WST-1 assay. Data are pooled from three independent experiments, and the values represent mean ± SD.
(B) C57BL/6J (left) or Rag1−/− (right) mice were injected with 5 x 10^5 MC38 or MC38-PD-L1-KO cells, subcutaneously (s.c.). Tumor growth was monitored at indicated time points by measuring three perpendicular diameters. MC38 or MC38-PD-L1-KO in C57BL/6J mice n = 20 and 21/group, respectively, in Rag1−/− mice, n = 6/group. Data are pooled from four and two independent experiments, and the values represent mean ± SEM.
(C) C57BL/6J mice were injected with 5 x 10^5 MC38 cells or MC38-PD-L1-KO cells s.c. and then treated with anti-PD-1 and anti-CTLA-4 on day 7, 10, and 13. Tumor growth was monitored at indicated time points by measuring three perpendicular diameters. (n = 17/group). Data are pooled from six independent experiments, and the values represent mean ± SEM.
(D) The frequency of immune cells in tumor sites was analyzed at day 15 after tumor inoculation by flow cytometric analysis. The values represent mean ± SD (n = 4/group). Closed and open bar indicated MC38 and MC38-PD-L1-KO tumor-bearing mice, respectively; ns indicates not significant.
(E) Tumor antigen-specific T cell response in ICB-treated MC38 or MC38-PD-L1-KO tumor-bearing mice. Frequency of MutAdpgk tetramer+ in CD8+ T cells was plotted. Data are pooled from four independent experiments; n = 10/group. Data were analyzed by unpaired Student’s t test, *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 3. Neoantigen Screening from PD-L1-KO Tumors

(A) Circos plot showing the detected mutations in the MC38 tumor. Tumor tissues were sampled at day 15 after tumor inoculation. Mutations of MC38 were identified by whole-exome sequence and RNA sequencing (RNA-Seq). FPKM value and MHC-I affinity values were also plotted.

(B) Venn diagram of identified missense mutations between MC38 WT and PD-L1-KO tumors. Reactive peptide sequences and their IC50 values as calculated by NetMHCpan ver3.0. H2-Kb-restricted peptides for MC38 neoantigen candidates were synthesized and prepared.

(C) MC38 PD-L1 KO (5x10^6 cells) s.c. Day7,10,13 (200 μg αPD-1, αCTLA-4 i.p.) Peptides stimulation screen H2-Kb prediction (IC50~200)

(D) IFN-γ Production

| Peptide No # | Gene Base | Mutation Base/Amino Acid | WT seq | WT IC50 | MUT seq | MUT IC50 |
|--------------|-----------|--------------------------|--------|---------|---------|---------|
| 1            | Ztb40     | G2303C/R768P KSFHFYCPPL  | 2.9    | 3.3     | 609.4   | 118.9   |
| 36           | Dparg1    | G637T/V213L ASIIVFNLL    | 781.2  | 164.7   | 809.4   | 118.9   |
| 42           | Cry1      | G1246T/V416L QFFHCYCPLY  |        |         |         |         |
induction and PD-L1 expression on tumors, we evaluated the combined ICB treatment (anti-PD-1 Ab plus anti-CTLA-4 Ab) against MC38 or MC38-PD-L1-KO tumor cells in vivo. The tumor regression was more effective in ICB-treated MC38-PD-L1-KO tumor-cell-bearing mice than ICB-treated MC38 tumor-bearing mice or non-treated MC38-PD-L1-KO tumor-cell-bearing mice (Figure 2C).

Due to the enhanced antitumor effect in MC38-PD-L1-KO mice, we hypothesized two possibilities: (1) the balance of immune responses was altered in MC38-PD-L1-KO and (2) T cells responded to neoantigens that were more expressed in MC38-PD-L1-KO. As such, we analyzed the immune responses two weeks later in MC38 or MC38-PD-L1-KO mice when no difference in tumor size was observed. As shown in Figure 2D, there was no statistical difference in the frequency of various types of immune cells including T and natural killer (NK) cells. Next, we assessed the CTL function. As shown in Figure 2C, growth of MC38-PD-L1-KO cells in ICB-treated mice progressed slowly and retarded two weeks after subcutaneous tumor inoculation. Therefore, we analyzed the T cell response for the previously identified H2-Db restricted antigen, mutant (Mut) Adpgk, and H2-Kb restricted antigen, p15E, on day 15. In ICB-treated MC38-PD-L1-KO bearing mice, we detected the apparent MutAdpgk-specific CD8+ T cell infiltration in the tumor (Figure 2E). We also assessed their functions using exhaustion/activation markers and found the differential presence of PD-1+Tim-3+ or PD-1+CD69+ responsive CD8+ T cells in MC38-KO tumor compared with MC38 WT tumor (Figure S3). Moreover, CD8+ T cells in the spleen and lymph nodes of these mice responded well to p15E and MutAdpgk antigens (Figure S4). Thus, the ICB-treated mice injected with MC38-PD-L1-KO enhanced the apparent antigen-specific T cell response.

Screening of H2-Kb-Restricted Neoantigens by Exome and Transcriptome Analyses

MutAdpgk was previously reported as a type of neoantigen, expressed on H2-Db of MC38 (Yadav et al., 2014). We speculated that neoepitopes on other MHCs could be identified, and thus a mixture of antigens, composed of different MHC-restricted neoantigens, would be more efficient as vaccines. Therefore, we conducted exome and transcriptome sequences of MC38 and MC38-PD-L1-KO tumor tissues to identify tumor-specific point mutations with amino acid substitutions, including stop gain, frameshift, and missense mutations (Figure 3A). Further, putative neoepitopes were found to be randomly distributed throughout the genome. First, to identify single-nucleotide variants (SNVs), data derived from the exome and RNA sequences obtained from MC38 or MC38-PD-L1-KO were compared with normal mouse blood (Figure S5A). As a result, 7,683 and 6,876 coding variants with almost similar mutation signature were identified by exome sequencing (Figure S5B), and subsequently 826 and 775 by RNA-seq, which overlapped with the exome-based variants, in MC38 and MC38-PD-L1-KO, respectively. Among these, 808 and 755 missense mutations were found in MC38 and MC38-KO, respectively. Common major mutated epitopes in MC38 and MC38-PD-L1-KO were observed to harbor 645 potential epitopes that might be stable or universal (Figure 3B). By contrast, mutated epitopes in MC38 alone or MC38-PD-L1-KO alone were 163 and 110, respectively (Figure 3B). Nevertheless, the rates of mutation detected in both transcriptome and exome analyses were similar: 826/7683 = 0.107 (WT) and 755/6876 = 0.113 (PD-L1-KO). Although some mutations disappeared and other novel mutations possibly appeared in PD-L1-KO tumor probably due to immunoeediting or clonal evolution in a broad sense, majority of the missense mutations was shared. Therefore, we hypothesized that common CTL responses against parental tumors may be present in PD-L1-KO tumor-bearing mice, by which augmented CTL responses enable high sensitive screening of tumor-specific response as shown in Figures 2E and S4.

High Immunogenic Neoantigens Identified by Immunome Analyses

SNVs were analyzed for their potential to generate MHC-I-restricted epitopes of the murine H2-Kb alleles using the NetMHCpan algorithm. We selected neoantigen candidates presented on H2-Kb (predicted IC50 < 200, rank < 1) from common missense mutations in both WT and PD-L1-KO tumors using the NetMHCpan (ver 3.0) algorithm (Nielsen and Andreatta, 2016). Overall, 49 peptides derived from abundant transcripts were more likely to be presented on MHC-I of MC38 cells. Next, we evaluated the
immunogenicity of mutated tumor antigens in vivo. By utilizing PD-L1-KO tumors, one week after tumor inoculation, the tumor-bearing mice were treated three times with ICB combination therapy. CD8+ T cells from the spleen and lymph nodes were harvested and stimulated with or without 49 types of epitope candidate peptides for 72 h. IFN-γ production as an indicator of CD8+ T cell responses was measured (Figure 3C). With this screening, significant IFN-γ production was shown by culturing in the presence of #1 (Zbtb40 R768P), #36 (Dpagt1 V213L), and #42 (Cry1 V416L) (Figure 3D).

To examine whether these three predicted peptides could elicit CD8+ T cell responses, we administered each peptide to WT mice together with anti-CD40 Ab plus poly(I:C). We demonstrated that CD8+ T cells specific for MutZbtb40, MutDpagt1, and MutCry1 antigens, respectively, produced IFN-γ, TNF-α, and IL-2 (Figure 4Ai) and expressed CD137+PD1+ CD8+ T cells in an antigen-specific manner (Figure 4Aii). We also demonstrated that antigen-specific CD8+ T cells responded to MutZbtb40 or MutDpagt1 neoantigen by multimer staining (Figure 4Bi, ii) and ELISA or ELISPOT assay (Figure 4C), but they did not respond

**Figure 4. Bioactive Neoantigen Epitopes**

Analysis of neoantigen-specific T cells in vaccinated mice. C57BL/6J mice were administered intravenously (i.v.) with neoantigen peptide (Zbtb40, Dpagt1, and Cry1) together with poly(I:C) and anti-CD40 Ab. (A) One week later, neoantigen-specific T cell activation in these mice was assessed by intracellular staining for IFN-γ and TNF-α production (i) and upregulation of PD-1 and CD137 (ii) after culturing with or without each peptide for 6 h (i) or 24 h (ii). (B) As shown in (A), neoantigen-specific T cell proliferating responses in these mice were assessed by multimer-APC, CD8α-FITC, and TCR-β-PE. Representative flow cytometry analysis (i) and each value was plotted (ii). (C) Neoantigen-specific T cell response was quantified. Whole splenocytes (ELISA) or isolated CD8+ T cells (ELISPOT) from mice given each neoantigen were cultured with each neoantigen peptide or non-mutated relevant peptide for 24 h and assessed for IFN-γ production. Data are pooled from two independent experiments, and the values represent mean ± SD. Circles and dot lines link respective mice.
to the non-mutated original antigen. Thus, through a computational approach and analysis of their immunogenic antigen-specific properties and biological analyses, these three predicted neoantigen peptides were determined to be immunogenic in the context of MC38 tumors.

Vaccination with Neoantigen-Peptide-pulsed DCs Demonstrates Tumor Protection

Different from self-antigen-derived, conventional tumor antigens, neoantigens are specific and powerful. Because DCs are known as the most powerful antigen-presenting cells (APCs) for priming T cells in murine and humans, ex vivo-expanded DCs are of interest. We initially assessed the antitumor effect using neoantigen-pulsed DC therapy in prophylactic models. To determine if CD8+ T cells induced against neoepitopes could provide protective anti-tumor immunity, C57BL/6J mice were administered with the mutated peptide (Zbtb40, Dpagt1, and Cry1)-pulsed DCs and then subsequently challenged with 1 × 10^6 MC38 tumor cells. Tumor growth was monitored at indicated time points (mean ± SEM, n = 8/group).

Figure 5A. Immunogenicity of neoantigen in vivo using prophylactic tumor model. Bone-marrow-derived dendritic cells (BM-DCs) were generated as previously reported. Each peptide-pulsed BM-DC (1 × 10^6/mouse) was intravenously injected twice to WT mice at day 14 and 7. Immunized mice were challenged with 1 × 10^6 MC38 tumor cells s.c. Tumor growth was monitored at indicated time points (mean ± SEM, n = 8/group).

