CRISPR-Cas9-mediated disruption of PD-1 on human T cells for adoptive cellular therapies of EBV positive gastric cancer

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ABSTRACT
The successful use of immune cell checkpoint inhibitors PD-1 and PD-L1, over the past 5 y has raised the concern of using immunotherapy to treat various cancers. Epstein-Barr virus-associated gastric cancer (EBVaGC) exhibits high infiltration of lymphocytes and high amplification of immune-related genes including PD-L1 as distinguished from Epstein-Barr virus-non-associated gastric cancer (EBVnGC). Here, we presume that this PD-1/PD-L1 pathway may hinder the efficacy of adoptive T cell therapy toward EBVaGC. These studies reveal possibility of generating PD-1-disrupted CTL by CRISPR-Cas9 system and demonstrate enhanced immune response of these PD-1-disrupted CTLs to the EBV-LMP2A antigen and superior cytotoxicity to the EBV-positive gastric cancer cell. In addition, when combined with low-dose radiotherapy, these PD-1-disrupted CTLs mediated an impressive antitumor effect in a xenograft mouse model of EBVaGC. Taken together, these studies illustrate PD-1/PD-L1-mediated immune tolerance of EBVaGC and provide a new strategy for targeting immune checkpoints to break the tolerance for the T cell-based adoptive therapy.

ARTICLE HISTORY
Received 1 August 2016
Revised 1 October 2016
Accepted 13 October 2016

KEYWORDS
Adoptive cell therapy; checkpoint blockade; CRISPR; EBV-associated gastric cancer

Introduction
The Epstein-Barr virus (EBV), a type of herpes virus, is one of the most common viral infections in humans; it is estimated that 90% of the world population have been exposed to this virus before adolescence. EBV is associated with a variety of malignancies such as Hodgkin’s disease, Burkitt’s lymphoma, nasopharyngeal carcinoma, and gastric carcinoma. EBV-associated gastric cancer (EBVaGC) comprises nearly 10% of all gastric carcinomas worldwide, and it is estimated that over 80,000 new cases of EBVaGC yearly worldwide. Study suggested that EBV infection might be involved in the early stages of gastric carcinogenesis.\textsuperscript{1}

It is suggested that the infection with EBV correlates with the degree of immune cell infiltration into tumor areas, suggesting that host inflammatory immune cells play a beneficial role in the development of the disease.\textsuperscript{2} The successful use of T cell checkpoint inhibitors, especially PD-1, in clinical trials over the past 5 y has raised the great concern of immunotherapy of various cancers.\textsuperscript{3-8} Recent literatures have discussed the stratification of the tumor microenvironment (TME) into four types and reported that the type I TME of patients who respond to anti-PD-1 mAb contain tumor-specific CD8\textsuperscript{T} cells expressing PD-1 in close proximity to PD-L1-expressing cells. These types of tumors, such as melanomas, are more sensitive to anti-PD-1/PD-L1 therapy.\textsuperscript{9} Study also showed that mismatch-repair status predicted clinical benefit of immune checkpoint blockade with anti-PD-1 mAb.\textsuperscript{10} Therefore, it is of great importance to define a population of gastric cancer patients who could benefit most from the immune checkpoint blockade therapy.

Recently, emerging evidence showed that EBV-associated malignancies had high level of PD-L1 gene amplification, indicating that these tumors may be candidates for PD-1/PD-L1-targeted therapies. Previous studies demonstrated that EBV-induced latent membrane protein 1 (LMP1) and IFN\gamma pathways cooperate to regulate programmed cell death protein 1 ligand (PD-L1) in nasopharyngeal carcinomas (NPC) and that PD-L1 expression was detected in the malignant cells of nearly all EBV-associated NPC.\textsuperscript{11,12} Investigation showed that PD-1 blockade can restore functions of T cells in EBV-positive diffuse large B cell lymphoma and it appears that infection-associated cancers are most likely create an “immune-privileged” milieu by upregulating PD-L1.\textsuperscript{13}

Similarly, most changes in EBVaGC occur in immune response genes. One of the characteristic was CD274 (encoding for PD-L1) and CD273 (encoding for PD-L2) gene amplification. Upon ligation with PD-L1 and PD-L2, PD-1 suppresses downstream PI3K-Akt signaling, which inhibits T cell proliferation.\textsuperscript{14} Prior studies of lymphoma malignancies suggested EBV-associated upregulation

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of PD-L1 on the cell surface, which is IFNγ-mediated, and thus inhibits killing of infected cells by cytotoxic T cells expressing PD-1. Therefore, we hypothesized that the function of EBV-specific CTLs may be affected by the acquired immune resistance indicated by the upregulation of PD-1 on CTLs and we assumed that blocking the interaction between PD-1 and PD-L1/L2 could augment the antitumor immune responses in EBVaGC.

