ORIGINAL ARTICLE

EBV-associated gastric cancer evades T-cell immunity by PD-1/PD-L1 interactions

Sho Sasaki[1] · Jun Nishikawa[2] · Kohei Sakai[3] · Hisashi Iizasa[4] · Hironori Yoshiyama[4] · Masashi Yanagihara[2] ·
Takuya Shuto[2] · Kanami Shimokuri[2] · Teru Kanda[5] · Yutaka Suehiro[3] · Takahiro Yamasaki[3] · Isao Sakaida[1]

Abstract

Background Epstein–Barr virus (EBV) is an oncogenic human herpesvirus involved in the development of around 10% of gastric cancers. The overexpression of PD-L1 is one of the features of EBV-associated gastric cancer (EBVaGC); however, the function of PD-L1 has not been studied in EBVaGC.

Methods We used three EBVaGC cell lines, SNU719 cells, NCC24 cells, and YCCEL1 cells, to evaluate the PD-L1 expression and function in EBVaGC. Jurkat T-lymphocytes expressing PD-1 were co-cultured with NCC24 and YCCEL1 cells and the cell cycles were analyzed. To study the regulatory mechanism for PD-L1 expression, the 3′UTR of PD-L1 was sequenced, and the effect of inhibitors of the IFN-γ signaling pathway was evaluated.

Results All of the EBVaGC cell lines expressed PD-L1, and its expression was further enhanced by stimulation with IFN-γ. In Jurkat T-cells co-cultured with IFN-γ-stimulated NCC24 and YCCEL1 cells, the number of cells in the G0/G1 phase was significantly increased. This G0/G1 arrest was partially released by administration of anti-PD-L1 antibody. We found SNPs in PD-L1 3′UTR nucleotide sequences that were located at seed regions for microRNAs. Treatment of EBVaGC cell lines with JAK2-inhibitor, PI3K-inhibitor, and mTOR inhibitor reduced the level of PD-L1 expression to the same level as cells without IFN-γ stimulation.

Conclusions EBVaGC cells expressing high levels of PD-L1 suppress T-cell proliferation, and the IFN-γ signaling pathway is involved in the expression of PD-L1.

✉ Jun Nishikawa
  junnis@yamaguchi-u.ac.jp

1 Department of Gastroenterology and Hepatology, Yamaguchi University Graduate School of Medicine, Ube, Japan

2 Department of Laboratory Science, Yamaguchi University Graduate School of Medicine, Minamikogushi 1-1-1, Ube, Yamaguchi 755-8505, Japan

3 Department of Oncology and Laboratory Medicine, Yamaguchi University Graduate School of Medicine, Ube, Japan

4 Department of Microbiology, Shimane University Faculty of Medicine, Shimane, Japan

5 Division of Microbiology, Faculty of Medicine, Tohoku Medical and Pharmaceutical University, Sendai, Japan

Introduction

Gastric cancer is the fourth most frequent cancer in the world and is the second leading cause of cancer deaths. More than 95,000 new patients are diagnosed each year, and there were 72,000 gastric cancer-related deaths worldwide in 2012. The prognosis of unresectable and recurrent gastric cancer with distant metastasis is poor, and thus the development of effective chemotherapy is desired. *Helicobacter pylori* infection is the most important cause of gastric cancer, whereas Epstein–Barr virus (EBV) is also known to be involved in the development of gastric cancer [1].

EBV is strongly associated with Burkitt lymphoma and nasopharyngeal carcinoma [2]. EBV-associated gastric cancer (EBVaGC) results from the monoclonal proliferation of EBV-infected cells [3, 4], which suggests that EBV plays an important role in the development of cancer. According

to a characterization based on molecular biology, The Cancer Genome Atlas (TCGA) divided gastric cancer into four types: EBVaGC, microsatellite unstable tumors, genomically stable tumors, and chromosomally unstable tumors. EBVaGC shows recurrent PIK3CA mutations, extreme DNA hypermethylation, and the amplification of JAK2, PD-L1, and PD-L2 overexpression [5].

The pathological features of EBVaGC show outstanding lymphocytic infiltration. Tumor tissues of EBVaGC are accompanied by massive lymphocyte infiltration containing a large amount of IFN-γ and PD-L1 is an IFN-γ responsive gene [6, 7]. EBVaGC is always exposed to the IFN-γ released from T-lymphocytes and is stimulated to express high levels of PD-L1. The expression of PD-L1 is observed more frequently in EBVaGC lesions than in the lesions of EBV-negative controls [8–12]. It thus seems that EBVaGC evades immunological surveillance by T-lymphocytes via the PD-1/PD-L1 pathway.

Treatment by blocking PD-1/PD-L1, which is an immunoinhibitory checkpoint molecule, is currently showing good prognosis in various cancers [13–19]. The anti-PD-1 antibody nivolumab is reported to have therapeutic effects in malignant melanoma, non-small cell lung cancer, renal cell carcinoma, and Hodgkin's lymphoma. Recently, patients with advanced or recurrent gastric cancer are being treated with nivolumab because they cannot undergo standard therapy due to their physical condition or intolerance. These patients have experienced significantly prolonged overall survival in comparison to those who received a placebo [20]. Panda et al. recently showed a patient with EBVaGC who received clinical benefit from treatment with the anti-PD-L1 antibody avelumab, although the tumor did not have a high mutation burden or mismatch repair defect [21].

Immune checkpoint blockade therapy can be applied in the treatment of EBVaGC because EBVaGC shows overexpression of PD-L1 and a lymphocyte-rich histology. In this study, we examined the function of the PD-1/PD-L1 pathway and the mechanisms of PD-L1 expression in EBVaGC.

Materials and methods

Cell cultures

The EBVaGC cell lines SNU719 and NCC24 were obtained from the Korea Cell Line Bank (Seoul, South Korea), and the YCCEL1 cell line was obtained from Prof. SY Rha from the Yonsei Cancer Center [22–24]. The EBV-negative gastric cancer cell lines AGS and NUGC3 and the T-cell lymphoma cell line Jurkat were obtained from ATCC.

The SNU719, AGS, NUGC3, and Jurkat cells were cultured in RPMI-1640; 25 mM HEPES and 25 mM NaHCO3 were added to the RPMI-1640 for NCC24. YCCEL1 was cultured in MEM. All cultures were supplemented with 10% heat-inactivated FBS (Sigma-Aldrich, St. Louis, MO, USA) and cultured at 37 °C in a humidified incubator with 5% CO_2.

Expression of PD-L1, PD-1 and IFN-γ receptors

The expression of PD-L1 was evaluated by flow cytometry. After washing with PBS containing 10% fetal calf serum, cells were incubated with anti-PD-L1 antibody (ab109052, Abcam) or matching isotype control antibodies for 1 h, washed, and fluorescent staining was carried out by the avidin–biotin method (Anti-mouse immunoglobulins/Biotinylated [Dako, Denmark], Streptavidin-PerCP/Cy 5.5 [BioLegend]). A total of 20,000 gated events were analyzed by flow cytometry using a FACSCaliber™ system (Becton Dickinson Labware, Bedford, MA, USA). In addition, the gastric cancer cell lines were treated with recombinant human IFN-γ 100 U/mL (PeproTech, Rocky Hill, NJ, USA) and reacted for 24 h to evaluate the expression of PD-L1.