(B) MutCry1-peptide-pulsed DCs (1 × 10^6/mouse) were administered to WT mice. Vaccinated mice were challenged with 1 × 10^6 MC38 tumor cells. In some experiments, mice were treated with anti-CD4 Ab or anti-CD8 Ab 2 days before tumor injection and repeatedly during tumor monitoring. Tumor growth was monitored at indicated time points (mean ± SEM, n = 7/group). Data are pooled from two independent experiments and analyzed by one-way ANOVA followed by Turkey’s multiple comparison test. **p < 0.001, ***p < 0.001.

Next, we evaluated the therapeutic potential of each single peptide by comparing mixed neoantigens in the antitumor-specific T cell response. For this study, the treatment with a single neoantigen-pulsed DCs was followed by peptide with anti-CD40 Ab and Poly(I:C), which induces robust T cell responses (Nimanong et al., 2017). We monitored the number of tumor-bearing mice that did not exceed 400 mm^3 of tumor size until day 25 and found that 40% of mice (i.e., 5/13, 6/13, and 7/13) treated with each of the neoantigen-pulsed DCs had smaller tumors on day 25 (Figure 6A). In addition, vaccinated mice with mixed peptide (Z + D + C)-pulsed DCs showed a greater antitumor effect, similar to MutAdpgk (Figure 6A). This protection in mice immunized with MutCry1-peptide-pulsed DCs was absent in anti-CD8 Ab-treated mice, but not anti-CD4 Ab-treated mice, strongly supporting that CD8+ T cell responses specific to mutated peptides conferred protection (Figure 6B).

Next, we evaluated the therapeutic potential of each single peptide by comparing mixed neoantigens in the antitumor-specific T cell response. For this study, the treatment with a single neoantigen-pulsed DCs was followed by peptide with anti-CD40 Ab and Poly(I:C), which induces robust T cell responses (Nimanong et al., 2017). We monitored the number of tumor-bearing mice that did not exceed 400 mm^3 of tumor size until day 25 and found that 40% of mice (i.e., 5/13, 6/13, and 7/13) treated with each of the neoantigen-pulsed DCs had smaller tumors on day 25 (Figure 6A). In addition, vaccinated mice with mixed peptide (Z + D + C)-pulsed DCs showed a greater antitumor effect, similar to MutAdpgk (Figure 6A). Thus, vaccination with neoantigens showed remarkable and sustainable inhibition of tumor growth. In addition, we found that the frequency of the three mixed-peptide-reactive CD8+ T cells that were capable of producing IFN-γ (left), IFN-γ/TNF-α (middle), or IL-2/IFN-γ/TNF-α (right) were increased at the tumor site (Figures 6B, 6C, 6D, and 6E). On the other hand, we detected the slight levels of MutAdpgk-specific IFN-γ/TNF-α-producing T cells (Figures 6B and 6C). Thus, we observed an enhancement of the multifunctional CD8+ T cell response to three peptides in mice vaccinated with three neoantigen-peptide DCs. In addition, the CD8+ T cell response against MutAdpgk was generated in mice vaccinated with MutAdpgk-peptide DCs to a similar extent as with three peptide-pulsed DCs. These suggest that neoantigen-responding T cells by vaccination with peptide-pulsed DCs are multifunctional in an antigen-specific manner and imply that even when with neoantigens of low antigenicity, the mixed-peptide-pulsed DC therapy would be useful.
Figure 6. Comparison of Therapeutic Effect by Single Epitope or Multiple Epitopes of Neoantigen

(A) Tumor therapeutic model. C57BL/6J mice were s.c. injected with MC38 tumor cells and treated with neoantigen single-peptide- or multipeptide-pulsed DCs at a week later and again with the relevant peptide together with poly(I:C) and anti-CD40 Ab. Tumor growth was monitored at indicated time points. Tumor growth curve in each group of mice was plotted. As shown by the red line, tumor volume <400 mm³ at day 25 was consider as a partial response. Data are pooled from five independent experiments (n = 11-13).

(B and C) Analysis of TIL in mice treated with multipeptides of neoantigens. TILs were stimulated by peptides in the presence of anti-CD28 and brefeldin. The percentages of IFN-γ single-producing T cells (left), IFN-γ+TNF-α+producing T cells (middle), and IFN-γ+TNF-α+IL-2+producing T cells (right) were plotted. Data are from three independent experiments (n = 6). (C) Representative flow cytometry analysis of CD8+ TILs shows IFN-γ and TNF-α. (D) Therapeutic effect by neoantigen-pulsed DCs. MC38 cells were administered s.c. One week later, these mice were treated with neoantigen-pulsed DCs twice at 1-week intervals. Anti-PD-1 Ab was injected intraperitoneally (i.p.) at day 7, 10, and 13 (n = 10 and 11/group). Data are pooled from four independent experiments, and the values represent mean ± SEM. Data were analyzed by one-way ANOVA followed by Turkey’s multiple comparison test. *p < 0.05.

Neoantigen-Reactive TCR Recognizes Parental MC38 Tumor Cells

Because we showed that mutation-specific peptide selected by biological assay induced tumor-reactive T cells, we next attempted to identify the T cell receptor (TCR) repertoire of antigen-specific T cell clones that could be elicited by each neoantigen. A detailed analysis of the phenotypes of these T cell clonotypes and the biophysical properties of the TCR may shed light on the extent, depth, and requirements for efficient T cell responses against tumors. We isolated neoantigen-reactive T cells by FACS sorting of the CD8+ multimer + CTL reactive for MutDpagt1 or CD8+ PD-1+CD137+ T cells reactive for MutCry1 (Figure S7A, left). We cloned each TCRα and TCRβ sequence into a retroviral vector (pMXs), followed by transfection into the TG40 cell line (Figure S7A, right). The top five sequences of repertoire analysis were selected, and orange- and green-colored ones were subcloned into the expression vector (Figure S7B). To verify that the sequences of the α and β chain were indeed candidates for neoantigen-reactive TCRα and β, we cocultured the TCR-gene-modified TG40 cells with peptide-pulsed EL4. As shown in Figures S7C and S7D, we found one combination of TCRα and TCRβ in MutDpagt1, whereas two combinations of TCRα and TCRβ were found in MutCry1 for CD69 upregulation, suggesting that they are unique and clonal TCRs. Because TCRs shared the conserved CDR3 sequences for recognizing the same MHC-I epitopes, clustering TCRs based on sequence motif enables epitope-specific recognition and diversity. We parsed randomly extracted repertoire sequences by TCRdist (Dash et al., 2017) for TCRB clustering. We observed the homology-based hierarchy and found that neoantigen reactive TCRs occurred in groups (red triangle) and these cluster-relevant newly established sequences in CDR3 during VDJ recombination (framed red; Figure 7A).

These new motifs were shown to frequently interact with MHC-I epitopes by crystal structure analysis. Therefore, to confirm that these neoantigens could induce specific responses, we synthesized mutated di-amino acids sequences, AlaAla, into the CDR3 region. As expected, AlaAla mutation-harboring TCRs drastically lost their reactivity to neoantigens. Moreover, we performed mutant and WT peptide titration assays (Figure S8) and confirmed strong specificity of the neoantigen mutation for TCR8 and TCR10, which was weak for TCR36 (Figures 7B, 7C, and S8). We observed a slight, but apparent, specificity of TCR36 (for Dpagt1) and TCR8 and 10 (for Cry1) by a mutant and WT peptide titration. These suggested that several clonotypes could be generated from the same mutated specific peptide (Cry1 peptide).

Finally, we examined whether antigen-specific TCRα and β chain-bearing T cells could respond to each peptide antigen and parental MC38 tumor cells. In primary T cells transduced with TCRα and TCRβ gene, even without neoantigens peptides, TCRs reacted with MC38 and produced IFN-γ (Figure 7D). Regarding the tumor response, not only TCR36 but also TCR8 and TCR10 could respond to MC38. Thus, tumor neoantigen-specific T cell clones exhibited antigen-specific responses against MC38.
Figure 7. Primary T Cell Expressing Neoantigen-Specific TCR Recognizes Tumor

(A) TCRdist analysis of TCRB repertoire sequences showing the tree diagram of TCRB CDR3 homology. Clonality was not reflected. Red arrows indicate neoantigen peptide reactive CDR3 sequence. As a negative control, rearranged CDR3-derived di-amino acids (red rectangle) were mutated into AlaAla.

(B and C) TCRαβ derived from CTL responding to Dpagt1 pep included TCR36, and TCRαβ derived from CTL responding to Cry1 pep is termed as TCR8 and TCR10. Reactive TCRα and β chains were selected, and they were tandemly linked with Furin-SGSG-P2A sequence and cloned into pMXs-iRES-GFP. Selected combination of TCRα and TCRβ were transfected to TG40 CD8A/B (TCR36, B; TCR8, TCR10, C). These TG40 cells were cocultured with each peptide-pulsed EL4 for 24 h. The percentage of CD69 upregulation by TCR36 or TCR8 and their AA mutation transduction in GFP+ cells for MutDapgt1 peptide or MutCry1 peptide is shown. Data were pooled from six or three independent experiments, and the values represent mean ± SD. Each black circle shows independent values.

(D) IFN-γ Production

The percentage of CD69 upregulation by TCR36 or TCR8 and their AA mutation transduction in GFP+ cells for MutDapgt1 peptide or MutCry1 peptide is shown. Data were pooled from six or three independent experiments, and the values represent mean ± SD. Each black circle shows independent values.
Figure 7. Continued
(D) TCR36-transduced or TCR8- and TCR10-transduced GFP+CD8+ T cells were cocultured with MC38 in the presence or absence of neoantigens peptide for 48 h. Subsequently, supernatants were measured for IFN-γ by ELISA. Data were pooled from seven to ten independent experiments, and the values represent mean ± SD. Data were analyzed by unpaired Student’s t test, *p < 0.05, **p < 0.01.

DISCUSSION

The TME is associated with response to ICB therapy. Particularly, PD-L1 expression, the frequency of CD8+ T cell infiltration, and the mutation burden in tumors correlate well with the likelihood of response during or after ICB therapy. However, the biological relevance of each of these factors has to be clarified for effective ICB usages. In this study, we showed that the difference between resistance and sensitivity of ICB tumors depended on the responsiveness of T cells to neoantigens as a key T cell response in tumor sites. Particularly, we demonstrated the relationship between PD-L1 expression on tumors and the sensitivity to ICB therapy by comparing ICB-treated MC38-PD-L1-KO (non-innate resistant type) and parental MC38 (innate resistant type). In fact, when tumors express PD-L1, even if they have the potential to generate an antitumor T cell response by ICB therapy, these T cells are weak, resulting in faster tumor growth. In contrast, when tumors lack PD-L1, the antitumor T cell immunity against neoantigens can be significantly elicited by ICB, resulting in slowed growth of the tumor.