EBVaGC belongs to latency type I or II, in which EBERs, EBNA-1, LMP2A, BARTs, and BART miRNAs are expressed. Among them, EBNA-1 and LMP2A mostly expressed on tumor cells and LMP2A protein is expressed in more than half of tumor cells of EBVaGC patients. Although EBVaGC has been shown to express EBNA1, it may not be a suitable target due to its internal Gly/Ala repeat domain which can prevent EBNA-1 from processing through the classical HLA class I pathway. Previous studies also showed that the sensitization of peripheral blood lymphocytes with LMP2A-derived peptide was able to induce CTL response against EBVaGC cells. Thus, EBVaGC is susceptible to the LMP2A-targeting immunotherapy. Here, in this study, we focused on LMP2A as a target molecule for immunotherapy of EBVaGC. We found upregulation of PD-1 on the LMP2A-specific T cells, indicating the feasibility of targeting PD-1/PD-L1 for the EBV-LMP2A-CTLs adoptive cell therapy. Also, in this study, we examined the protein expression of PD-L1 by immunohistochemistry in the tumor micro-environment of EBVaGC and EBVnGC. Although working with a relatively small research sample, we found higher expression of PD-L1 on the tumor membrane of EBVaGC, indicating the role of PD-1/PD-L1 pathway in immune tolerance of EBVaGC.

Our laboratory pioneered in a gene editing approach by using CRISPR-Cas9 to disrupt PD-1 on primary T cells. By electroporation of plasmids encoding sgRNA and Cas9 DNA, we achieved this genome disruption in primary human T cells. And we found the disruption sustained over prolonged in vitro culture in the presence of antigen stimulation. Here, in this study, we further evaluate the possibility of PD-1 disruption by CRISPR-Cas9 in the generation of EBV-LMP2A-CTL derived from healthy donors or EBVaGC patients for the adoptive cell therapy. Function of these gene-modified CTLs cells was characterized by upregulated cytokine production and enhanced cytotoxicity. The in vitro injection of these gene-modified T cells was safe and improved the survival of tumor-bearing mice. More importantly, these PD-1-disrupted T cells mediated efficient antitumor efficacy in the xenograft model when co-administered with low-dose radiotherapy. These results suggest that the PD-1 disruption by CRISPR-Cas9 could provide a new strategy to improve the efficacy of T cell-based adoptive therapies for targeting EBVaGC in future clinical studies.

Results

PD-L1 is upregulated in the tumor microenvironment of EBVaGC

It is reported that EBVaGC is always accompanied by high infiltration of TILs and is suggested to be more immunotherapy-sensitive than other types of gastric cancer. Importantly, PD-1 ligands are induced in response to inflammatory cytokines such as IFNγ, and represent a major mechanism for tissue protection in the setting of T cell-mediated inflammation. However, the upregulation of PD-L1 by IFNγ-mediated immune resistance, which is termed as adaptive immune resistance. We therefore sought to determine whether expression of PD-1/PD-L1, is linked to infiltrating TILs in EBVaGC. To do so, we collected 34 tumor tissue samples from patients with lymphocyte-infiltrated gastric cancer (Table S1).

Among these tumor tissues, 14 were EBVaGC and 20 were EBVnGC as determined by EBER+/-ISH. Of the 14 EBVaGC samples examined in our study, 64% (9 of 14) expressed PD-L1 and displayed tumor cell surface staining on ≥5% of tumor cells. In order to determine if PD-L1 expression was specific to EBVaGC, we evaluated 20 EBER-negative gastric cancers which were also infiltrated with T cells and found that only 15% (3 of 20) expressed PD-L1 (Table 1). PD-L1 expression in EBV-negative cancers was membranous and at the tumor periphery juxtaposed to immune infiltrates, similar to EBVaGC.

Furthermore, we evaluated the intensity of these PD-L1 positive EBVaGC tumors and found 22% (2 of 9) were 1+ intensity, 33% (3 of 9) were 2+ intensity, and 45% (4 of 9) were 3+ intensity (Fig. 1). Furthermore, we compared the frequency of PD-1 expression on TILs from patients with EBVaGC versus patients with EBVnGC. We found that the frequency of PD-1 expression on the TILs of EBVaGC was 3 in 9 (33%) in PD-L1+ patients and was 1 in 5 (20%) on PD-L1− patients. While we did not find any on the TILs of EBVnGC in PD-L1+ patients (0/3, 0%), and we detected 3 in 17 (18%) PD-L1− patients (Table 1). A 1% threshold of cell surface PD-1 expression on lymphocytes was defined as positive.