The expression of IFN-γ receptor 1 (IFNGR1) was evaluated in the EBVaGC cell lines NCC24, SNU719, and YCCEL1 and in the EBV-negative cell lines AGS and NUGC3. Cells were collected and reacted with anti-IFNGR1 antibody (ab11286, Abcam) or matching isotype control antibodies at 4 °C for 1 h. After washing, the cells were evaluated by flow cytometry. The expression of PD-1 in Jurkat cells was similarly examined [25, 26].

Co-culture and the cell cycle analysis

NCC24 and YCCEL1 cells (EBVaGC cell lines) were seeded at a density of 1×10^6 cells/mL. After 24 h of incubation, the cells were treated in the presence or absence of IFN-γ 100 U/ml for 24 h. After washing the cells twice with PBS, the cells were co-cultured with Jurkat cells at a density of 5×10^6 cells/mL for 24 h. Jurkat cells co-cultured with NCC24 and YCCEL1 were collected, centrifuged, washed, and a cell cycle assay was performed. To measure the DNA content, treated cells were collected and fixed with 70% ethanol. After resuspension, the fixed cells were washed and incubated with propidium iodine (final concentration, 10 μg/mL) (Merck Millipore) and RNase (final concentration, 0.2 mg/mL). After staining, flow cytometry was performed to analyze the cell cycle phase. The cell number ratio of each cell cycle phase was calculated based on the results of 4 independent experiments. Furthermore, to evaluate the function of PD-L1, co-culturing was performed by administering 20 μg/mL of PD-L1 antibody (Anti-Human CD 274 [B7-H1] Functional Grade Purified 16-5983 [eBioscience]).
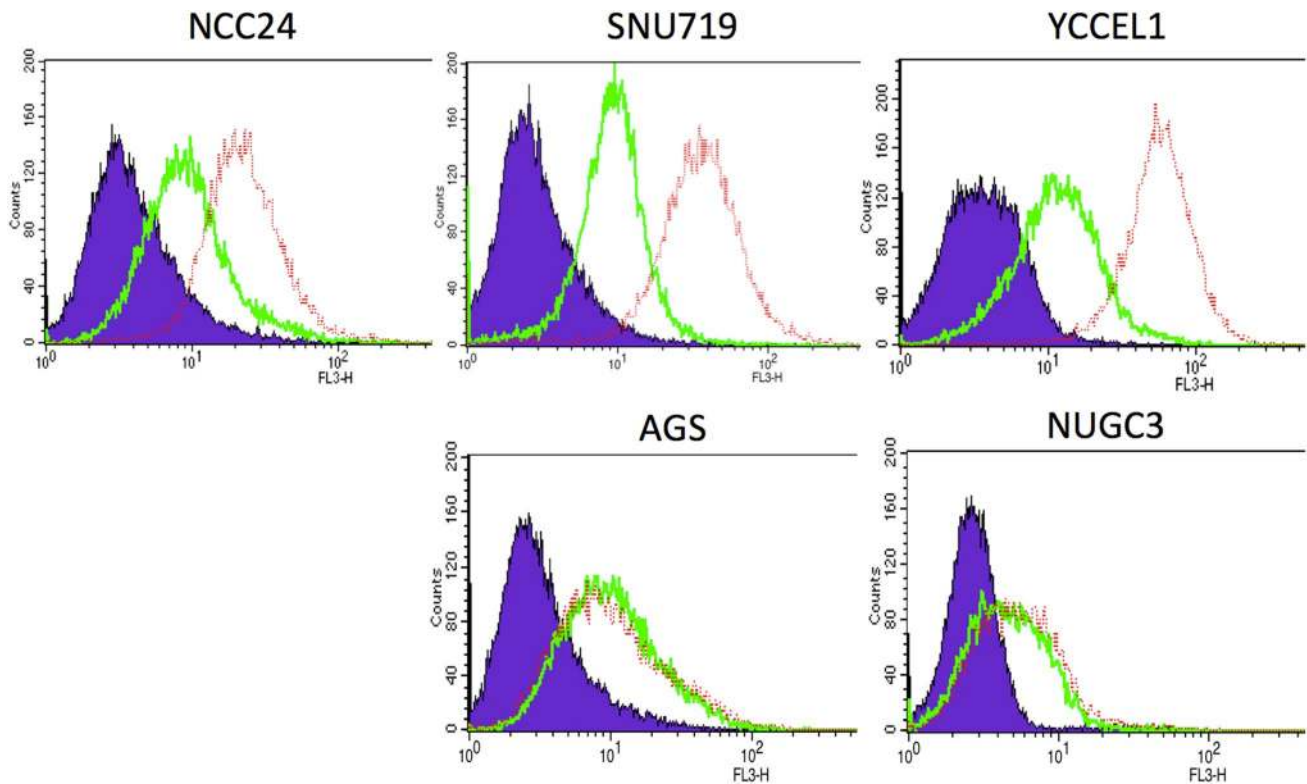
Fig. 1 The expression of PD-L1 in gastric cancer cell lines. NCC24, SNU719, and YCCEL1 are EBVaGC cell lines, and AGS and NUGC3 are EBV-negative gastric cancer cell lines. The violet solid line shows the PD-L1 expression of the isotype control, the green line shows that of cells without IFN-γ treatment, and the red line shows that of cells treated with IFN-γ for 24 h. The vertical axis indicates the number of cells, and the horizontal axis indicates the fluorescence intensity

## Single nucleotide polymorphisms (SNPs) of PD-L1 3′UTR

The sequence of the PD-L1 3′UTR was analyzed to study the mechanisms involved in the expression of PD-L1. rs4143815 and rs2297136 were seed regions binding microRNAs that inhibit the expression of PD-L1 [27]. The following primers were used for the amplification of the PD-L1 3′UTR: 5′-GAT ACA CAT TTG GAG GAG ACG-3′ (forward) and 5′-CAA ATA CTC CAT GTT TTA CTA G-3′ (reverse). Briefly, each 50 μL reaction contained 20 mM Tris–HCl (pH 9.0), 100 mM KCl, 2.0 mM MgCl₂, 200 μM of each dNTP, 0.2 μM of each primer, 1.25 U of Taq DNA polymerase, and 0.1–0.2 μL genomic DNA (10–30 ng). Thermal cycling was performed in an iCycler Thermal Cycler (Bio-Rad) with an initial denaturation of 5 min at 94 °C, followed by 35 cycles of 94 °C for 20 s, 55 °C for 30 s, and 72 °C for 1 min. Final extension was completed at 72 °C for 7 min. The PCR products were used as templates for Sanger DNA sequencing at GENEWIZ (Saitama, Japan). The SNPs (rs2297136 and rs4143815) located in the PD-L1 3′UTR were examined.