A tumor-intrinsic role of PD-L1 is to promote cancer initiation toward CSC metastasis, progression, and resistance to therapy (Dong et al., 2018; Fabrizio et al., 2018). In in vitro studies, disruption of intrinsic PD-L1 by CRISPR/Cas9 technique led to suppression of progressive cancer cells, inhibition of spheroid formation of osteosarcoma, and increased anticancer drug sensitivities to doxorubicin and paclitaxel (Liao et al., 2017). PD-L1 transactivation pathway is likely abnormal in many cancers. In intrinsic mechanisms underlying aberrant PD-L1 activation, genomic alterations (i.e., copy number amplification of CD274 residues (Cancer Genome Atlas Research Network, 2014; Straub et al., 2016; George et al., 2017) and 3'UTR disruption (Schoenberger and Maquat, 2012; Kataoka et al., 2016)), constitutive oncogenic signaling activation (via PI3K/AKT or RAS/MAPK (Crane et al., 2009; Coelho et al., 2017)), and epigenetic changes (e.g., expression of microRNA 197 during PD-L1 inhibition in lung cancer and aberrant DNA methylation (Fujita et al., 2015; Dong et al., 2016)) have been described. Whereas, as extrinsic factors, IFN-γ (Garcia-Diaz et al., 2017) and other inflammatory cytokines (e.g., IL-17 and TNF-α (Wang et al., 2017) and HIF-1α (Noman et al., 2014) can activate PD-L1. Hence, what determines the low PD-L1 expression in highly immunogenic TME, which presumably drives best clinical benefits from ICB therapy, needs to be investigated in the future studies.

Effective antitumor vaccines using neoantigens are often the most immunogenic. In the current study, we identified novel three neoantigens on MC38 in an H2-Kb-restricted manner and showed the potential utility of a neoantigen-mixed peptides-pulsed DC vaccine beyond the PD-L1-restricted mechanism. DCs are essential in immunity owing to their role in activating T cells, thereby promoting antitumor responses. Compared with the clinical success of ex vivo DC therapy based on conventional tumor antigens, neoantigen-pulsed DCs have been anticipated for showing potent efficacy (Carreno et al., 2015; Chen et al., 2019). In fact, in this study, we exhibited the efficacy of four types of neoantigen-responding T cells infiltrating in the tumor in mice treated with four neoantigen-peptide-pulsed DCs. Moreover, we demonstrated that neoantigen-specific T cells in TME by DC vaccination possess multifunctionality and an antitumor effect similar to ICB therapy. It is known that anti-CTLA-4 Ab acts through APCs, particularly on DCs, leading to CTL generation, whereas anti-PD-1 Ab therapy can reactivate an impaired CTL. Effectiveness of ICB therapy must depend on DC function. Further, neoantigen-pulsed DC vaccines improved T cell responses in tumors, at levels similar to those by ICB therapy. These support ICB sensitivity dependences on CD8+ T cell responses specific to “multiple” neoantigens, conferring protection against tumors. Combination therapy using ICB may have clinical benefits, but a major limitation remains its characteristic antigen loss. Particularly, it is known that ICB efficacy can be impaired by deleting neoantigens on tumors, which results in tumor progression (Tran et al., 2016). In this study, using different MHC-restricted tumor-associated neoantigens simultaneously with mature DCs, we suggested that starting therapy using multiple neoantigen-peptide-pulsed DCs at early phase generates clinically relevant neoantigen-specific T cells before possible deletion and immune evasion.

Taken together, our findings provide tumor immunological evidence that the level of T cell responses to neoantigens and PD-L1 expression on tumor determine the positive and negative cancer immunity cycle, and therefore, may shape immunoediting during tumor occurrence, which must be optimally targeted for
clinical responses. In addition, our results suggested that PD-L1-KO tumor cells may be useful in isolating neoantigen-specific T cells and that identified neoantigen vaccination could block immune escape, highlighting the recently refocused development in DC cancer vaccines against immune checkpoint ligand-expressing tumors (Figure S9).

Limitation of the Study
In this study, we clarified the innate resistance roles of PD-L1-expressing tumor, which dampens tumor-specific CTLs responses including neoantigens. The fact that PD-L1-deficient tumors treated with anti-PD-1Ab plus anti-CTLA-4 Ab facilitated the neoantigens screen with higher sensitivity may indicate the usefulness of applicable DC-based vaccines. This approach may extend to the clinical application. However, in the current study, the identification of neoantigens were derived from in vivo studies in murine. There are still several problems to identify the neoantigens in human. We need to make new in vitro protocol or new method to use humanized mice for identification of neoantigens.

Resource Availability
Lead Contact
Further information is available from the Lead Contact, Shin-ichiro Fujii (shin-ichiro.fujii@riken.jp).

Materials Availability
Requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact.

Data and Code Availability
The accession number for the Exome and RNA sequence datasets reported in this paper is DNA Data Bank of Japan (DDBJ) Sequence Read Archive (DRA):DRA010264. The request for additional data or codes not infringing on ethics restrictions is available to Lead Contact.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101238.

ACKNOWLEDGMENTS
This work was supported by JSPS KAKENHI Grant Number JP19K07653.

AUTHOR CONTRIBUTIONS
S.F., M.O., and K.S. conceived and designed the experiments. Y.M., T.W., and O.O performed bioinformatics analysis for potential neoepitopes. T.W. conducted and analyzed RNA sequencing data, and M.O. detected the TCR repertoire. J.S. established the cassette of TCRA and TCRB genes, and M.O. generated the final TCRA and TCRB. M.O. mainly, K.S., S.U., and T.I. performed the biological studies, and S.F., M.O., K.S., and T.I conducted data analysis. S.F. supervised the overall project. S.F. and M.O. wrote the manuscript with input from all the other authors.

DECLARATION OF INTERESTS
The authors have no financial conflicts of interest related to this manuscript.

Received: January 7, 2020
Revised: May 4, 2020
Accepted: June 2, 2020
Published: June 26, 2020
REFERENCES

Ahmadzadeh, M., Johnson, L.A., Heemskerk, B., Wellner, R.J., Dudley, M.E., White, D.E., and Rosenberg, S.A. (2009). Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. Blood 114, 1537–1544.

Bezu, L., Kepp, O., Cerrato, G., Pol, J., Fucikova, J., Spisek, R., Zitvogel, L., Kroemer, G., and Galluzzi, L. (2018). Trial watch: peptide-based vaccines in anticancer therapy. Oncoimmunology 7, e1511566.

Bol, K.F., Fidgert, C.G., Aarnzen, E.H., Welzen, M.E., van Rossum, M.M., Bloks, W.A., de van der Kuit, M.W., Scharenborg, N.M., de Boer, A.J., Pots, J.M., et al. (2015). Intranalional vaccination with mRNA-optimized dendritic cells in metastatic melanoma patients. Oncoimmunology 4, e1019197.

Bottcher, J.P., and Reis, E.S.C. (2018). The role of type 1 conventional dendritic cells in cancer immunity. Trends Cancer 4, 784–792.

Brahmer, J., Reckamp, K.L., Baas, P., Crino, L., Eberhardt, W.E., Poddubskaya, E., Antonia, S., Brahmer, J., Reckamp, K.L., Baas, P., Crino, L., and Reis, E.S.C. (2018). The role of e1019197.

Bottcher, J.P., and Reis, E.S.C. (2018). The role of type 1 conventional dendritic cells in cancer immunity. Trends Cancer 4, 784–792.

Castle, J.C., Kreiter, S., Diekmann, J., Lower, M., van de Roemer, N., de Graaf, J., Selmi, A., Diken, Y., Rutter, S., and Parsa, A.T. (2009). PI(3) kinase is immunoresistance by stabilizing PD-L1 mRNA. Nature 464, 1083–1099.e6.

Chen, F., Zou, Z., Su, S., Shao, J., Meng, F., Yang, J., Xu, Q., Ding, N., Yang, Y., et al. (2019). Neoantigen identification strategies enable the mutanome for tumor vaccination. Cancer Res. 79, 306–312.

Coelho, M.A., de Carne Trecesson, S., Rana, S., Belizon, D., Moore, C., Molina-Arcas, M., East, P., Spencer-Dene, B., Nye, E., Barnouin, K., et al. (2017). Oncogenic RAS signaling promotes tumor immunoresistance through PD-1/PD-L1 disruption in melanoma. Immunity 47, 1083–1099.e6.

Crane, C.A., Panner, A., Murray, J.C., Wilson, S.P., Xu, H., Chen, L., Simko, J.F., Waldman, F.M., Pieper, R.O., and Parsa, A.T. (2009). PI3K kinase is associated with a mechanism of immunoresistance in breast and prostate cancer. Oncogene 28, 306–312.

Dash, P., Fiore-Gartland, A.J., Hertz, T., Wang, G.C., Sharma, S., Souquet, A., Crawford, J.C., Clemens, E.B., Nguyen, T.H.O., Kedzierska, K., et al. (2017). Quantifiable predictive features define epitope-specific T cell receptor repertoires. Nature 547, 89–93.

Dong, P., Xiong, Y., Watabi, H., Hanley, S.J., Konno, Y., Ihiu, K., Suzuki, F., Yamada, T., Kudo, M., Yue, J., et al. (2016). Suppression of IASP-dependent aggressiveness in cervical cancer through reversal of mesialoning of silencing mRNA-124A. Sci. Rep. 6, 35480.

Eberhardt, W.E., Poddubskaya, E., Antonia, S., Brahmer, J., Reckamp, K.L., Baas, P., Crino, L., Lie, W.R., Hildebrand, W.H., Mardis, E.R., et al. (2015). Cancer immunotherapy. A dendritic cell network of PD-L1/ligand 1 by CRISPR/Cas9 in osteosarcoma cells. Oncotarget 6, 1189–1201.

Fujii, S., Fujimoto, K., Shimizu, K., Ezaki, T., Kawan, F., Takatsuki, K., Kawakita, M., and Matsuno, K. (1999). Presentation of tumor antigens by phagocytic dendritic cell clusters generated from human CD34+ hematopoietic progenitor cells. J. Exp. Med. 190, 2150–2158.

Fujii, S., Liu, K., Smith, C., Bonito, A.J., and Steinman, R.M. (2004). The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. J. Exp. Med. 199, 1607–1618.

Fujita, Y., Yagishita, S., Hagiwara, K., Yoshioka, Y., Okazaki, T., Nakanishi, T., Kamisaka, Y., and Stein, R.M. (2007). Innate Valpha(14)+/natural killer T cells mature dendritic cells, leading to strong adaptive immunity. Immunity 20, 183–194.

Fuji, S.I., and Shimizu, K. (2019). Immune networks and therapeutic targeting of iNKT cells in cancer. Trends Immunol. 40, 984–997.

Garcia-Diaz, A., Shin, D.S., Moreno, B.H., Saco, J., Garcia-Diaz, A., Shin, D.S., Moreno, B.H., Saco, J., Yarbrough, J., Ely, J., and Steinman, R.M. (2017). Interferon receptor signaling pathways regulating PD-L1 and PD-L2 expression. Cell Rep. 21, 1197–1201.

George, J., Saito, M., Tsuta, I., Ikawa, R., Shirasaki, K., Suzuki, F., Shigeoka, A.H., Uchida, S., Watanabe, S., and Noguchi, M. (2017). Genomic correlation between CD274 (PD-L1) in small-cell lung cancer. Clin. Cancer Res. 23, 1220–1226.

Greyle, R., Kim, H., Hsieh, E.T., Aronson, M.D., Holowaty, E.J., Bull, S.B., Redston, M., and Gallinger, S. (2000). Tumor microsatellite instability and clinical outcome in young patients with colorectal cancer. N. Engl. J. Med. 342, 69–77.

Herbst, R.S., Soria, J.C., Kowanetz, M., Fine, G.D., Hamid, O., Gordon, M.S., Sosman, J.A., McDonald, T.D., Powles, Y., et al. (2014). Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature 515, 563–567.

Hodi, F.S., Cheson, J., Pavlick, A.C., Robert, C., Grossman, K.F., McDermott, D.F., Linette, G.P., Meyer, N., Giguere, J.K., Agarwala, S.S., et al. (2010). Combined nivolumab and ipilimumab versus ipilimumab alone in patients with advanced melanoma: 2-year overall survival outcomes in a multicentre, randomised, controlled, phase 2 trial. Lancet Oncol. 11, 1558–1568.