Among those EBVaGC tumors, 71% (10 in 14) were EBV-LMP2A-positive-staining, and 70% (7 in 10) of these LMP2A-

| Table 1. PD-L1 expression of EBVaGC and EBVnGC tumor tissues. |
|---------------------------------------------------------------|
|                  | BBER (+) (N = 14) |          | BBER (−) (N = 20) |          |
|                  | Positive | Negative | Positive | Negative |
| PD-L1 expression | 9/14(64%) | 5/14(36%) | 3/20(15%) | 17/20(85%) |
| 1+ intensity     | 2/9(22%) | —        | 0/3(0%)  | —        |
| 2+ intensity     | 3/9(33%) | —        | 2/3(66%) | —        |
| 3+ intensity     | 4/9(45%) | —        | 1/3(33%) | —        |
| Presence of TILs | 9/9(100%) | 5/5(100%) | 3/3(100%) | 17/17(100%) |
| Presence of PD-1 | 3/9(33%) | 1/5(20%) | 0/3(0%)  | 3/17(18%) |
| Presence of LMP2A| 7/9(76%) | 3/5(60%) | —        | —        |

The expression and the intensity of PD-L1 were analyzed on tumor tissues of 14 EBVaGC and 20 EBVnGC patients as determined by IHC. A ≥5% tumor cell surface staining of PD-L1 was determined as PD-L1 positive staining. Among PD-L1 positive or PD-L1 negative staining tissues, the presence of CD3, PD-1, and LMP2A were further analyzed.
positive tumors co-expressed PD-L1 (Table 1). Taken together, these data may indicate that PD-L1 expression in gastric cancer infiltrated by lymphocytes is correlated with the presence of EBV infection as well as LMP2A expression in the presence of TILs.

**PD-1 expression is upregulated in EBV-LMP2A-specific CD8^+ T cells and associated with impaired function**

It has been proved that sensitization of peripheral blood lymphocytes with LMP2A peptide could induce specific T cell response against EBVaGC cells, and we previously demonstrated that the specific killing of LMP2A peptide-induced CTL on EBV positive AGS-EBV cells (Fig. S1). However, these LMP2A-specific T cells do not always confer solid cytotoxicity toward the target cells. We hypothesized that these tumor cells were able to escape immune surveillance by inhibitory pathways such as PD-1/PD-L1, and immunotherapy targeting EBV antigens may be hindered by the immune resistance. We obtained LMP2A-specific CTLs by stimulating PBMC derived from HLA-A^0201 positive healthy donors with autologous DCs loaded with a well-defined HLA-A^0201 immunogenic epitope LMP2A 356–364 (FLYALALLL). We measured the PD-1 expression on these in vitro cultured T cells and found an increase of PD-1 expression after long-term culturing with antigens or irradiated EBV^+ tumor cells (data not shown). We next sorted these LMP2A-CD8^+ T cells into LMP2A-tetramer^+ and LMP2A-tetramer^- T cells as indicated (Fig. 2A) and examined the PD-1 expression. PD-1 expression was detected in both groups of cells, but the frequency of PD-1 expression was much higher on LMP2A-tetramer^+ T cells as compared with negative cells (48.7% vs. 7.08%) (Fig. 2B). Moreover, we measured the cytotoxicity of these T cells toward an EBV^+ gastric cell line AGS-EBV and found that the LMP2A-tetramer^+ T cells did not exhibit an enhanced cytotoxicity to AGS-EBV cells as compared with LMP2A-tetramer^- T cells (Fig. 2C). These data suggested that PD-1 is upregulated on EBV-LMP2A-specific CD8^+ T cells and associated with impaired function of CTLs.