PD-L1 3′UTR luciferase reporter assay

Genomic DNAs were extracted from SNU719 (CC at rs4143815) and NUGC3 (GG at rs4143815). The PD-L1 3′UTR was amplified using specific primers, 5′-CTCGAG TTTAGGGGTTCATCGGGG-3′ (forward) and 5′-GCGGCC GCTGTCCCGTTCCAACACTGA-3′ (reverse). The products of PCR were cloned in TA vector (Promega Corporation, WI, Madison). Two sequences containing the potential biding site for miR-570 were cloned into the psiCHECK2 vector (Promega) downstream of the luciferase gene. For the luciferase assays, the vectors were transfected to SNU719, YCCEL1, and AGS cells. Following incubation for 48 h, the cells were collected and analyzed for luciferase activity using the dual-luciferase reporter assay system (Promega).

Drug treatment and the expression of PD-L1

The following reagents were used to investigate the mechanisms involved in the expression of PD-L1 in EBVaGC cell lines: fedratinib JAK2-inhibitor (1 μM), AZD1480 JAK2-inhibitor (10 0 nM; Selleck Chemicals, Houston, TX, USA),
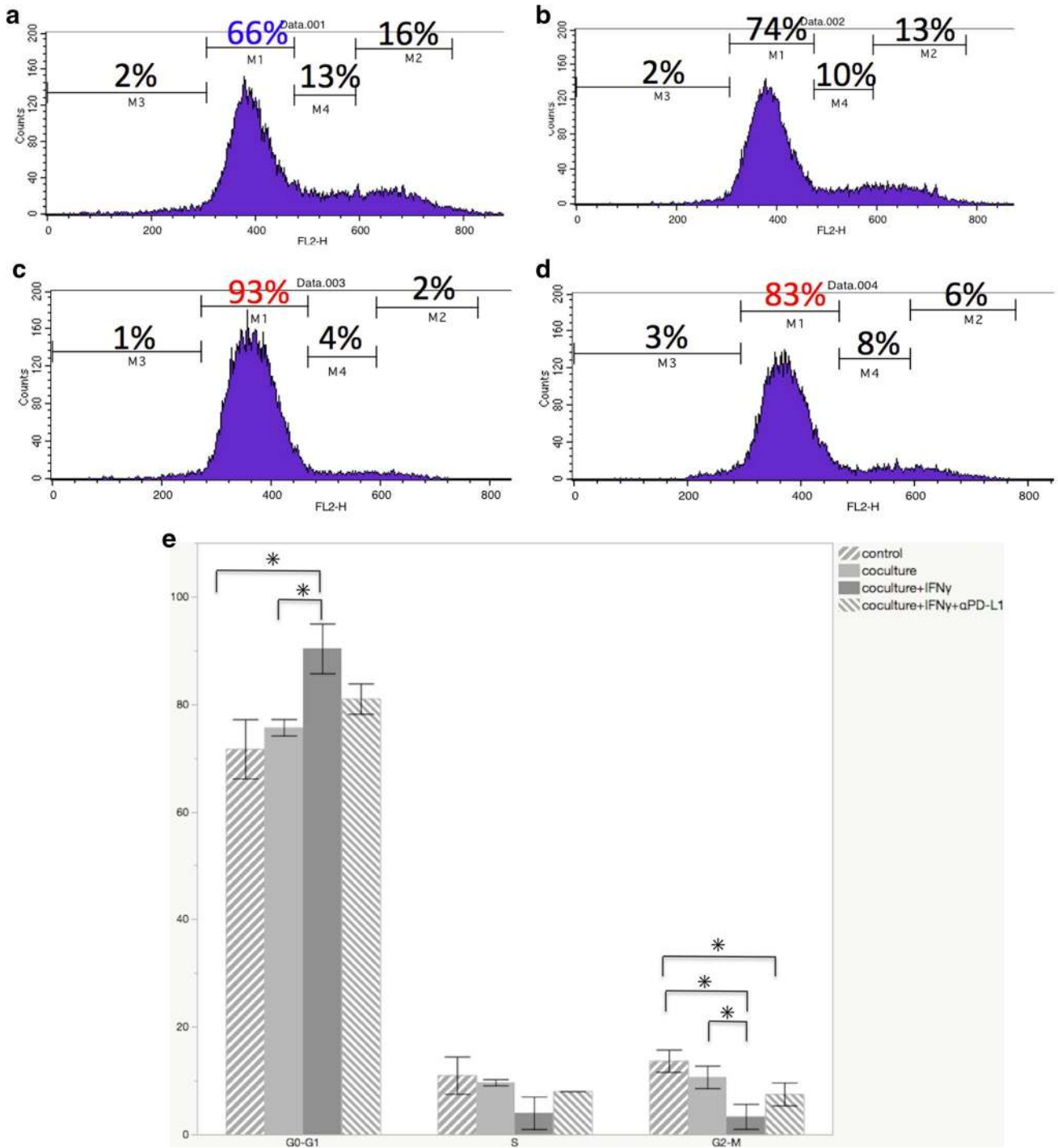
Fig. 2 The cell cycle of Jurkat cells co-cultured with NCC24 cells (an EBVaGC cell line). **a** Jurkat cells alone, **b** Jurkat cells co-cultured with NCC24 cells, **c** Jurkat cells co-cultured with IFN-γ-treated NCC24 cells, and **d** Jurkat cells co-cultured with IFN-γ-treated NCC24 cells, which were also treated with αPD-L1. Representative results of one experiment are shown. The vertical axis indicates the number of cells, and the horizontal axis indicates the fluorescence intensity. **e** The cell cycle of Jurkat cells co-cultured with NCC24 cells. The ratio of the number of cells in each phase is represented by a bar graph in the order of **a–d**. The mean and standard deviation of four independent experiments are represented by bars. Asterisks indicate the values for which there were statistically significant differences between the two groups ($p < 0.05$)

LY294002 PI3K-inhibitor (50 μM) and Wortmannin PI3K-inhibitor (1 μM) (Sigma-Aldrich), and rapamycin mTOR inhibitor (25 nM; Cell Signaling Technology, Danvers, MA, USA). The NCC24, SNU719, and YCCEL1 EBVaGC cell lines were administered at a density of 1×10^6 cells/mL, and EBVaGC cell lines were treated with the abovementioned reagent at 24 h after 100 U/mL IFN-γ treatment. After 24 h of incubation, the cells were collected and centrifuged. Thereafter, the change in PD-L1 expression was evaluated by flow cytometry according to the method described above.

Statistical analysis

The results were statistically analyzed using Tukey's honestly significant difference test. P values of < 0.05 were considered to indicate statistical significance. All statistical analyses were performed using the JMP software program (version 9 for Mac; SAS Institute, Cary, NC, USA).