Hugo, W., Zaretsky, J.M., Sun, L., Song, C., Moreno, B.H., Li, S., Monget-Maoz, B., Pang, J., Chmielowski, B., Cherry, G., et al. (2016). Genomic and transcriptomic features of response to anti-PD-1 therapy in metastatic melanoma. Cell 165, 35–44.

Juneja, V.R., McGuire, K.A., Manguo, R.T., LaFleur, M.W., Collins, N., Haining, W.N., Freeman, G.J., and Sharpe, A.H. (2017). PD-L1 on tumor cells is sufficient for immune evasion in immunogenic tumors and inhibits CD8 T cell cytotoxicity. J. Exp. Med. 214, 895–904.

Karakas, T., Nagayama, K., Kuwano, H., Naito, J.I., Sato, M., Anraku, M., Hosoi, A., Matsushita, H., Takazawa, M., Ohara, O., et al. (2017). Prediction and prioritization of neoantigens: integration of RNA sequencing and data with whole-exome sequencing. Cancer Sci. 108, 170–177.

Kataoka, K., Shinjishi, Y., Takeda, Y., Sakata, S., Matsumoto, M., Nagano, S., Maeda, T., Nagata, Y., Kitakan, A., Mizuno, S., et al. (2016). Aberrant PD-L1 expression through 3’UTR disruption in multiple cancers. Nature 534, 402–406.

Kleffel, S., Posch, C., Barthel, S.R., Mueller, H., Schlapbach, C., Guenova, E., Elco, C.P., Lee, N., Juneja, V.R., Zhan, Q., et al. (2015). Melanoma cell-intrinsic PD-1 receptor functions promote tumor growth. Cell 162, 1242–1256.

Kowanetz, M., Zou, W., Gettinger, S.N., Koeppe, H., Kockx, M., Schmid, P., Kadel, F.E., 3rd, Westuba, I., Chaft, J., Rizvi, N.A., et al. (2018). Differential regulation of PD-L1 expression by immune and tumor cells in NSCLC and the response to treatment with atezolizumab (anti-PD-L1). Proc. Natl. Acad. Sci. U S A 115, E10119–E10126.

Le, D.T., Uram, J.N., Wang, H., Bartlett, B.R., Kemberling, H., Eyring, A.D., Skora, A.D., Luber, B.S., Azad, N.S., Laheru, D., et al. (2015). PD-1 blockade in tumors with mismatch-repair deficiency. N. Engl. J. Med. 372, 2509–2520.

Liao, Y., Chen, L., Feng, Y., Shen, J., Gao, Y., Cote, G., Choy, E., Harmon, D., Mankin, H., Hornick, F., et al. (2017). Targeting programmed cell death ligand 1 by CRISPRi/Cas9 in osteosarcoma cells. Oncotarget 8, 30276–30287.
Lin, H., Wei, S., Hurd, E.M., Green, M.D., Zhao, L., Vatan, L., Szelig, W., Herbst, R., Harms, P.W., Fecher, L.A., et al. (2018). Host expression of PD-L1 determines efficacy of PD-L1 pathway blockade-mediated tumor regression. J. Clin. Invest. 128, 805–815.

McGranahan, N., Furness, A.J., Rosenthal, R., Ramskov, S., Lyngaa, R., Saini, S.K., Jamal-Hanjani, M., Wilson, G.A., Birkbak, N.J., Hiley, C.T., et al. (2016). Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. Science 353, 1463–1469.

Motzer, R.J., Tannir, N.M., McDermott, D.F., Ares F., Frontera, O., Melichar, B., Choueiri, T.K., Pilepich, E.R., Barthelemy, P., Porta, C., George, S., et al. (2018). Nivolumab plus ipilimumab versus sunitinib in advanced renal-cell carcinoma. N. Engl. J. Med. 378, 1277–1290.

Nielsen, M., and Andreottla, M. (2016). NetMHC-pAn-3.0; improved prediction of binding to MHC class I molecules integrating information from multiple receptor and peptide length datasets. Genome Med. 8, 33.

Nimanong, S., Ostroumov, D., Wingerath, J., Knoke, S., Wolter, N., Gurleiv, E., Falk, C.S., Manns, M.P., Kuhnel, F., and Wirth, T.C. (2017). CD40 signaling drives potent cellular immune responses in heterologous cancer vaccinations. Cancer Res. 77, 1918–1926.

Nomaz, M.Z., Desant, S., Jani, B., Hasmin, M., Karry, S., Dessen, P., Bronte, V., and Chouaib, S. (2014). PD-L1 is a novel direct target of IFN-alpha, and its blockade under hypoxia-enhanced MDSC-mediated T cell activation. J. Exp. Med. 211, 781–790.

Riaz, N., Morris, L., Havel, J.J., Makev, V., Desrichrad, A., and Chan, T.A. (2016). The role of neoantigens in response to immune checkpoint blockade. Int. Immunol. 28, 411–419.

Rittmeyer, A., Barlesi, F., Waterkamp, D., Park, K., Ciardiello, F., von Pawel, J., Gadgeel, S.M., Hida, T., Karray, S., Dessen, P., Bronte, V., and Chouaib, S. (2018). Nivolumab plus ipilimumab versus sunitinib in advanced renal-cell carcinoma. J. Clin. Invest. 128, 805–815.

Royer, M.S., Shukla, S.A., Wu, C.J., Getz, G., and Hacohen, N. (2015). Molecular and genetic properties of tumors associated with local immune cytolitic activity. Cell 160, 48–61.

Roufas, C., Chasiotis, D., Makris, A., Efthathiades, C., Dimopoulos, C., and Zaravinos, A. (2018). The expression and prognostic impact of immune cytolitic activity-related markers in human malignancies: a comprehensive meta-analysis. Front. Oncol. 8, 27.

Saxena, M., Balan, S., Roudvko, V., and Bhardwaj, N. (2018). Towards superior dendritic cell vaccines for cancer therapy. Nat. Biomed. Eng. 2, 341–346.

Schendel, D.R., and Maquart, L.E. (2012). Regulation of cytotoxic micRNA decay. Nat. Rev. Genet. 13, 246–259.

Schumacher, T.N., and Schreiber, R.D. (2015). Neoantigens in cancer immunotherapy. Science 348, 69–74.

Shimizu, K., Iyoda, T., Okada, M., Yamasaki, S., and Fuji, S.I. (2016). Immune suppression and reversal of the suppressive tumor microenvironment. Int. Immunol. 28, 449–454.

Snyder, A., Makarov, V., Merghoub, T., Yuan, J., Zaretsky, J.M., Desrichrad, A., Walsh, L.A., Postow, M.A., Wong, P., Ho, T.S., et al. (2014). Genetic basis for clinical response to CTLA-4 blockade in melanoma. N. Engl. J. Med. 371, 2189–2199.

Spranger, S., Spassan, R.M., Zha, Y., Williams, J., Meng, Y., Ho, T.T., and Gajewski, T.F. (2013). Up-regulation of PD-L1, Ido, and Tregs in the melanoma tumor microenvironment is driven by CDB(+) T cells. Sci. Transl. Med. 5, 200ra116.

Steinman, R.M. (2012). Decisions about dendritic cells: past, present, and future. Annu. Rev. Immunol. 30, 1–22.

Steinman, R.M., and Banchereau, J. (2007). Taking dendritic cells into medicine. Nature 449, 419–426.

Straub, M., Drecoll, E., Pfr, N., Weichert, W., Langer, R., Hafelmeier, A., Gotz, C., Wolff, K.D., Kolk, A., and Specht, K. (2014). CD274/PD-L1 gene amplification and PD-L1 protein expression are common events in squamous cell carcinoma of the oral cavity. Oncotarget 7, 12024–12034.

Tanegashima, T., Togashi, Y., Okada, M., Yamasaki, S., Kawahara, A., Ideguchi, K., Sugiyama, D., Nishida, T., Kowalski, D.M., Ols, M.C., et al. (2017). Atezolizumab versus docetaxel in patients with previously treated non-small-cell lung cancer (OAK): a phase 3, open-label, multicentre randomised controlled trial. Lancet 389, 255–265.

Rav, N.A., Hellmann, M.D., Snyder, A., Kvistborg, P., Makev, V., Havel, J.J., Lee, W., Yuan, J., Weng, P., Ho, T.S., et al. (2015). Cancer immunology: Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science 348, 124–128.

Robert, C., Schachter, J., Long, G.V., Arance, A., Grob, J.J., Mortier, L., Daud, A., Carlino, M.S., McNeil, C., Lotem, M., et al. (2015). Pembrolizumab versus ipilimumab in advanced melanoma. N. Engl. J. Med. 372, 2521–2532.

Rojas-Sepeulveda, D., Tittarella, A., Gleisner, M.A., Avalos, I., Pereida, C., Gallegos, J., Gonzalez, F.E., Lopez, M.N., Butte, J.M., Roa, J.C., et al. (2018). Tumor lysate-based vaccines: on the road to immunotherapy for gialbladder cancer. Cancer Immunol. Immunother. 67, 1897–1910.

Yang, W., Lee, K.W., Srivastava, R.M., Kuo, F., Krishna, C., Chowell, D., Makarov, V., Hoen, D., Dalin, M.G., Wexler, L., et al. (2019). Immunogenic neoantigens derived from gene fusions stimulate T cell responses. Nat. Med. 25, 767–775.

Zou, W., Wolchok, J.D., and Chen, L. (2016). PD-L1 (B7-H1) and PD-1 pathway blockade: a common denominator approach to cancer therapy. Cancer Cell 27, 450–461.
Supplemental Information

PD-L1 Expression Affects Neoantigen Presentation

Masahiro Okada, Kanako Shimizu, Tomonori Iyoda, Shogo Ueda, Jun Shinga, Yoshiki Mochizuki, Takashi Watanabe, Osamu Ohara, and Shin-ichiro Fujii
Supplementary Figure 1. TCGA survival analysis focusing on PD-L1 expression with CYT score. Related to Figure 1. Kaplan-Meier survival curves of patients with PD-L1 expression with high versus low CYT score.
Supplementary Figure 2. Genetic PD-L1 deletion in tumor and effect on antitumor responses. Related to Figure 2.

(A) Flow cytometry analysis of PD-L1 and H-2Kb of MC38-PD-L1-KO cells. (B) Tumor growth of E0771 murine breast cancer. C57BL/6J (left) or Rag1−/− (right) mice were subcutaneously (s.c.) injected with 5 × 10⁵ parental or E0771-PD-L1-KO cells, respectively. Tumor growth was monitored at indicated time points by measuring three perpendicular diameters. E0771 and E0771-PD-L1-KO in C57BL/6J mice n = 8/group **p < 0.01, in Rag1−/− mice n = 3 and 4/group, respectively; mean ± SEM. (C) Tumor growth of B16F10 murine melanoma. Tumor volume was measured every three days from seven days after injection of 1 × 10⁵ parental or B16F10-PD-L1-KO cells in C57BL/6J mice. The values represent mean ± SEM. n = 10 to 11/group. (D) Antitumor effect of anti-PD-1 Ab on PD-L1 KO tumor. C57BL/6J mice were s.c. injected with 5 × 10⁵ MC38 or MC38-PD-L1-KO cells and then treated with anti-PD-1 on day 7, 10, and 13. Tumor growth was monitored at indicated time points by measuring three perpendicular diameters. n = 9 to 10/group. Data were analyzed by unpaired Student’s t-test, **p < 0.01. N.S., non-statistical difference.
Supplementary Figure 3. Neoantigen specific T cells in tumor infiltrating lymphocytes under ICB therapy. Related to Figure 2.