**The generation of PD-1-disrupted LMP2-CTL by CRISPR-Cas9 system**

We have achieved efficient gene disruption of primary human T cells by CRISPR-Cas9 system.21 In this study, PBMC from healthy donors was isolated and activated for nucleofection for 3 d. To determine the transfection efficiency, we used reporter plasmids pST1374-Cas9-GFP encoding green fluorescent protein (GFP) tagged Cas9 to evaluate the transfection efficacy through the co-electroporation with pST1374-Cas9-GFP and pGL3-U6-hPD-1-sgRNA, as described previously,21 and GFP-enhanced blank plasmid was used as a positive control. GFP expression was determined 24 h later by fluorescence microscope (Fig. 3A) and by flow cytometry (Fig. 3B). Then, we assessed the proliferation capacity of sgRNA:Cas9-treated T cells in vitro upon stimulation with LMP2A-pulsed DCs for a total period of 21 d. We found the proliferation of primary T cells of these donors was neither significantly affected by the disruption of PD-1 during the prolonged culture period, nor affected by the electroporation as shown by the un-transfected control T cells (Fig. 3C). During the culturing of LMP2A-CTL, the surface expression of PD-1 was examined by flow cytometry. The expression of PD-1 on CD3^+ T cells increased from
1.32% at the baseline to 3.53% of the PD-1-disrupted group while it increased from 4.65% to 13.6% in the control group (Fig. 3D). Moreover, we further evaluated PD-1 upregulation upon the stimulation of gastric cancer cell lines with or without EBV expression and found similar results (Fig. S2), which indicated an efficient and sustained disruption of PD-1 by CRISPR-Cas9. We then evaluated the phenotype and activation status of these gene-modified CTL. The T cell subsets were characterized with memory or activation markers. In accordance with our previous findings, we did not find any significant change to the memory markers on CD8+ T cells, including central memory CD8+CD45RO+CD62L+ T cell. Also, no significant difference in the activation markers CD28, CD27, CD69, and HLA-DR on CD8+ T cells was detected between the sgRNA-hPD-1 T cells and control T cells (data not shown). These results indicated that PD-1 disruption on culturing LMP2A-CTLs could be achieved by CRISPR-Cas9 system.

**The disruption of PD-1 by CRISPR-Cas9-enhanced cellular immune response and cytotoxicity toward EBV+ gastric cancer cell line in vitro**

We have previously shown that the effector capabilities of reprogrammed primary T cells were enhanced upon antigen stimulation. In this study, we further assessed effector capabilities of PD-1-disrupted LMP2A-CTLs upon antigen re-stimulation. sgRNA hPD-1: Cas9-modified CTLs and control CTLs from healthy donors were stimulated by HLA-A*0201 or HLA-A*2402 restricted LMP2A epitope in the presence of autologous DCs for 7 ~14 d after electroporation and re-stimulated by the antigen for 20 h. Interferon-γ secretion was measured by Elispot assay. Among these healthy donors, higher IFN-γ was detected from PD-1-disrupted CTLs as compared with control CTLs by the stimulation of HLA-A*0201 matched epitopes LMP2A 356, LMP2A 426 or HLA-A*2402 matched LMP2A 419 on day 14 or day 21 of culturing, (HD#01: LMP2A 356, Day 14: p > 0.05, Day 21: p < 0.005; LMP2A 419, Day 14: p < 0.005, Day 21: p < 0.005; HD#02: LMP2A 356, Day 14: p < 0.05, Day 21: p < 0.005; LMP2A 426, Day 14: p < 0.005, Day 21: p < 0.005, LMP2A 419, Day 14: p < 0.005, Day 21: p < 0.005; HD#03: LMP2A 356, Day 14: p < 0.05, Day 21: p < 0.005; LMP2A 426, Day 14: p < 0.05, Day 21: p < 0.05) (Fig. 4A). We subsequently sought to determine if the lytic activity of the modified CTLs could be augmented. As expected, the cytotoxicity of the PD-1-disrupted CTLs was shown to be enhanced in a dose dependent manner toward AGS-EBV cells, while not toward AGS cells (Fig. 4B). We further examined the PD-L1 expression of these cells and found both AGS and AGS-EBV cells showed weak expression of PD-L1 at the base level. However, PD-L1 expression was only moderate upregulated on AGS cells (16%) but more aggressive upregulated on AGS-EBV cells (51%) by the stimulation of IFNγ and TNF-α (Fig. 4C). All together these results showed that AGS-EBV is more sensitive to the IFNγ and TNF-α mediated PD-L1 upregulation in vitro, and the acquired immune resistance mediated by PD-1/PD-
Figure 3. The generation of PD-1-disrupted LMP2-CTL by CRISPR-Cas9 system. Human PBMC were activated in vitro for 2 d and were co-transfected with pST1374-Cas9-GFP and pGL3-U6-hPD-1-sgRNA plasmids or GFP-enhanced pMAX-EGFP control plasmid for each reaction. (A) The GFP expression was evaluated by fluorescence microscope 24 h after electroporation, (B) The percentage of GFP positive cells were analyzed 24 h after electroporation by flow cytometry. (C) The total cell numbers of sgRNA:Cas9-treated T cells, Cas9-treated T cells, and un-transfected T cells stimulated with LMP2A-peptide pulsed DCs were recorded every 7 d post-transfection for a total period of 21 d. (D) The expression of PD-1 on CD3+ T cells stimulated by LMP2A-peptide pulsed autologous DCs was determined by flow cytometry 7 and 21 d post-transfection. We depicted a representative out of three experiments yielding similar results.
Figure 4. The enhanced cellular response and cytotoxicity of PD-1-disrupted CTL toward EBV+ gastric cancer cell line. PD-1-disrupted human T cells and control T cells were cultured in IL-2, IL-7, and IL-15 for 14 d or 21 d. (A) 1 x 10^5 cultured CTLs were stimulated by LMP2A 356 and LMP2A 426 (HLA-A02 restricted) or LMP2A 419 (HLA-A24 restricted) in the presence of 1 x 10^4 autologous DCs for 20 h. The IFN-γ secreting cells were evaluated by Elispot. (B) PD-1 disrupted CTLs or control CTLs were co-cultured with CFSE labeled AGS or AGS-EBV cells at ratio (E:T) of 5:1, 10:1, 20:1, 40:1, respectively. After 6 h, PI was added and the PI^+ CFSE^− T cells were analyzed by flow cytometry. (C) AGS and AGS-EBV cell lines were cultured in the absent or presence of IFN-γ (25 ng/mL) and TNF-α (20 ng/mL) for 48 h, PD-L1 expression was determined by flow cytometry.
L1 axis could be reversed through the disruption of PD-1 by CRISPR-Cas9 system.