Results

Expression of PD-L1 in EBVaGC

PD-L1 was expressed on the EBVaGC cell lines NCC24, SNU719, and YCCEL1. The expression was further enhanced by stimulation with IFN-γ for 24 h (Fig. 1). The expression of PD-L1 was also verified in NUGC3 cells and AGS cells, which are EBV-negative gastric cancer cell lines. However, the expression of PD-L1 was not enhanced when NUGC3 cells and AGS cells were stimulated with IFN-γ for 24 h (Fig. 1).

The expression of IFN-γ receptor 1 (IFN-γR1) was observed in all gastric cancer cell lines, but the expression level was lower in AGS cells than in EBV-positive cells. Jurkat cells also expressed PD-1 (data not shown) [25, 26].

Cell cycle analysis of Jurkat cells co-cultured with EBVaGC

When Jurkat cells were co-cultured with IFN-γ-treated NCC24 and YCCEL1 cells, the number of cells in the G0/G1 phase was significantly increased. However, when Jurkat cells were co-cultured with NCC24 and YCCEL1 cells without IFN-γ treatment, cell cycle arrest was not observed. The addition of anti-PD-L1 antibody to the co-culture of Jurkat cells with NCC24 or YCCEL1 cells, partially released the G0/G1 arrest. Representative results are shown in Figs. 2a–d and 3a–d. The cell number ratio of each cell cycle phase was statistically evaluated based on the results obtained from 4 independent experiments. Asterisks indicate the values showing statistically significant differences between the two groups in Figs. 2e and 3e.

Analysis of PD-L1 3′UTR

The sequence of the PD-L1 3′UTR was analyzed to study the mechanisms involved in the expression of PD-L1. rs4143815 and rs2297136 which were seed regions binding microRNAs that inhibit the expression of PD-L1 [27]. A G-C mutation was found at rs4143815 in all of the EBVaGC cell lines. In addition, SNU719 and YCCEL1 cells showed a G-A mutation at rs2297136 (Fig. 4).

The effect of the rs4143815 SNP on the interaction between the *PD-L1* 3′-UTR and miR-570 was also examined. The luciferase activity of CC and GG allele-specific psiCHECK2 constructs did not show any significant difference between the three cell lines (Fig. 5).

Expression of PD-L1 is suppressed by JAK2, PI3K, and mTOR inhibitors

EBVaGC cell lines were treated with (a) JAK2-inhibitor fedratinib (1 μM), (b) JAK2-inhibitor AZD1480 (100 nM), (c) PI3K-inhibitor LY294002 (50 μM), (d) PI3K-inhibitor wortmannin (1 μM), and (e) mTOR inhibitor rapamycin (25 nM) at 24 h after IFN-γ treatment. When JAK2-inhibitors were administered in the EBVaGC cell cultures, the expression of PD-L1 was suppressed to a similar level as the cells untreated with IFN-γ. The expression of PD-L1 was similarly suppressed by the treatment of cells with PI3K inhibitors LY294002 (50 μM) and Wortmannin (1 μM) or rapamycin (25 nM), an mTOR inhibitor. Figure 6 shows a representative result from the experiments using NCC24 cells. Similar results were obtained from the experiments using SNU719 and YCCEL1 cells (data not shown). None of the inhibitors showed any effect on PD-L1 expression in the NCC24, SNU719, and YCCEL1 cells untreated with IFN-γ (data not shown).

Discussion

The overexpression of PD-L1 has been reported as one of the features of EBVaGC [5]. Numerous reports have also shown that PD-L1 is expressed in EBVaGC tumors [8–12]. Using EBVaGC cell lines, we first reported that EBVaGC cells expressing PD-L1 strongly suppressed T-cell proliferation and that the IFN-γ signaling pathway is involved in the expression of PD-L1.

The pathological characteristics of EBVaGC are shown by its infiltration of lymphocytes [28, 29]. Approximately, 50% of EBVaGC expresses LMP2A, which is a latent EBV gene but is recognized and targeted by cytotoxic T-lymphocytes [30]. We verified the PD-L1 expression in EBVaGC cell lines. We then co-cultured EBVaGC cell lines with PD-1-positive T-cells to reproduce the lymphocytic infiltration
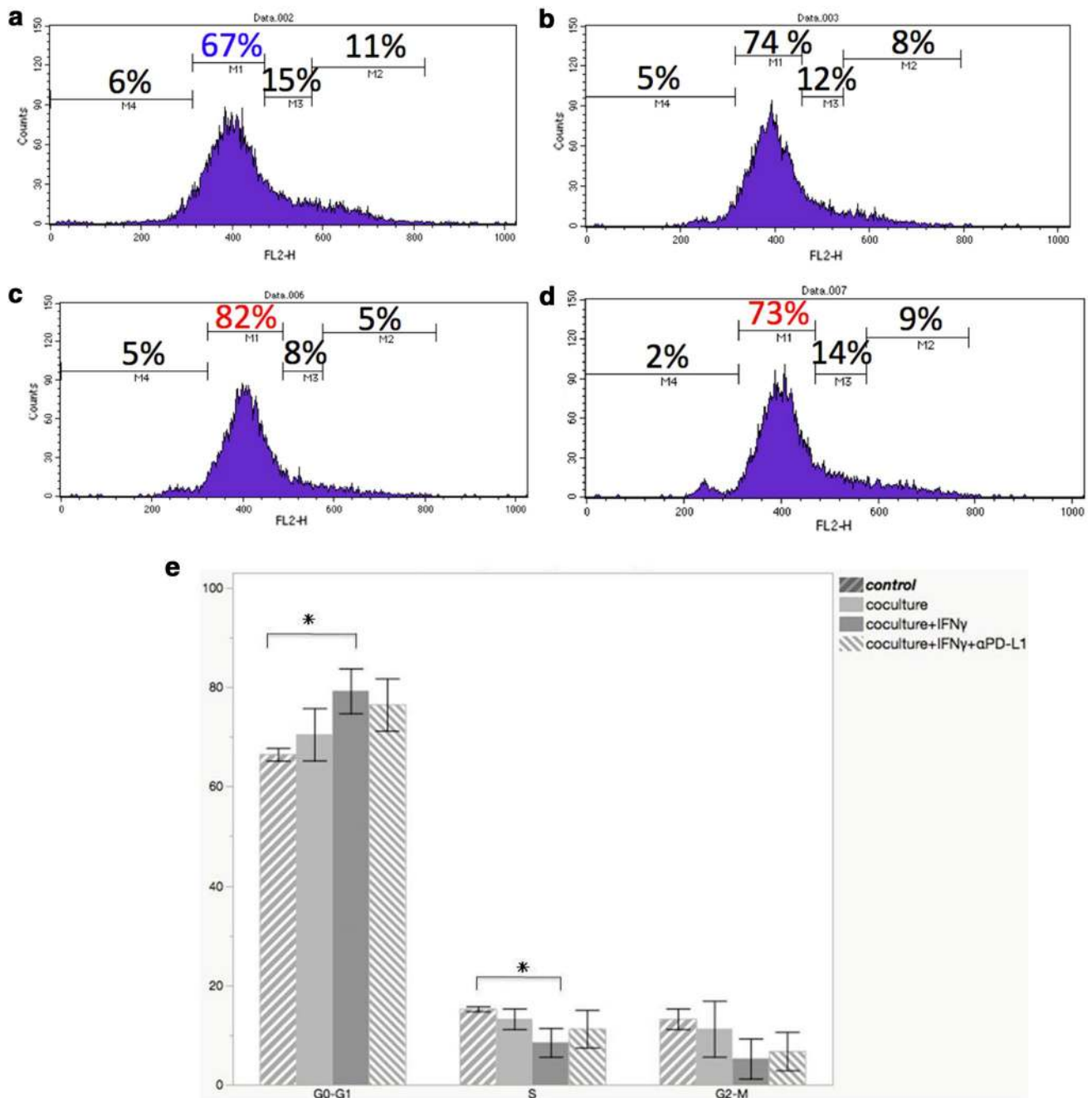
Fig. 3 The cell cycle of Jurkat cells co-cultured with YCCEL1 cells (an EBVaGC cell line). **a** Jurkat cells alone, **b** Jurkat cells co-cultured with YCCEL1 cells, **c** Jurkat cells co-cultured with IFN-γ-treated YCCEL1 cells, and **d** Jurkat cells co-cultured with IFN-γ-treated YCCEL1 cells, which were also treated with αPD-L1. Representative results of one experiment are shown. The vertical axis indicates the number of cells, and the horizontal axis indicates the fluorescence intensity. **e** The cell cycle of Jurkat cells co-cultured with YCCEL1 cells. The ratio of the number of cells in each phase is represented by a bar graph in the order of **a–d**. The mean and standard deviation of four independent experiments are represented by bars. Asterisks indicate the values for which there were statistically significant differences between the two groups ($p < 0.05$)