(A) Gating strategy and representative flowcytometry analysis. (B) The percentages of PD-1^+Tim-3^+ or PD-1^+CD69^+ CD8^+ T cells among total CD8 (non-gated) and MutAdpgk tetramer^+ cells were plotted. Circles and lines link the respective mice, n=10.
Supplementary Figure 4. Antigen-specific T cell response in ICB-treated mice. Related to Figure 2.
MC38 or MC38-PD-L1-KO tumor-bearing mice were treated with anti-PD-1 and anti-CTLA-4 on day 7, 10, and 13. Fifteen days after tumor inoculation, CD8⁺ T cells were purified from splenocytes and the tumor draining lymph node, and cocultured with 30 Gy-irradiated splenocytes in the presence or absence of 10 μg/mL p15E or MutAdpgk peptide for 72 h. The culture supernatants were measured for IFN-γ levels by ELISA. Data are pooled from eight or five independent experiments.

| WT / KO | W | W | K | K | W | W | K | K | W | W | K | K |
|---------|---|---|---|---|---|---|---|---|---|---|---|---|
| ICB     | - | + | - | + | - | + | - | + | - | + | - | + |
| ex vivo | DMSO | p15E | MutAdpgk |
Supplementary Figure 5. Neoantigen identification pipeline. Related to Figure 3.

(A) Mutations in tumors were identified by whole-exome sequence, RNA sequencing, and accompanying bioinformatics approaches using MHC-I-binding algorithms. Reactive peptides sequences and their IC50 values were calculated by NetMHCpan ver3.0. (B) Mutation signature analysis of exome sequence from MC38 WT and MC38-PD-L1-KO tumors.
Supplementary Figure 6. Analysis of TIL in mice treated with neoantigen multi-peptides. Related to Figure 6.

(A) Analysis of TIL in mice treated with neoantigen multi-peptides. TILs from the treated mice were analyzed for cytokine production at day 25. TILs were stimulated by peptides in the presence of anti-CD28 and brefeldin. The frequency of IFN-γ single-producing T cells or IFNγ+IL-2+ producing T cells was plotted. Data are pooled from three independent experiments (n = 6). (B) FlowSOM analysis representing the multifunctionality of cytokine production in CD8 TILs (IFN-γ+ TNFα+ and IL-2+).
**A**

Gated on live cell, TCRb(+), CD8a(+)

#MutDpagt1 Splenocyte post sort

Multimer

CD8a

#MutCry1 Splenocyte

DMSO stimulation post sort

CD137

PD-1

**B**

| Repertoire Analysis | V gene | D gene | J gene | CDR3 counts | CDR3 Amino Acids Sequence |
|---------------------|--------|--------|--------|-------------|---------------------------|
| TCRA                |        |        |        |             |                           |
| 17164               | CATD\_NAYKVIF TRAV8\_1*02 |
| 6354                | CAMRD\_TGYQNYVF TRAV16\_D/V11\_03 |
| 5448                | CASSDGGITYLKF TRAV12\_2*03 |
| 5448                | CTA\_EGYKLTF TRAV12\_2*03 |
| 64404               | CAA\_SLNTGNYKF TRAV8\_2*02 |
| 5262                | CAA\_STGQNYVF TRAV19\_1*03 |
| 5353                | C\_SDPDGKLIF TRAV12\_2*03 |
| 2561                | CAL\_LNGKSLST TRAV12\_1*02 |
| 2557                | CAV\_OGNKLTF TRAV12\_2*03 |

| TCRA                |        |        |        |             |                           |
| 15473               | CA\_WDRGTNTEVF TRBV3\_1*01 |
| 8780                | CASS\_PTRSQNTLYF TRBV3\_4*01 |
| 8339                | CASS\_CGGITYLKF TRBV13\_1*02 |
| 8067                | CASS\_LTLT\_EYVF TRBV3\_2*01 |
| 6702                | CASS\_LQLNSDYTF TRBV2\_2*01 |

| TCRB                |        |        |        |             |                           |
| 14053               | CASS\_DVEQVF TRBV13\_3*01 |
| 8496                | CASS\_TRYEYVF TRBV8\_4*01 |
| 5739                | CASS\_QQSWQNYLTF TRBV13\_2*01 |
| 5549                | CASS\_QDRGYEYVF TRBV13\_2*01 |
| 4359                | CASS\_PGTQSNLYTF TRBV13\_1*01 |

| TCR36               |        |        |        |             |                           |
| 14053               | CASS\_DVEQVF TRBV13\_3*01 |
| 8496                | CASS\_TRYEYVF TRBV8\_4*01 |
| 5739                | CASS\_QQSWQNYLTF TRBV13\_2*01 |
| 5549                | CASS\_QDRGYEYVF TRBV13\_2*01 |
| 4359                | CASS\_PGTQSNLYTF TRBV13\_1*01 |

| TCR10               |        |        |        |             |                           |
| 14053               | CASS\_DVEQVF TRBV13\_3*01 |
| 8496                | CASS\_TRYEYVF TRBV8\_4*01 |
| 5739                | CASS\_QQSWQNYLTF TRBV13\_2*01 |
| 5549                | CASS\_QDRGYEYVF TRBV13\_2*01 |
| 4359                | CASS\_PGTQSNLYTF TRBV13\_1*01 |

| TCR8                |        |        |        |             |                           |
| 14053               | CASS\_DVEQVF TRBV13\_3*01 |
| 8496                | CASS\_TRYEYVF TRBV8\_4*01 |
| 5739                | CASS\_QQSWQNYLTF TRBV13\_2*01 |
| 5549                | CASS\_QDRGYEYVF TRBV13\_2*01 |
| 4359                | CASS\_PGTQSNLYTF TRBV13\_1*01 |

**C**

CD69 [%]

EL4-#MutDpagt1

EL4-#MutCry1

**D**

CD69 [%]

CAW\_DRGTNTEVF TRBV3\_1*01 TRBJ1-1*01

CASS\_LGKQGYEYVF TRBV2\_1*01 TRBJ1-1*01

CASS\_LGKQGYEYVF TRBV2\_1*01 TRBJ1-1*01

CASS\_LGKQGYEYVF TRBV2\_1*01 TRBJ1-1*01
Supplementary Figure 7. Identification of neoantigen-reactive TCR. Related to Figure 7.

(A) Cell sorting of neoantigen-responding CTL. Splenic antigen-specific T cells were isolated as CD8⁺ multimer⁺ cells for MutDpagt1 and 1-day cultured CD8⁺PD-1⁺CD137⁺ cells for MutCry1 from C57BL/6J mice one week after immunization with neoantigen single peptide (MutDpagt1 or MutCry1) with poly(I:C) and Anti CD40 Abs. Repertoire analyses have been made for each mouse group as shown in the experimental scheme (right). (B) Five major TCRα and TCRβ from each group were shown by repertoire analysis. (C and D) As shown in the right panel of Fig. S7A, various combinations for TCRα and TCRβ selected from 3–4 major types have been cloned to pMXs-IRES-TdTomato (TCRA) or GFP (TCRB) and transduced to TG40 CD8A/B cells. TG40-bearing TCRA/B (+/+) cells were selected as candidates. Subsequently, these were cocultured with each peptide-pulsed EL4 for 24 h. The percentage of CD69⁺ cells in TG40 TdTomato/GFP(+/+) cells for MutDpagt1 peptide (C) or MutCry1 peptide (D) is shown. Data are representative of two independent experiments.
Supplementary Figure 8. Peptide concentration titration of TCRs. Related to Figure 7.

TCR36 (for Dpagt1), TCR8 or TCR10 (for Cry1)-transduced-TG40 cells were cocultured with EL4 in the presence of indicated concentrations of each peptide for 24 h. The percentage of upregulated CD69 of TCR-transduced TG40 cells are shown. Data were pooled from (i) six or (ii) four independent experiments, and the values represent mean ± SD. Data were analyzed by unpaired Student’s t-test, *p < 0.05, ***p < 0.001.
Supplementary Figure 9. Scheme of the study. Related to Figure 2, 3, 5, and 6.

(1) After exome and RNA sequencing, neoantigen candidates were predicted by NetMHCpan. (2) Neoantigens were screened by using activated CTLs from ICB-treated PD-L1 deficient tumor. (3) Vaccination with a mixture of neoantigen-pulsed DCs generated CTLs against PD-L1 positive tumors.
| Peptide No | Sequence | IC50(WT) | Rank(WT) | Gene Name | Mutation Information | WT Sequence | IC50(WT) |
|------------|----------|----------|----------|-----------|----------------------|-------------|----------|
| 33         | AS1IVPFL | 60.9     | 7        | Zbtb40    | NM_198248 c.G2830T protein-altering (position 768 changed from R to F) | AS1IVPFL   | 60.9     |
| 34         | AS1IVPFL | 60.1     | 6        | Zbtb40    | NM_01295073 c.G502T protein-altering (position 1674 changed from R to L) | AS1IVPFL   | 60.1     |
| 35         | AS1IVPFL | 60.2     | 5        | Zbtb40    | NM_001939 c.G2017T protein-altering (position 673 changed from A to S) | AS1IVPFL   | 60.2     |
| 36         | AS1IVPFL | 60.3     | 4        | Zbtb40    | NM_013935 c.G648T protein-altering (position 216 changed from M to I) | AS1IVPFL   | 60.3     |
| 37         | AS1IVPFL | 60.4     | 3        | Zbtb40    | NM_01293715 c.G635T protein-altering (position 212 changed from C to F) | AS1IVPFL   | 60.4     |
| 38         | AS1IVPFL | 60.5     | 2        | Zbtb40    | NM_194355 c.G118T protein-altering (position 40 changed from D to Y) | AS1IVPFL   | 60.5     |
| 39         | AS1IVPFL | 60.6     | 1        | Zbtb40    | NM_015827 c.A2210C protein-altering (position 737 changed from K to T) | AS1IVPFL   | 60.6     |
| 40         | AS1IVPFL | 60.7     |          | Zbtb40    | NM_021523 c.C1066A protein-altering (position 356 changed from H to N) | AS1IVPFL   | 60.7     |
| 41         | AS1IVPFL | 60.8     |          | Zbtb40    | NM_008793 c.C1691A protein-altering (position 564 changed from T to K) | AS1IVPFL   | 60.8     |
| 42         | AS1IVPFL | 60.9     |          | Zbtb40    | MM8570 c.G167C protein-altering (position 56 changed from C to S) | AS1IVPFL   | 60.9     |
| 43         | AS1IVPFL | 61.0     |          | Zbtb40    | NM_026079 c.A843T protein-altering (position 315 changed from T to S) | AS1IVPFL   | 61.0     |
| 44         | AS1IVPFL | 61.1     |          | Zbtb40    | NM_178066 c.G457T protein-altering (position 153 changed from V to F) | AS1IVPFL   | 61.1     |
| 45         | AS1IVPFL | 61.2     |          | Zbtb40    | NM_019092 c.A311T protein-altering (position 104 changed from Q to L) | AS1IVPFL   | 61.2     |
| 46         | AS1IVPFL | 61.3     |          | Zbtb40    | NM_133966 c.G171T protein-altering (position 573 changed from A to S) | AS1IVPFL   | 61.3     |
| 47         | AS1IVPFL | 61.4     |          | Zbtb40    | NM_009195 c.G2017T protein-altering (position 673 changed from A to S) | AS1IVPFL   | 61.4     |
| 48         | AS1IVPFL | 61.5     |          | Zbtb40    | NM_010902 c.G2017T protein-altering (position 673 changed from A to S) | AS1IVPFL   | 61.5     |
| 49         | AS1IVPFL | 61.6     |          | Zbtb40    | NM_025970 c.G167C protein-altering (position 56 changed from C to S) | AS1IVPFL   | 61.6     |

Supplementary Table S1. Neoantigens candidate peptides. Related to Figure 3.
Transparent Methods

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

Pathogen-free, 6–8 week old C57BL/6J female mice were purchased from Charles River Japan, and Rag1 knockout mice were purchased from The Jackson Laboratory. All mice were maintained under specific pathogen-free conditions. All mice experiments were approved by and performed in compliance with Institutional Animal Care and Use Committee of RIKEN Yokohama Branch.