**The evaluation of CRISPR-Cas9-mediated disruption of PD-1 on CTLs of EBVaGC patient**

To evaluate the potential advantages of the CRISPR-Cas9-mediated disruption of PD-1 on CTLs in clinical settings, we prepared LMP2A-CTL from patient with LMP2A positive EBVaGC described above. The tumor tissue sample showed strong PD-L1 staining on tumor membrane as well as high level of immune cell infiltration. Also, a correlation of co-localization of PD-L1 and PD-1 was observed in the patient (Fig. 5A). LMP2A-specific response of the patient’s PBMC was confirmed by IFNγ assay (data not shown). We isolated patient’s PBMCs and generated CTLs through stimulating PD-1-disrupted T cells or control T cells with autologous DCs loaded with responded epitopes LMP2A 356 and LMP2A 426 as well as IL-7, IL-15, and IL-2. We observed higher level of IFNγ produced by the PD-1-disrupted group as compared with control CTLs by Elispot (LMP2A 356, p < 0.005; LMP2A 426, p < 0.005) (Fig. 5B). To decide whether the enhanced IFNγ production was CD8+ T cell dependent, we further isolated CD8+ T cells from the bulk PBMC and found a significant enhancement of the response toward LMP2A epitopes (LMP2A 356, p < 0.005; LMP2A 426, p < 0.005) (Fig. 5C). Besides, cytokines important for T cell antitumor function in the cell culture supernatant were detected by multiple cytokine assays. Three cytokines that are defined as important for T cell cytotoxicity were also significantly upregulated in PD-1-disrupted group than in the control group, they were IFNγ (LMP2A 356, p < 0.05; LMP2A 426, p < 0.005), TNF-α (LMP2A 426, p < 0.0001), and IL-2 (LMP2A 426, p < 0.05). We also noticed upregulated production of IL-6 (LMP2A 426, p < 0.05) and IL-13 (LMP2A 426, p < 0.05), which are non-Th1 cytokines but also play some role in the inflammation and activation of cytotoxic T cells. As for the immune suppressive function, we noticed that the secretion of cytokine IL-10 in the PD-1-disrupted group was much lower than in the control group (LMP2A 356, p < 0.05) (Fig. 5D). Relatively low amount of IL-4 (data not shown) were found in both groups by the stimulation of LMP2A epitopes.

In line with the cellular responses of gene-modified T cells, we also observed an enhanced cytotoxicity of PD-1-disrupted CTLs toward PD-L1 hi AGS-EBV cells at a ratio of 20:1 (E:T), but not toward PD-L1 lo AGS cells (Fig. 5E, left). Enhanced cytotoxicity was also shown by PD-1 disruption of pure CD8+ T cells at a ratio of 20:1 (Fig. 5E, right), suggesting the superior antitumor efficacy of gene-modified T cells and its dependency on CD8+ T cells to EBV positive gastric cancer cell line in vitro.