observed in EBVaGC in the in vitro culture system. As a result, G0/G1 arrest was induced in T-cells surrounding the EBVaGC cells. The EBVaGC cells, which highly expressed PD-L1, suppressed the proliferation of T-cells, and the G0/G1 arrest of the T-cells was partially cancelled by treating EBVaGC with anti-PD-L1 antibody. These findings showed the possible use of anti-PD-L1 antibodies in the treatment of EBVaGC.

The effects of anti-PD-1/PD-L1 antibody treatment have been reported in various cancers, including melanoma

Fig. 4 The sequence data of the PD-L1 3′UTR in the gastric cancer cell lines. NCC24, SNU719, and YCCEL1 are EBVaGC cell lines, and AGS and NUGC3 are EBV-negative gastric cancer cell lines. The results of sequencing include rs4143815 on the left side and rs2297136 on the right side
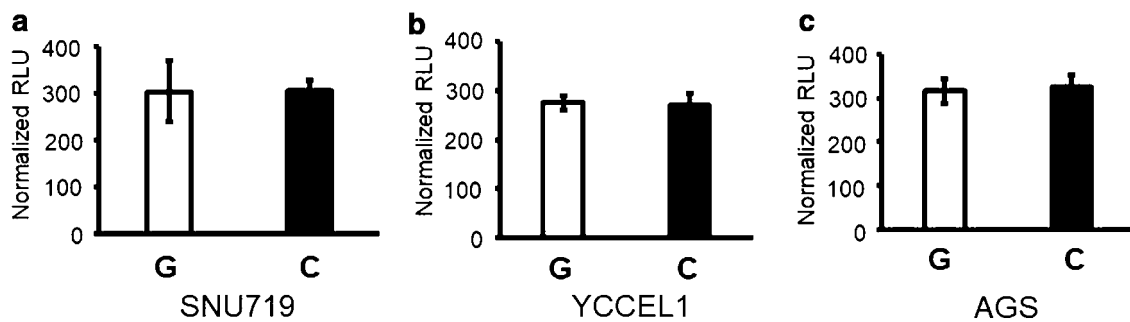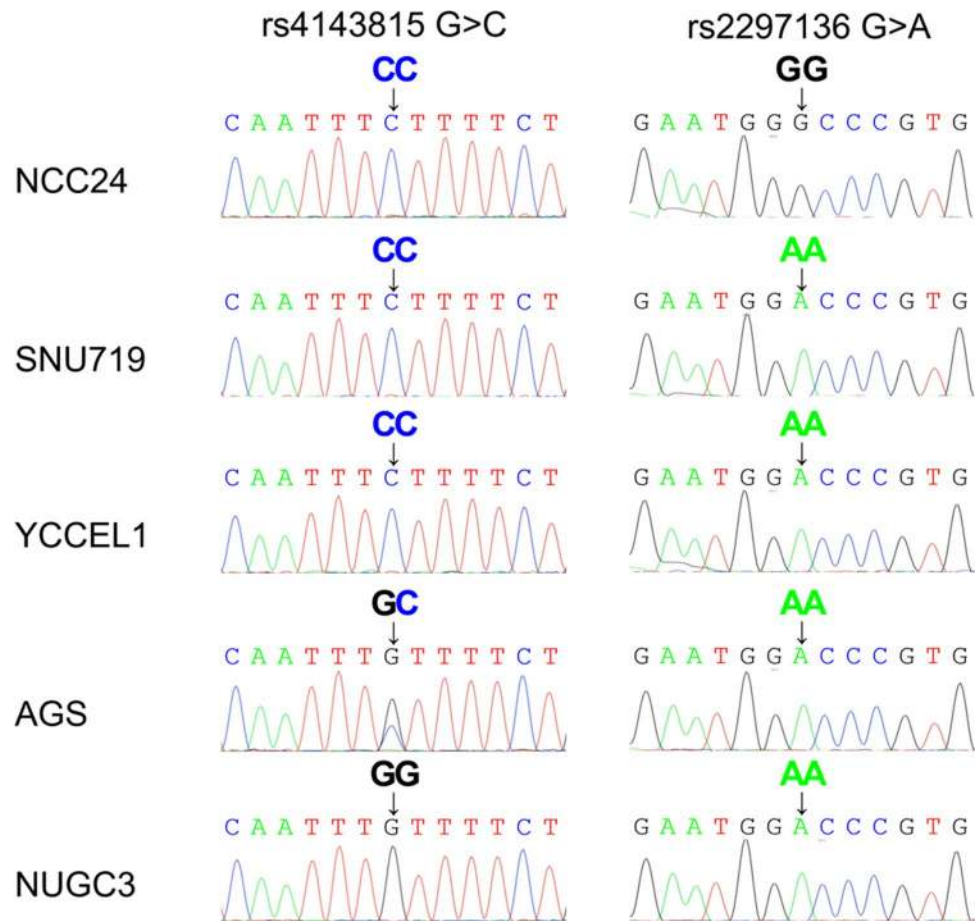