Reagents

InVivoMAb Anti-mouse PD-1 (RMP1.14) and Anti-mouse CTLA-4 (9D9) were purchased from BioXCell (West Lebanon, NH, USA). H2Kb MuLV p15E peptide (KSPWFTTL) was purchased from MBL (Aichi, Japan).

Flow cytometry

The following monoclonal antibodies (mAbs) were purchased from BD Bioscience, BioLegend, eBioscience, or MBL: anti-CD274 (10F.9G2), anti-H2-Kb (AF6-88.5), anti-CD4 (GK1.5), anti-TCRβ (H57-597), anti-CD8a (53-6.7), anti-TNF-α (MP6-XT22), anti-IL-2 (JES6-5H4), anti-IFN-γ (XMG1.2), anti-CD44 (IM7), anti-CD137 (17B5), anti-CD62L (MEL-14), anti-PD-1 (29F.1A12), anti-CD8 (KT-15), anti-CD3e (145-2C11), anti-CD45 (30-F11), anti-CD8b (YTS156.7.7), anti-CD69 (H1.2F3), anti-CD11b (M1/70), anti-CD366 (RMT3-23), anti-Ly-6C (HK1.4), anti-CD24 (M1/69), anti-Siglec-F (E50-2440), anti-F4/80 (BM8), anti-I-A/I-E (M5/114.15.2), anti-CD103 (2E7), anti-Ly-6G (1A8), anti-CD11c (HL3), anti-CD19 (1D3), anti-CD25 (PC61), anti-NK-1.1 (PK136), anti-CD4 (RM4-5), and anti-FOXP3 (FJK-
H2-D<sup>b</sup> Adpgk neoepitope tetramer and H2-K<sup>b</sup> Zbtb40 and Dpagt1 neoepitope multimers were purchased from MBL and Immudex (Copenhagen, Denmark), respectively.

**Cell culture**

MC38 (a kind gift from Dr. MT Lotze, University of Pittsburgh), HEK293T cells and B16F10 cells from ATCC were maintained in Dulbecco’s Modified Eagle Medium (DMEM, 4500 mg/L glucose) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco). E0771 cells from CH3 BioSystems, EL-4 cells (a kind gift from Dr. Steinman, The Rockefeller University), and TG40 cells expressing CD8A and CD8B (a kind gift from Dr. T Saito, RIKEN) (Yokosuka et al., 2002) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS, 55 µM 2-ME, and 1% penicillin/streptomycin. TG40 cells were constantly sorted to maintain CD8A and CD8B expression at > 95%. Primary T cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 55 µM 2-ME, 1% penicillin/streptomycin, 1% non-essential amino acid solution, and 2 mM L-glutamine. Bone marrow cells were cultured in RPMI-1640 medium supplemented with 5% FBS, 55 µM 2-ME, 1% penicillin/streptomycin, and 1 mM HEPES with GM-CSF Sup derived from J558L-GM-CSF. All cells were cultured in a humid, 5% CO<sub>2</sub>, 37°C incubator.

**Bone marrow-derived dendritic cell culture and vaccination**

Bone marrow-derived DCs were generated in the presence of GM-CSF and matured using LPS on day 6 as previously described (Shimizu et al., 2006). On the following day, mature DCs were harvested and pulsed with 10 µg/mL of indicated peptides for 2 h. After washing with PBS, peptide-pulsed DCs (1 × 10<sup>6</sup>) were intravenously injected for vaccination.
**MC38 transplantation mouse model**

Briefly, 6 to 10-week-old female mice were shaved on the right flank, and MC38 (1 or 5 × 10⁵ cells) was subcutaneously injected. Tumor volume, calculated as 0.52 × Length × Width × Height [mm³], was measured at day 7 and every three days. For ICB treatment, 200 µg anti-mouse PD-1 (RMP1.14) and 200 µg anti-mouse CTLA-4 (9D9) were intraperitoneally injected on day 7, 10, and 13. For the CD4 or CD8 T cell-depletion, 250 µg anti-mouse CD4 (GK1.5) or anti-mouse CD8 (53-6.7) were injected intraperitoneally on day -2, 0, 2 and 4, and moreover every 3 to 4 days.

**Poly(I:C), anti-CD40 antibody and peptide immunization**

10 µg PolyIC, 30 µg anti-CD40 antibody (1C10) (BioLegend), and 100 µg indicated peptides dissolved in 200 µL PBS were intravenously injected for vaccination (33.3 µg each of peptide #1, #36, and #42 immunization was used) into 6 to 10-week-old female mice. Or 50 µg PolyIC, 50 µg anti-CD40 antibody, and 100 µg peptide were used for immunization into tumor bearing mice in TCR isolation experiments. 1 week after immunization, splenocytes were further analyzed for multimer staining, intracellular cytokine staining after 6 h culture in the presence of GolgiPlug (BD Bioscience), and activation marker staining and ELISA after 24 h culture with 10 µg/ml peptide.

**Neoantigen prediction**

Missense mutation-containing amino acid sequences, positioned at 9 in 17 aa, near the first Met, or near the stop codon, were investigated for their potential loading to MHC class I H2-Kb using the NetMHCpan (version 3.0) (Nielsen and Andreatta, 2016) algorithm provided by Immune Epitope DataBase and Analysis Resource (http://tools.iedb.org/mhci/). Predicted peptide length was set as 9 mer. Top 48 candidate peptides (IC50 < 200 nM) were narrowed
down for further biological investigation. Candidate peptides (Table S1) and MutAdpgk (ASMTNMELM) were synthesized (purity > 90%) by GenScript Japan (Tokyo, Japan).

**Flow cytometry**

Fixable Violet or Aqua Dead Cell stain kit (Invitrogen) was used to eliminate dead cells. Cytokine expression by CD8 T cells was determined using a protocol for intracellular cytokine staining (Shimizu and Fujii, 2009). Briefly, splenic cells were incubated in the presence of Golgi Plug (BD Bioscience) for 6 h with or without 10 μg/mL indicated peptide, followed by incubation with antibodies to the surface markers. Isolated TILs (~1 × 10^6 cells/200 μL) were seeded in 96-well round bottom plates and stimulated with 10 μg/mL indicated peptides and soluble 2 μg/mL anti-CD28 (clone 53.67) (BioLegend) in the presence of GolgiPlug for 6 h. Each 3.3 μg/mL #1, #36, and #42 peptide was used for mixed peptides stimulation. Cells were treated with the anti-mouse CD16/32 antibody (clone 93) (BioLegend) and then stained using cell surface antibodies. Subsequently, the cells were permeabilized in Cytofix-Cytoperm Plus (BD Biosciences) and stained with anti-IFN-γ, -TNF-α, and -IL-2 mAb. Splenocytes (0.5~1 × 10^7 cells) or TILs were treated with anti-mouse CD16/32 antibody in a 50 μL volume, followed by addition of ~10 μL volume of MHC dextramer or MutAdpgk tetramer.

**CD69 upregulation assays**

TCR retroviruses were transduced into TG40 CD8A/B cells in the presence of 5 μg/mL polybrene (Nacalai) by centrifugation at 2300 rpm for 90 min at 35°C. Further, 2–3 × 10^4 Bulk TG40 cells were cocultured with the same number of EL-4 in the presence or absence of 10 μg/mL corresponding peptides for 24 h in a 96-well flat plate. CD69 upregulation in TdTomato’GFP^+ or GFP^+ cells was analyzed by FACS.
**TCR-T assay**

MACS-sorted 2 × 10⁵ CD8 T cells were cultured with 2 × 10⁵ mouse CD3/28 beads using the Treg expansion kit (Miltenyi Biotec) and 100 U Immunace (recombinant human IL-2) (Shionogi, Osaka, Japan) in a 24-well plate. Two days after stimulation, TCR retroviruses were transduced into cells in the presence of 6 µg/mL polybrene by centrifugation at 2300 rpm for 90 min at 35°C. On the following day, the cells were harvested and resuspended in fresh medium containing IL-2 after magnetic removal of CD3/28 beads. Two days after transduction, CD8⁺GFP⁺ cells were sorted. Approximately 5 × 10⁴ CD8 T cells were cocultured with 1 × 10⁴ MC38 cells with or without 10 µg/mL peptide for 48 h in the presence of 100 U IL-2 in 96-well flat plates. Culture supernatants were harvested and subjected to IFN-γ ELISA.

**TCGA Analysis**

Gene expression data (FPKM values) were downloaded from the TCGA GDC portal site (https://portal.gdc.cancer.gov/repository) using gdc-rnaseq-tool (https://github.com/cpreid2/gdc-rnaseq-tool), and clinical data were obtained using R package “TCGAbiolinksGUI” (Silva et al., 2018) on October 2019. FPKM values were converted to TPM values. CYT scores were defined as geomeans of GZMA and PRF1. Overall survival of sub-grouped patients was parsed by R package “survival” and R package “survminer”.

**Establishment of PD-L1 Knockout cells**

gRNA targeting mouse PD-L1 (chr19:29373571-93) expression vector was generated by annealing of oligonucleotides, followed by ligation into BsmBI and BamHI-restricted sites of pCas-Guide-EF1a-GFP (OriGene #GE100018). Primers (forward; gatcgGGCTCCAAAGGACTTGTACGg and reverse;
aaaacGTCAGTCCTTGGAGCCc) were used for construction. CRISPR-Cas9 #PD-L1 vector was transfected into MC38, B16F10, and E0771 cells using the Lipofectamine Plus Reagent and LTX reagent, followed by a medium change to remove the transfection reagents. Three days after transfection, GFP+ cells were sorted using ARIA3 (BD Biosciences, San Jose, CA, USA) and further cultured. Seven days after transfection, cells were detached using Accutase (Nacalai). Following, PD-L1(-)GFP(-) cells were sorted and further expanded. Several sorting was performed consecutively after expansion to yield completely PD-L1 knockout cells.