Additionally, we found a more activated status of PD-1-disrupted LMP2A-CTLs-treated group and higher in CD8+ T cells were found in the tumor parenchyma of the radiotherapy (14 d after start of therapy: C9+RT vs. C9+sg+RT, p < 0.05; C9+sg vs. C9+sg+RT, p < 0.005. Figs. 6C and D, right). However, we observed that PD-1-disrupted LMP2A-CTLs improved survival of tumor-bearing mice and similar results were shown when it combined with radiotherapy. The mice survived after 60 d of tumor inoculation was the one with PD-1-disrupted LMP2A-CTLs in combination with radiotherapy(Fig. 6B). Radiotherapy has been reported to modulate the immunogenicity of tumor cells and improve the infiltration of immune cells into the tumor tissue.

We next investigated the mechanisms underlying the advantage of PD-1-disrupted LMP2A-CTLs in vivo and the maximum benefit with radiotherapy. Twenty days after the start of therapy, tumors were excised and used for IHC analysis. CD3+ T cells were found to be enriched in PD-1-disrupted LMP2A-CTLs-treated group and higher infiltration of CD3+ T were found in the tumor parenchyma of the radioimmunotherapy group (Fig. 6E). We produced single-cell suspension of these tumors and analyzed the infiltration of adoptive transferred CD3+ T cells by flow cytometry. Similarly, we observed a higher proportion of CD3+ T cells in the PD-1-disrupted LMP2A-CTLs-treated group (Fig. 6F). This suggests of EBVaGC may be achieved by CRISPR-Cas9-mediated PD-1-disrupted strategy.
Figure 5. The evaluation of CRISPR-Cas9-mediated disruption of PD-1 on CTLs of EBVaGC patient. PBMC were isolated from HLA-A02 EBVaGC patient and used for the generation of PD-1-disrupted LMP2A-CTL. (A) The tumor tissue of the patient was analyzed for the expression of PD-1, PD-L1, and CD3 by IHC. (B) 1 x 10^5 PD-1-disrupted CTLs or control CTLs generated from the patient were re-stimulated with HLA-A02-restricted LMP2A peptides and 1 x 10^4 autologous DCs for 20 h. IFNγ production was evaluated by Elispot. (C) CD8+ T cells were isolated from bulk PBMC to generate PD-1-disrupted CTLs and re-stimulated with LMP2A peptides and 1 x 10^4 autologous DCs for 20 h. IFNγ production was evaluated by Elispot. (D) The PD-1-disrupted LMP2A-CD8+ T cells were re-stimulated with HLA-A02-restricted LMP2A peptides and 1 x 10^4 autologous DCs for 24 h, the cell culture supernatant were harvested and used for Th1/Th2 cytokine detection by FACS. (E) PD-1-disrupted CTLs or control CTLs were co-cultured with CFSE labeled AGS or AGS-EBV cells at ratio (E:T) of 5:1, 10:1, 20:1, respectively. After 6 h, PI was added and the PI^+CFSE^+ T cells were analyzed by FACS (left: bulk CTL, right: CD8+ CTL).
that modified T cells are able to traffic to and infiltrate into EBVaGC and that PD-1 disruption could endow T cells with improved persistence. To evaluate the cytokine level of adoptive transferred T cells in the in vivo environment, we than evaluated three important antitumor cytokines: IFNγ, IL-2, and TNF-α. In accordance with the in vitro assay, the mice treated with PD-1-disrupted LMP2A-CTLs retained a higher level of IL-2 and IFNγ. However, we did not observe further enhancement of the cytokine level when combined with radiotherapy (Fig. 6G). In conclusion, PD-1-disrupted T cells demonstrated a higher cytotoxic potential when combined with low-dose radiotherapy in the xenograft model of EBVaGC.