Fig. 5 Analysis of the PD-L1 3′UTR by luciferase reporter assay. SNU-719 (**a**) and YCCEL1 (**b**) are EBVaGC cell lines, and AGS is an EBV-negative gastric cancer cell line. White bars show the luciferase activity from the reporter construct containing GG at rs4143815 in the PD-L1 3′UTR. Black bars show the results from the reporter construct containing CC at rs4143815

[13–19]. Recently, the anti-PD-1 antibody nivolumab was reported to show good prognosis, with prolonged overall survival in patients with unresectable and recurrent gastric cancers refractory to conventional chemotherapy [20]. In comparison to melanoma, there are too many patients suffering from lung cancer and gastric cancer to reduce the cost of

anti-PD-1 treatment, which puts added pressure on medical economics. It is important to search for biomarkers that predict the effects of anti-PD-1/PD-L1 antibody therapy [31]. Previous studies reported that tumors with high expression levels of PD-L1 and those with lymphocytic infiltration in the stroma are more likely to respond to anti-PD-1 antibody
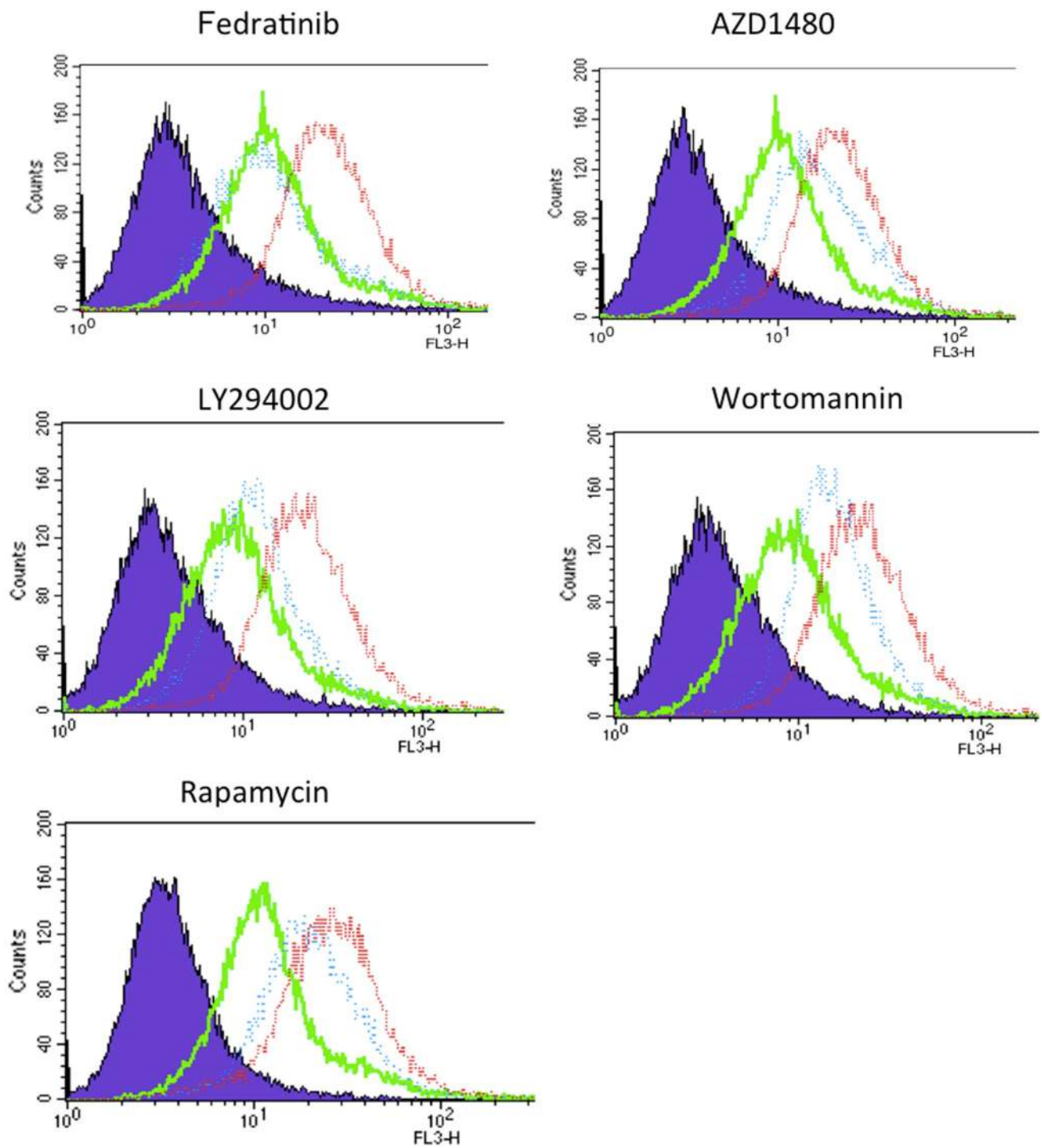
Fig. 6 The suppression of PD-L1 expression in EBVaGC cell lines by drug treatment. NCC24 cells (an EBVaGC cell line) were treated with **a** JAK2-inhibitor fedratinib (1 μM), **b** JAK2-inhibitor AZD1480 (100 nM), **c** PI3K-inhibitor LY294002 (50 μM), **d** PI3K-inhibitor wortmannin (1 μM), and **e** mTOR inhibitor rapamycin (25 nM). Representative results of one experiment with NCC24 are shown. The results of SNU 719 and YCCEL1 were similar (data not shown). The violet solid area shows the isotype control, the green line shows the expression of PD-L1 without IFN-γ treatment, and the red line shows the expression of PD-L1 at 24 h after IFN-γ treatment. The blue line shows the expression of PD-L1 at 24 h after IFN-γ treatment and then with treatment with the abovementioned drugs. The vertical axis indicates the number of cells, and the horizontal axis indicates the fluorescence intensity

therapy [31]. The dramatic response of PD-1 inhibitor in MSI-High GC or EBVaGC was recently demonstrated. There was a high correlation between PD-L1 positivity and EBV+/MSI-high in the cohort [32]. There are several reports that PD-L1 was overexpressed in GC with EBV or MSI-high [8–12, 33, 34]. Both EBVaGC and MSI-high GC are accompanied by abundant lymphocyte infiltration which can produce IFN-γ [28, 29, 34–37]. Though it might be possible that MSI-high GC uses the same mechanism as EBVaGC, further studies are needed to clarify the role of PD-1/PD-L1 interaction in MSI-high GC. Based on our research, we consider EBVaGC to be a suitable target for immune checkpoint inhibitor treatment.

It is reported that carcinomas for which immune checkpoint inhibitors are effective have numerous genetic mutations and thus express diverse neoantigens. In melanoma, numerous genetic mutations are present due to exposure to ultraviolet radiation; similarly, lung cancers in smokers show large numbers of genetic mutations. Gastric cancer is also known to show high antigenicity in general because of frequent somatic mutations [38]. Although EBVaGC does not have a high mutation burden or mismatch repair defects according to TCGA data, EBVaGC expresses EBV latent and lytic genes, which may also act as neoantigens.