**IFN-γ ELISA and ELISPOT assay**

CD8 T cells were positively selected by mouse CD8a (Ly-2) MicroBeads (Miltenyi Biotec) and MACS LS column. The purity was almost > 90%. For ELISA analysis, 2×10^5 CD8 T cells were MACS purified from the spleen and draining lymph node in tumor-bearing mice, and 2×10^5 30 Gy-irradiated splenocytes were cocultured in the presence of 10 μg/mL peptides in a total volume of 200 μL/well for 72 h. Following, the culture supernatants were harvested and stocked. Technical-duplicate wells were prepared for assays, except the neoantigen peptides screen. IFN-γ production in the frozen and thawed samples were examined using Mouse IFN-γ Duo Set ELISA (R&D systems, Minneapolis, MN, USA) and High Binding Coaster Assay Plates (Corning). ELISPOT assays for antigen-specific IFN-γ secreting cells were performed on 96-well filtration plates (Merck Millipore) coated with rat anti-mouse IFN-γ capture antibody at 10 μg/mL (BD Biosciences R4-6A2) as previously described (Shimizu et al., 2007). Splenic and lymph node CD8 T cells were isolated from immunized mice or naïve mice using CD8+ MACS Beads. Further, 5×10^5 CD8 T cells were cocultured with 3×10^5 irradiated splenic cells pulsed with the indicated peptides for 40–48 h. Biotinylated anti-mouse IFN-γ detection antibody was added at 2 μg/mL (BD Biosciences,
XMG1.2) for 2 h, and spots were developed with Streptavidin-HRP (BD Biosciences) for 1 h and stable DAB substrate (FALMA, Tokyo, Japan). Finally, cells were counted microscopically.

**Exome-seq and RNA-seq**

MC38 WT and PD-L1 KO tumor tissues were resected and divided into two samples for exome-seq and RNA-seq, then snap frozen in liquid nitrogen. Whole blood was sampled with heparin, and centrifuged PBMCs were prepared for normal tissues. For exome-seq, genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega). DNA was sheared by Covaris (Covaris), and the exome was captured using the SureSelectXT mouse all exon system (Agilent Technologies, 5190-4641) for library preparation. For RNA-seq, RNA was extracted using the Trizol LS Reagent and isolated with chloroform. Following, RNA was precipitated with glycogen and isopropanol, followed by 75% ethanol wash. mRNA was retrieved with oligo dT beads. The RNA sequencing library was prepared using the SureSelect Strand-Specific RNA Library Prep kit (Agilent Technologies, G9691A). Library preparation and pair-end sequencing (100 cycles for RNA sequencing;125 cycles for exome sequencing) on a HiSeq2500 (Illumina) were performed by the Kazusa DNA Research Institute.

**Variant calling**

Exome-seq data were mapped onto the reference mouse genome (MM9) by BWA-MEM (Li, 2013; Vasimuddin et al., 2019) with default parameters. After removal of unmapped reads, the rest of sequence reads were sorted with a Samtools (Li et al., 2009; Li, 2011). Subsequently, the bam files were processed for removal of PCR duplicates (Picard) and indel realignment, followed by base recalibration referring SNP (number) (GATK) (McKenna et al., 2010). Mutect2 (https://software.broadinstitute.org/gatk/documentation/tooldocs/3.8-)
0/org_broadinstitute_gatk_tools_walkers_cancer_m2_MuTect2.php) and VarScan 2 (Koboldt et al., 2012) were used to detect tumor specific variants in comparison with those found by germline blood exome-seq and the variants detected by both of the detection tools were taken as candidate ones in this study. Mutation signature analysis was performed by signeR. RNA-seq data were mapped onto mouse reference genome (MM9) by STAR with two-pass alignment (Dobin et al., 2013). The bam files were processed for removal of PCR duplicates (Picard) and inaccurate spliced reads, and indel realignment, followed by base recalibration referring SNP (number) (GATK). Variants were called using GATK HaplotypeCaller.

**T cell receptor repertoire assay**

Total RNA was extracted from TrizolLS lysed highly sorted multimer^CD8^+ T cells or peptide stimulated PD-1^CD137^CD8^+ T cells and reverse transcribed for first-strand cDNA synthesis using a SMARTer Mouse TCR a/b Profiling kit (Clontech). Both universal mix primer and primers specific for the T cell receptor constant region sequence were used for second-strand amplifications, resulting in TCR PCR products of high purity, which were then submitted for high-throughput DNA sequencing using an Illumina Miseq sequencing system. The sequencing was performed with the paired end (Read1: 300 nt, Read2: 300 nt) using the MiSeq Reagent Kit v3 (MS-102-3003, Illumina). All reads of the TCRα and TCRβ repertoire sequence were analyzed using LymAnalyzer ver1.2.2 (Yu et al., 2016), or randomly extracted 1000 sequences of TCRβ by SeqKit (Shen et al., 2016) were parsed by TCRdist (Dash et al., 2017).

**TCR expression vector cloning**

The VDJ regions were amplified by PCR using the second PCR products described above as templates, and cloned together with the corresponding C region into pMXs retroviral vectors.
Peptide-reactive pairs of TCRA and TCRB were concatenated with Furin (RAKR)-SGSG-P2A and cloned into pMXs-IRES-GFP. Site-directed mutagenesis was carried out by PCR.

**Retrovirus production**

pMXs and pCL-Eco were co-transfected into HEK293T cells using FuGene6 (Promega), followed by a medium change to remove the transfection reagents. Virus-containing medium was harvested after 48 h.

**WST-1 Assay**

Briefly, $1 \times 10^3$ MC38 cells were seeded in triplicated well on each 96-well plate before using the Premix WST-1 Cell Proliferation Assay System (Takara Bio Inc, Shiga, Japan) at 24, 48, and 72 h culture. Absorbance was measured after a 1-h incubation. Relative proliferation on day 1 was monitored.

**Statistical analysis**

Statistical analysis was performed using Microsoft Excel, R, and StatMate. Unpaired student’s t-test were used for two experimental groups’ comparison. One-way ANOVA followed by Tukey-test were used for multiple experimental groups’ comparison. Kaplan-Meier survival curves were analyzed by the log-rank test. P values under 0.05 were considered statistically significant and indicated with *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$. 
### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies (for FACS, ELISPOT, Biological Analysis) | BioLegend | Cat# 400104, RRID:AB_326427 |
| Biotin Mouse IgG1, k Isotype Ctrl Antibody, MOPC-21 | BioLegend | Cat# 101824, RRID:AB_1595491 |
| Biotin anti-mouse CD274 (B7-H1, PD-L1) antibody, 10F9G2 | BioLegend | Cat# 124306, RRID:AB_961220 |
| eBioscience™ Streptavidin PE Conjugate | eBioscience™, MBL | Cat# 12-4317-87 |
| Mouse IgG1, kappa Isotype Control, PE Conjugated, Clone MOPC-21 | BioLegend | Cat# 400113, RRID:AB_326435 |
| PE anti-mouse H-2Kb antibody, AF6-88.5 | BioLegend | Cat# 116508, RRID:AB_313735 |
| PerCP anti-mouse CD4 antibody, GK1.5 | BioLegend | Cat# 100432, RRID:AB_893323 |
| PE/Cy7 anti-mouse TCRb chain antibody, H57-597 | BioLegend | Cat# 109222, RRID:AB_893625 |
| APC/Cyanine7 anti-mouse CD8a antibody, 53-6.7 | BioLegend | Cat# 100714, RRID:AB_312753 |
| Alexa Fluor® 488 anti-mouse TNF-a antibody, MP6-XT22 | BioLegend | Cat# 506313, RRID:AB_493328 |
| PE anti-mouse IL-2 antibody, JES6-5H4 | BioLegend | Cat# 503808, RRID:AB_313502 |
| Brilliant Violet 421™ anti-mouse IFN-g antibody, XMG1.2 | BioLegend | Cat# 505830, RRID:AB_2563105 |
| Rat Anti-CD44 Monoclonal Antibody, FITC Conjugated, Clone IM7 | BD Biosciences | Cat# 553133, RRID:AB_2076224 |
| PE anti-mouse CD137 antibody, 17B5 | BD Biosciences | Cat# 106106, RRID:AB_2287565 |
| CD62L (L-Selectin) Monoclonal Antibody (MEL-14), APC, eBioscience™ | Invitrogen | Cat# 17-0621-82, RRID:AB_469410 |
| Brilliant Violet 421™ anti-mouse CD279 (PD-1) antibody, 29F.1A12 | BioLegend | Cat# 135221, RRID:AB_2562568 |
| Anti-CD8 (Mouse) mAb-FITC, KT15 | MBL | Cat# D271-4, RRID:AB_10597265 |
| FITC anti-mouse CD8a antibody, 53-6.7 | BioLegend | Cat# 100706, RRID:AB_312745 |
| CD3e Monoclonal Antibody (145-2C11), PerCP-Cyanine5.5, eBioscience™ | Invitrogen | Cat# 45-0031-82, RRID:AB_1107900 |
| BV510 Rat Anti-Mouse CD45, 30-F11 | BD Biosciences | Cat# 563991, RRID:AB_2734134 |
| APC Rat Anti-Mouse IFN-γ, XMG1.2 | BD Biosciences | Cat# 554413, RRID:AB_398551 |
| PE-Cy™7 Rat Anti-Mouse TNF, MP6-XT22 | BD Pharmingen | Cat# 557644, RRID:AB_396761 |
| PerCP/Cy5.5 anti-mouse CD8b (Ly-3) antibody, YTS156.7.7 | BioLegend | Cat# 126609, RRID:AB_961304 |
| PE/Cy7 anti-mouse CD69 antibody, HI.2F1 | BioLegend | Cat# 104512, RRID:AB_493564 |
| Pacific Blue™ anti-mouse CD3e antibody, 145-2C11 | BioLegend | Cat# 100334, RRID:AB_2028475 |
| APC/Fire™750 anti-mouse/human CD11b antibody, M1/70 | BioLegend | Cat# 101262, RRID:AB_2572122 |
| Biotin anti-mouse CD366 (Tum-3) antibody, RMT3-23 | BioLegend | Cat# 119720, RRID:AB_2571936 |
| Brilliant Violet 650™ Streptavidin | BioLegend | Cat# 405232 |
| Brilliant Violet 785™ anti-mouse CD3e antibody, 145-2C11 | BioLegend | Cat# 100355, RRID:AB_2565969 |
| BV605 Rat Anti-Mouse CD45, 30-F11 | BD Biosciences | Cat# 563053, RRID:AB_2737976 |
| BUV395 Rat Anti-Mouse CD44, IM7 | BD Biosciences | Cat# 740215, RRID:AB_2739963 |
| BUV737 Rat Anti-Mouse CD8a, 53-6.7 | BD Biosciences | Cat# 564297, RRID:AB_2722580 |
| FITC anti-mouse Ly-6c antibody, HK1.4 | BioLegend | Cat# 128006, RRID:AB_1186135 |
| PerCP/Cyanine5.5 anti-mouse CD24 antibody, M1/69 | BioLegend | Cat# 101824, RRID:AB_1595491 |
| Alexa Fluor® 647 Rat Anti-Mouse Siglec-F, ES0-2440 | BD Biosciences | Cat# 562680, RRID:AB_2687570 |
| Brilliant Violet 650™ anti-mouse F4/80 antibody, BM8 | BioLegend | Cat# 123149, RRID:AB_2564589 |
| Brilliant Violet 785™ anti-mouse 1-A/1-E antibody, M5/114.15.2 | BioLegend | Cat# 107645, RRID:AB_2565977 |
| PE anti-mouse CD103 antibody, 2E7 | BioLegend | Cat# 121406, RRID:AB_1133989 |
| BUV395 Rat Anti-Mouse Ly-6G, 1A8 | BD Biosciences | Cat# 563978, RRID:AB_2716852 |
| BUV737 Hamster Anti-Mouse CD11c, HL3 | BD Biosciences | Cat# 564986, RRID:AB_2739034 |
| FITC Rat Anti-Mouse CD44, IM7 | BD Biosciences | Cat# 553133, RRID:AB_2076224 |
| APC Hamster Anti-Mouse CD3e, 145-2C11 | BD Biosciences | Cat# 553066, RRID:AB_398529 |
| APC-Cy™7 Rat Anti-Mouse CD19, 1D3 | BD Biosciences | Cat# 557655, RRID:AB_396770 |
| Brilliant Violet 711™ anti-mouse CD25 antibody, PC61 | BioLegend | Cat# 102049, RRID:AB_2564130 |
| Brilliant Violet 785™ anti-mouse NK-1.1 antibody, PK136 | BioLegend | Cat# 108749, RRID:AB_2564304 |
| PE Rat Anti-Mouse CD62L, MEL-14 | BD Biosciences | Cat# 553131, RRID:AB_394666 |
| BUV395 Rat Anti-Mouse CD8a, 53-6.7 | BD Biosciences | Cat# 563786, RRID:AB_2732919 |
| Item                                                                 | Supplier            | Cat#/ID               |
|----------------------------------------------------------------------|---------------------|-----------------------|
| BUV737 Rat Anti-Mouse CD4, RM4-5                                      | BD Biosciences      | Cat# 564933, RRID:AB_2732918 |
| FOXP3 Monoclonal Antibody (FJK-16s), PE, eBioscience™               | Invitrogen          | Cat# 12-5773-82, RRID:AB_465936 |
| H-2D<sup>+</sup> Adpgk Neoepitope Tetramer-ASMTNELM-PE              | MBL                 | Cat# TB-5113-1         |
| H-2 Kb/SDKFHYCPL-APC                                                 | Immudex             | Cat# JD4508-APC (this study) |
| H-2 Kb/ASHFVNLL-APC                                                 | Immudex             | Cat# JD4118-APC (this study) |
| InVivoMab anti-mouse PD-1 (CD279) antibody, RMP1-14                 | Bio X Cell          | Cat# BE0146, RRID:AB_10949053 |
| InVivoMab anti-mouse CTLA-4 (CD152) antibody, 9D9                    | Bio X Cell          | Cat# BE0164, RRID:AB_10949069 |
| Ultra-LEAF<sup>®</sup> Purified anti-mouse CD40 antibody, 1C10       | BioLegend           | Cat# 102812, RRID:AB_2561489 |
| LEAF<sup>®</sup> Purified anti-mouse CD28 antibody, 37.51            | BioLegend           | Cat# 102112, RRID:AB_313723 |
| Purified anti-mouse CD16/32 antibody, 93                             | BioLegend           | Cat# 101302, RRID:AB_312877 |
| Rat Anti-IFN-gamma Monoclonal Antibody, Unconjugated, Clone R4-6A2  | BD Biosciences      | Cat# 551216, RRID:AB_394094 |
| Rat Anti-IFN-gamma Monoclonal Antibody, Biotin Conjugated, Clone     | BD Biosciences      | Cat# 554410, RRID:AB_395374 |
| H-2 Kb/Neoepitope candidates and counterparts synthetic peptides     | GenScript           | N/A (this study)       |
| LIVE/DEAD<sup>™</sup> Fixable Violet Dead Cell Stain Kit, for 405 nm excitation | Invitrogen       | Cat# L34964 |
| LIVE/DEAD<sup>™</sup> Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation | Invitrogen        | Cat# L34966 |
| Collagenase D                                                        | Roche               | Cat# 1108882001       |
| DNase I                                                             | Roche               | Cat# 11284932001      |
| Poly(IEC)                                                           | TOCRIS              | Cat# 4287             |
| BD GolgiPlug<sup>™</sup>                                              | BD Biosciences      | Cat# 555029           |
| Actinomycin D                                                        | Sigma               | Cat# A1410            |
| Polybrene Solution                                                   | Nacalai             | Cat# 12996-81         |
| FuGENE® 6 Transfection Reagent                                      | Promega             | Cat# E2691            |
| Lipofectamine<sup>™</sup> LTX Reagent with PLUS<sup>™</sup> Reagent  | ThermoFisher        | Cat# 15338100        |
| Critical Commercial Assays                                           |                     |                      |
| Mouse IFN-gamma DuoSet ELISA                                        | R&D Systems         | Cat# DY485-05         |
| SMARTer<sup>®</sup> Mouse TCR a/b Profiling Kit                      | TAKARA BIO INC      | Cat# Z4404N          |
| Wizard<sup>®</sup> Genomic DNA Purification Kit                      | Promega             | Cat# A1120            |
| SureSelect XT Mouse All exon                                         | Agilent             | Cat# 5190-4641       |
| SureSelect Strand Specific RNA Reagent Kit                           | Agilent             | Cat# G9091A           |
| Agencourt AMPure XP Kit                                             | Beckman Coulter Genomics | Cat# A63880   |
| TRIzol<sup>™</sup> LS Reagent                                       | Invitrogen          | Cat# 10296028        |
| BD Cytofix/Cytopermtm<sup>™</sup>                                    | BD Biosciences      | Cat# 554714           |
| eBioscience<sup>™</sup> Foxp3 / Transcription Factor Staining Buffer Set | Invitrogen       | Cat# 00-5523-00       |
| Premix WST-1 Cell Proliferation Assay System                        | Takara Bio          | Cat# MK400            |
| Deposited Data                                                       |                     |                      |
| C57BL/6J blood Exome-Seq                                            | DDBJ                | DRA010264            |
| MC38 WT or PD-L1 KO tumors Exome-Seq                                | DDBJ                | DRA010264            |
| MC38 WT or PD-L1 KO tumors RNA-Seq                                  | DDBJ                | DRA010264            |
| TCR Seq for MutDpagt1 and MutCry1                                    | DDBJ                | DRA010264            |
### Experimental Models: Cell Lines