Discussion

As we illustrated that it is of great importance to define a subpopulation of gastric cancer which could benefit most from PD-1/PD-L1 pathway inhibition. According to the related literature, we hypothesis that the function of EBV-specific CTLs may be affected by the acquired immune resistance induced by the upregulation of PD-1 and presumed that blocking the interaction of PD-1 and PD-L1 could augment immune response of EBV positive gastric cancer patients. In this manuscript, we first show that PD-L1 has varying degrees of expression on gastric cancer (Fig. 1) and is upregulated in gastric cancer with the presence of EBV infection (Table 1). Importantly, our study indicates that PD-1 is upregulated on EBV-LMP2A-specific CD8+ T cells and associated with impaired function of EBV-LMP2A-CTLs (Fig. 2). Based on these findings, we further explore the possibility of PD-1 disruption by CRISPR-Cas9 in the generation of EBV-LMP2A CTLs for adoptive cell therapy of EBV-positive gastric cancer. We demonstrated that PD-1 disruption on culturing LMP2A-CTLs could be achieved by CRISPR-Cas9 system (Fig. 3). The cellular immune response of these gene-modified CTLs is enhanced and the cytotoxicity of these cells to the tumor cells is improved which is closely correlated with the expression of PD-L1 in the target tumor cells (Fig. 4). Next, we transferred the system to PD-L1+PD-1+ EBVaGC patient and found that the disruption of PD-1 on EBV-LMP2A-CTLs derived from the patient by CRISPR-Cas9 could also enhance the cellular immune response and cytotoxicity toward EBV-positive gastric cancer cell line, suggesting that improvements of adoptive cell therapy of EBVaGC may be achieved by CRISPR-Cas9-mediated PD-1 disruption strategy (Fig. 5). More importantly, we applied this novel strategy to in vivo study. In a xenograft mouse model of EBVaGC, these PD-1-disrupted T cells improved local tumor control as well as survival when co-administered with low-dose radiotherapy (Fig. 6).

To our knowledge, this is the first pre-clinical study to examine the therapeutic effect of CRISPR-Cas9-modified T cells in the treatment of an established EBV-positive gastric cancer model. In our view, combining adoptive transfer of antigen-specific T cells with therapies that break immune tolerance to increase antitumor capacity is a promising strategy to improve treatment outcome. Recent genome editing technologies such as protein and RNA-guided endonucleases raise the possibility of disrupting gene expression in T cells in order to enhance effector functions or bypass tumor immune suppression. In this way, systemic administration of compounds that disrupt immune homeostasis could be avoided. The CRISPR-Cas9 system confers targeted gene editing through use of small guide RNAs that lead the Cas9 nuclease to the target site through base pairing. This new method has been demonstrated as an easy-to-handle, yet highly specific and efficient approach for engineering eukaryotic genomes. The application of CRISPR-Cas9 in immune cells is still in the early stage of exploration. Recently, several studies have reported the application of using CRISPR-Cas9 technology in T cells. Osborn, M. J successfully knocked endogenous TCR of T cells, solving the problem of transplant rejection. Chang Li also used this method with CD4+ T cells on CCR5 gene knockout to inhibit HIV replication. However, gene editing of immune checkpoints is rarely documented in relevant literature. In our previous studies, we described, for the first time, a new approach of inhibiting PD-1/PD-L1 co-stimulation by directly disrupting genome PD-1 expression on primary T cells from...
cancer patients through the CRISPR-Cas9 system. We are currently exploring PD-1 disruption of T cells by generating CTLs targeting on specific tumor antigen, as to solve the problem that continuous treatment to interrupt PD-1/PD-L1 co-stimulation is needed in the process of sustained expansion of PD-1-expressing antigen-specific CTLs.\(^{28}\)

In recent years, a number of studies confirmed PD-L1 expression on the surface of gastric cancer cells\(^ {29,30}\); PD-1 expression in peripheral circulating blood lymphocytes, tumor-infiltrating lymphocytes, and its ligands; and PD-L1 in serum and tumor tissue.\(^ {31}\) The expression of PD-L1 and PD-1 was closely related with tumor progression, invasion,
and the patient’s prognosis.\textsuperscript{32-36} Importantly, emerging evidence demonstrated that EBV is present in a considerable amount of tumor cells of gastric cancer patients worldwide. Studies confirmed that EBV-positive gastric cancer cell line (AGS-EBV) was resistant to the chemotherapy drugs docetaxel and 5-fluorouracil as compared with EBV-negative cells (AGS).\textsuperscript{37,38} At the same time, studies indicated that EBVaGC should be sensitive to immune checkpoint disruption therapy targeting PD-1, as it has been proved that the genes amplified in EBVaGC are associated with immune responses, among which PD-L1, PD-L2 had obvious amplification.\textsuperscript{14} This is in accordance with our demonstration that EBVaGC tumor cells had a much higher rate of PD-L1 expression compared to EBVnGC cells. Besides, the infiltrated T cells produced much more IFN$\gamma$ in EBV-high-load GC other than EBV-low or negative GC,\textsuperscript{59} therefore it is likely that EBVnGC is tend to acquire immune resistance through the upregulated IFN$\gamma$ production. In addition, EBVaGC is also characterized by a high frequency of gene mutation, such as PIK3CA (10.3\%\textasciitilde80\%), ARID1A (47\%\textasciitilde55\%), and AKT2 (38\%),\textsuperscript{14} tending to produce tumor neo-antigen. Studies confirmed that PD-1/PD-L1 and CTLA-4 inhibitor obtain better therapeutic efficacy in the tumors with the presence of a high mutation load, such as in malignant melanoma and non-small cell lung cancer.\textsuperscript{40,41}