Previous studies showed that DNA methylation is closely associated with carcinogenesis of EBVaGC [5, 39, 40]. Methylation of both viral and host DNA is important for the development of EBVaGC. Viral DNA methylation controls the expression of EBV latent and lytic genes. Methylation of host cell DNA inactivated expression of tumor suppressor genes and tumor-associated antigens [41, 42]. When EBVaGC cells were treated with DNA demethylating agents, the promoters of viral and host genes were demethylated, and their expression was upregulated [43]. EBV latent and lytic genes and tumor-associated antigens might act as neoantigens, thus can be targeted to immunotherapy including immune checkpoint inhibitors. The therapeutic application of immune checkpoint blockades with DNA demethylating agents is an attractive therapeutic approach for EBVaGC.

Tumor tissues of EBVaGC are accompanied by massive lymphocyte infiltration containing a large amount of IFN-γ [6]. EBVaGC is always exposed to the IFN-γ released from T-lymphocytes and is stimulated to express high levels of PD-L1. PD-L1 is an IFN-γ responsive gene, and response to IFN-γ is also regarded as a biomarker for predicting the effect of anti-PD-1 antibodies [31]. All EBVaGC cell lines strongly responded to IFN-γ treatment and the IFN-γ signaling pathway was involved in the expression of PD-L1.

We studied the mechanisms involved in the high expression of PD-L1 in EBVaGC cell lines. Abnormalities in the PD-L1 3′UTR are reported to be involved in the high expression of PD-L1 in various tumors [27, 44]. We focused on two SNPs corresponding to the seed regions of miRNAs.

The SNP in the seed region of miRNA 570 was found in all EBVaGC cell lines, but we could not show a difference in luciferase activity between the CC and GG allele-specific psiCHECK2 constructs. Function-acquired mutation of PI3KCA and amplification of 9p24.1, which encodes JAK2 and PD-L1 have been reported in EBVaGC [5, 11]. These genetic abnormalities may lead to the high expression of PD-L1 in EBVaGC.

The expression of PD-L1 is common in EBVaGC and EBV-associated posttransplant lymphoproliferative disorder, NK/T lymphoma, and nasopharyngeal carcinoma [8–12, 45]. The constitutive expression of PD-L1 might be involved in the development of these EBV-related tumors. In EBV-positive nasopharyngeal carcinoma, LMP1 enhances the expression of PD-L1 [46]; however, EBVaGC does not express LMP1. It is possible that other EBV latent genes are involved in the expression of PD-L1.

To our knowledge, we are the first to report the function of the PD-1/PD-L1 pathway and the IFN-γ signaling pathway is involved in the expression of PD-L1 in EBVaGC. We consider EBVaGC to be a suitable target for immune checkpoint inhibitor treatment.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The study was approved by the Institutional Review Board of Yamaguchi University Hospital (approval number H26-119).

References

1. Van Cutsem E, Sagaert X, Topal B, Haustermans K, Prenen H. Gastric cancer. Lancet. 2016;388:2654–64.
2. zur Hausen H, Schulte-Holthausen H, Klein G, Henle W, Henle W, Clifford P, et al. EBV DNA in biopsies of Burkitt tumours and anaplastic carcinomas of the nasopharynx. Nature. 1970;228:1056–8.
3. Fukayama M, Hayashi Y, Iwasaki Y, Chong J, Ooba T, Takizawa T, et al. Epstein–Barr virus-associated gastric carcinoma and Epstein–Barr virus infection of the stomach. Lab Invest. 1994;71:73–81.
4. Imai S, Koizumi S, Sugiura M, Tokunaga M, Uemura Y, Yamamoto N, et al. Gastric carcinoma: Monoclo- nal epithelial malignant cells expressing Epstein–Barr virus la- tent infection protein. Proc Natl Acad Sci USA. 1994;91:9131–5.
5. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of gastric adenocarcinoma. Nature. 2014;513:202–9.

6. Ohtani H, Jin Z, Takegawa S, Nakayama T, Yoshie O. Abundant expression of CXCL9 (MIG) by stromal cells that include dendritic cells and accumulation of CXCR3+ T cells in lymphocyte-rich gastric carcinoma. J Pathol. 2009;217(1):21–31.

7. Ayers M, Lunceford J, Nebozhyn M, Murphy E, Loboda A, Kaufman DR, et al. IFN-γ-related mRNA profile predicts clinical response to PD-1 blockade. J Clin Invest. 2017;127:2930–40.

8. Kawazoe A, Kuwata T, Kuboki Y, Shitara K, Nagatsuma AK, Aizawa M, et al. Clinicopathological features of programmed death ligand 1 expression with tumor-infiltrating lymphocyte, mismatch repair, and Epstein–Barr virus status in a large cohort of gastric cancer patients. Gastric Cancer. 2017;20(3):407–15.

9. Böger C, Behrens HM, Mathiak M, Krüger S, Kalthoff H, Röcken C. PD-L1 is an independent prognostic predictor in gastric cancer of Western patients. Oncotarget. 2016;7:24269–83.

10. Derks S, Liao X, Chiaravalli AM, Xu X, Camargo MC, Solcia E, et al. Abundant PD-L1 expression in Epstein–Barr virus-infected gastric cancers. Oncotarget. 2016;7:32925–32.

11. Ma C, Patel K, Singhi AD, Ren B, Zhu B, Shaikh F, et al. Programmed death-ligand 1 expression is common in gastric cancer associated with Epstein–Barr virus or microsatellite instability. Am J Surg Pathol. 2016;40:1496–506.

12. Saito R, Abe H, Kunita A, Yamashita H, Seto Y, Fukayama M. Overexpression and gene amplification of PD-L1 in cancer cells and PD-L1+ immune cells in Epstein–Barr virus-associated gastric cancer: the prognostic implications. Mod Pathol. 2017;30:427–39.

13. Hamid O, Robert C, Daud A, Hodi FS, Hwu WJ, Kefford R, et al. Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. N Engl J Med. 2013;369:134–44.

14. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, et al. Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med. 2013;369:122–33.

15. Robert C, Schachter J, Long GV, Arance A, Grob JJ, Mortier L, et al. Pembrolizumab versus ipilimumab in advanced melanoma. N Engl J Med. 2015;372:2521–32.

16. Reck M, Rodríguez-Abreu D, Robinson AG, Hui R, Csőszi T, Fülöp A, et al. KEYNOTE-024 Investigators. Pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer. N Engl J Med. 2016;375:1823–33.

17. Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, et al. Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. N Engl J Med. 2015;373:1627–39.

18. Motzer RJ, Escudier B, McDermott DF, George S, Hammers HJ, Srinivas S, et al. CheckMate 025 Investigators. Nivolumab versus everolimus in advanced renal-cell carcinoma. N Engl J Med. 2015;373(19):1803–13.