| Name          | Source | Catalogue Number |
|---------------|--------|------------------|
| HEK293T       | ATCC   | Cat# CRL-3216    |
| MC38          | Dr. MT Lotze | N/A              |
| EL4           | ATCC   | Cat# TIB-39      |
| B16F10        | ATCC   | Cat# CRL-6475    |
| E0771         | CH3 BioSystems | Cat# 940001     |
| TG40 mCD8A/mCD8B | Dr. Saito | N/A            |

### Experimental Models: Organisms/Strains

| Name          | Source | Catalogue Number |
|---------------|--------|------------------|
| Mouse: C57BL/6 | Charles River Japan | N/A              |
| Mouse: Rag1(-/-) | The Jackson Laboratory | N/A          |

### Recombinant DNA

| Name                          | Source | Catalogue Number |
|-------------------------------|--------|------------------|
| pMXs-IRES-GFP Retroviral Expression Vector | Cell Biolabs | Cat# RTV-013 |
| pCas-Guide-EF1a-GFP CRISPR Vector | OriGene | Cat# GE100018 |

### Software and Algorithms

| Software          | Version | Source                  |
|-------------------|---------|-------------------------|
| STAR              |         | Dobin et al., 2013      |
| NetMHCpan-3.0     |         | Nielsen et al., 2016    |
| samtools v1.9     |         | Li et al., 2009         |
| Picard Toolkit v2.20 |      | Broad Institute         |
| VarScan v2.4      |         | Koboldt et al., 2012    |
| bwa-mem2          |         | Vasmuddin et al., 2019  |
| bwa v0.7          |         | Li et al., 2009         |
| GATK v3           |         | Broad Institute         |
| ClicO FS          |         | Cheong et al., 2015     |
| R3.6.1            |         | The R Project           |
| RStudio Desktop   |         | RStudio Team            |
| TCGAbiolinksGUI   |         | Silva et al., 2018      |
| gdc-rnaseq-tool   |         | Dr. Reid                 |
| FlowJo v10.5.3    |         | BD                      |
| FlowJo plugins FlowSOM v1.5 |      |                         |
| Excel             |         | Microsoft               |
| StatMate V for Win&Mac Hybrid |    | ATMS Co., Ltd          |
| LymAnalyzer gui v1.2.2 |    | Yu et al., 2016         |
| TCRdist v0.0.2    |         | Dash et al., 2017       |
| SeqKit v0.8       |         | Shen et al., 2016       |
| signeR            |         | Rosales RA et al., 2016 |
References
Dash, P., Fiore-Gartland, A.J., Hertz, T., Wang, G.C., Sharma, S., Souquette, A., Crawford, J.C., Clemens, E.B., Nguyen, T.H.O., Kedzierska, K., et al. (2017). Quantifiable predictive features define epitope-specific T cell receptor repertoires. Nature 547, 89-93.
Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M. and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15-21.
Koboldt, D.C., Zhang, Q., Larson, D.E., Shen, D., McLellan, M.D., Lin, L., Miller, C.A., Mardis, E.R., Ding, L. and Wilson, R.K. (2012). VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome Res 22, 568-576.
Li, H. (2011). A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. Bioinformatics 27, 2987-2993.
Li, H. (2013). Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv preprint arXiv:1303.3997.
Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R. and Genome Project Data Processing, S. (2009). The Sequence Alignment/Map format and SAMtools. Bioinformatics 25, 2078-2079.
McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M., et al. (2010). The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 20, 1297-1303.
Nielsen, M. and Andreatta, M. (2016). NetMHCpan-3.0; improved prediction of binding to MHC class I molecules integrating information from multiple receptor and peptide length datasets. Genome Med 8, 33.
Shen, W., Le, S., Li, Y. and Hu, F. (2016). SeqKit: A Cross-Platform and Ultrafast Toolkit for FASTA/Q File Manipulation. PLoS One 11, e0163962.
Shimizu, K. and Fujii, S. (2009). DC therapy induces long-term NK reactivity to tumors via host DC. Eur J Immunol 39, 457-468.
Shimizu, K., Hidaka, M., Kadowaki, N., Makita, N., Konishi, N., Fujimoto, K., Uchiyama, T., Kawano, F., Taniguchi, M. and Fujii, S. (2006). Evaluation of the function of human invariant NKT cells from cancer patients using α-galactosylceramide-loaded murine dendritic cells. J. Immunol. 177, 3484-3492.
Shimizu, K., Kurosawa, Y., Taniguchi, M., Steinman, R.M. and Fujii, S. (2007). Cross-presentation of glycolipid from tumor cells loaded with α-galactosylceramide leads to potent and long-lived T cell mediated immunity via dendritic cells. J. Exp. Med. 204, 2641-2653.
Silva, T.C., Colaprico, A., Olsen, C., Malta, T.M., Bontempi, G., Ceccarelli, M., Berman, B.P. and Noushmehr, H. (2018). Tegabiolinksgui: a graphical user interface to analyze cancer molecular and clinical data. F1000Research 7.
Vasimuddin, M., Misra, S., Li, H. and Aluru, S. (2019) Efficient architecture-aware acceleration of BWA-MEM for multicore systems: IEEE. pp. 314-324.
Yokosuka, T., Takase, K., Suzuki, M., Nakagawa, Y., Taki, S., Takahashi, H., Fujisawa, T., Arase, H. and Saito, T. (2002). Predominant role of T cell receptor (TCR)-alpha chain in forming preimmune TCR repertoire revealed by clonal TCR reconstitution system. J Exp Med 195, 991-1001.
Yu, Y., Ceredig, R. and Seoighe, C. (2016). LymAnalyzer: a tool for comprehensive analysis of next generation sequencing data of T cell receptors and immunoglobulins. Nucleic Acids Res 44, e31.