19. Ansell SM, Lesokhin AM, Borrello I, Halwani A, Scott EC, Gutierrez M, et al. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. N Engl J Med. 2015;372:311–9.

20. Kang YK, Boku N, Satoh T, Ryu MH, Chao Y, Kato K, et al. Nivolumab in patients with advanced gastric or gastro-oesophageal junction cancer refractory to, or intolerant of, at least two previous chemotherapy regimens (ONO-4538-12, ATTRACTION-2): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet. 2017;390:2461–71.

21. Panda A, Mehnert JM, Hirshfield KM, Riedlinger G, Damare S, Saunders T, et al. Immune activation and benefit from avelumab in EBV-positive gastric cancer. J Natl Cancer Inst. 2018;110(3):316–20.

22. Ku JL, Kim KH, Choi JS, Kim SH, Shin YK, Chang HJ, et al. Establishment and characterization of six human gastric carcinoma cell lines, including one naturally infected with Epstein–Barr virus. Cell Oncol (Dordr). 2012;35:127–36.

23. Oh ST, Seo JS, Moon UY, Kang KH, Shin DJ, Yoon SK, et al. A naturally derived gastric cancer cell line shows latency I

24. Kim DN, Seo MK, Choi H, Kim SY, Shin HJ, Yoon AR, et al. Characterization of naturally Epstein–Barr virus-infected gastric carcinoma cell line YCCEL1. J Gen Virol. 2013;94(Pt 3):497–506.

25. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat Immunol. 2001;2(3):261–8.

26. Wu YY, Lin CW, Cheng KS, Lin C, Wang YM, Lin IT, et al. Increased programmed death-ligand-1 expression in human gastric epithelial cells in Helicobacter pylori infection. Clin Exp Immunol. 2010;161(3):551–9.

27. Wang W, Li F, Mao Y, Zhou H, Sun J, Li R, et al. A miR-570 binding site polymorphism in the B7-H1 gene is associated with the risk of gastric adenocarcinoma. Hum Genet. 2013;132:641–8.

28. Oda K, Tamaru J, Takenouchi T, Mikata A, Nunomura M, Saitoh N, et al. Association of Epstein–Barr virus with gastric carcinoma with lymphoid stroma. Am J Pathol. 1993;143(4):1063–71.

29. Murphy G, Pfeiffer R, Camargo MC, Rabkin CS. Meta-analysis shows that prevalence of Epstein–Barr virus-positive gastric cancer differs based on sex and anatomic location. Gastroenterology. 2009;137:824–33.

30. Ribeiro J, Oliveira C, Malta M, Sousa H. Epstein–Barr virus gene expression and latency pattern in gastric carcinomas: a systematic review. Future Oncol. 2017;13:567–79.

31. Choudhury N, Nakamura Y. Importance of immunopharmacogenomics in cancer treatment: Patient selection and monitoring for immune checkpoint antibodies. Cancer Sci. 2016;107(2):107–15.

32. Kim ST, Cristescu R, Bass AJ, Kim KM, Odegaard JI, Kim K, et al. Comprehensive molecular characterization of clinical responses to PD-1 inhibition in metastatic gastric cancer. Nat Med. 2018;24(9):1449–58.

33. Cho J, Chang YH, Heo YJ, Kim S, Kim NK, Park JO, et al. Four distinct immune microenvironment subtypes in gastric adenocarcinoma with special reference to microsatellite instability. ESMO Open. 2018;3(3):e000326.

34. De Rosa S, Sahnane N, Tibiletti MG, Magnoli F, Vanoli A, Sessa F, et al. EBV+ and MSI gastric cancers harbor high PD-L1/PD-1 expression and high CD8+ intratumoral lymphocytes. Cancers (Basel). 2018;10(4):E102.

35. Seruca R, Santos NR, David L, Constancia M, Barroca H, Carneiro F, et al. Sporadic gastric carcinomas with microsatellite instability display a particular clinicopathologic profile. Int J Cancer. 1995;64:32–6.

36. Chiaravalli AM, Feltri M, Bertolini V, Bagnoli E, Furlan D, Cerutti R, et al. Intratumour T cells, their activation status and survival in gastric carcinomas characterised for microsatellite instability and Epstein–Barr virus infection. Virchows Arch. 2006;448(3):344–53.

37. Gullo I, Carvalho J, Martins D, Lemos D, Monteiro AR, Ferreira M, et al. The transcriptomic landscape of gastric cancer: insights into Epstein–Barr virus infected and microsatellite unstable tumors. Int J Mol Sci. 2018;19(7):E2079.

38. Lawrence MS, Stojanov P, Polak P, Kryukov GV, Cibulskis K, Sivachenko A, et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature. 2013;499:214–18.

39. Kusano M, Toyota M, Suzuki H, Akino K, Aoki F, Fujita M, et al. Genetic, epigenetic, and clinicopathologic features of gastric carcinomas with the CpG island methylator phenotype and an association with Epstein–Barr virus. Cancer. 2006;106:1467–79.

40. Chang MS, Uozaki H, Chong JM, Ushiku T, Sakuma K, Ishikawa S, et al. CpG island methylation status in gastric carcinoma with

Epstein–Barr virus infection closely resembling EBV-associated gastric cancer. Virology. 2004;320:330–6.

and without infection of Epstein–Barr virus. Clin Cancer Res. 2006;12:2995–3002.

41. Chong JM, Sakuma K, Sudo M. Ushiku T. Uozaki H. Shibahara J, et al. Global and non-random CpG-island methylation in gastric carcinoma associated with Epstein–Barr virus. Cancer Sci. 2003;94:76–80.

42. Saito M. Nishikawa J. Okada T. Morishige A. Sakai K. Nakamura M, et al. Role of DNA methylation in the development of Epstein–Barr virus-associated gastric carcinoma. J Med Virol. 2013;85:121–7.

43. Nakamura M. Nishikawa J. Saito M. Sakai K. Sasaki S. Hashimoto S.et al. Decitabine inhibits tumor cell proliferation and up-regulates e-cadherin expression in Epstein–Barr virus-associated gastric cancer. J Med Virol. 2017;89:508–17.

44. Kataoka K, Shiraishi Y, Takeda Y, Sakata S, Matsumoto M, Nagano S, et al. Aberrant PD-L1 expression through 3′-UTR disruption in multiple cancers. Nature. 2016;534:402–6.

45. Green MR, Rodig S, Juszczynski P, Ouyang J, Sinha P, O'Donnell E, et al. Constitutive AP-1 activity and EBV infection induce PD-L1 in Hodgkin lymphomas and posttransplant lymphoproliferative disorders: implications for targeted therapy. Clin Cancer Res. 2012;18:1611–8.

46. Fang W, Zhang J, Hong S, Zhan J, Chen N, Qin T, et al. EBV-driven LMP1 and IFN-γ up-regulate PD-L1 in nasopharyngeal carcinoma: implications for oncotargeted therapy. Oncotarget. 2014;5:12189–202.