It is worth noted that the profound therapeutic efficacy of PD-1-disrupted T cells was achieved by combining this method with low-dose radiotherapy in our pre-clinical model. The immune-modulating effects of radiation therapy have gained considerable interest as multiple reports documented the apparent synergy between radiation and immunotherapy. The possible mechanisms undergirding these findings are that radiotherapy upregulates tumor-associated antigen–MHC complexes, enhances antigen cross-presentation in the draining lymph node, boosts T cell infiltration into tumors,\textsuperscript{42} and promotes tumor-associated macrophage transformation from M2 to M1.\textsuperscript{43} At the same time, emerging evidences indicated that low doses of fractionated radiotherapy led to PD-L1 upregulation on tumor cells by recruiting infiltrated lymphocytes in an IFN$\gamma$-dependent manner, and fractionated radiotherapy delivered in combination with anti PD-1 or anti PD-L1 mAbs generated efficacious CD8$^+$T-cell responses that improved local tumor control, long-term survival, and protection against tumor re-challenge.\textsuperscript{44,45} Although we showed that the PD-1-disrupted LMP2A-CTLs conferred enhanced cytotoxicity to the

\begin{figure}[h]
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\caption{(Continued).}
\end{figure}
EBV-positive gastric cancer cell line in vitro, in the xenograft model of EBVaGC, we did not observe augmented tumor regression following treatment of PD-1 disrupted LMP2A-CTL. The adoptive cell therapy showed therapeutic effect only when combined with low-dose radiotherapy (2 Gy), and the PD-1-disrupted LMP2A-CTLs showed maximum efficacy. Importantly, we found that CD3+ T cells in the tumor tissue of mice were enriched and infiltrated into the tumor parenchyma when treated by radiation. Therefore, we presumed that the function of transferred immune cells was hindered due to the poor infiltration into the solid cancer and that the immune-modulating effects of radiation could overcome this limitation and enhance the antitumor effect of immunotherapy. Additional studies to explore the optimal dosage and fractionation of radiotherapy are required. Scheduling of administration of adoptive cell transfer is also important for therapeutic outcome.

Altogether our results show that by the brake of immune tolerance, CRISPR-Cas9-modified CTLs ranked superiorly in terms of immune responses and cytotoxicity. Accounting for the immune privilege landscape of EBVaGC, PD-1-disrupted EBV-specific CTLs could serve as a potent approach for the treatment of EBVaGC. Collectively, these results provide validation for the utilization of CRISPR-Cas9 system in the gene editing of primary T cells for the immune checkpoint modified CTLs. Moreover, our findings lead us to recommend combining radiotherapy with immune checkpoint modified CTLs for clinical tumor treatment.

Materials and methods

Ethics statement

All the experimental methods were carried out in accordance with the approved guidelines. The blood collection procedure was carried out in accordance with the guidelines verified and approved by the Ethics Committee of Drum Tower Hospital. All donors signed an informed consent for scientific research statement.

Immunohistochemistry

PD-L1 expression analysis was performed using rabbit anti-human mAb EPR1161 (2) (Abcam). A 5% threshold of cell surface PD-L1 expression on tumor cells was defined as positive. For PD-1 immunostaining, the murine anti-human PD-1 mAb, clone NAT105 (Abcam), was used. A 1% threshold of cell surface PD-1 expression was defined as positive. Infiltrated human T cells were detected by rabbit anti-human CD3 mAb SP7 (Abcam). For the expression of EBV LMP2A, rat anti-human mAb 15F9 were used (Abcam). All slides were reviewed and interpreted independently by two pathologists (Y.F and L.L).

Plasmid construction and tumor cell lines

The Cas9 expression construct pST1374-Cas9-N-NLS-Flag-linker (Addgene 44758) and pGL3-U6-sgRNA-hPD-1 (Addgene 51133) vectors were constructed as previously described.21 The expression of EBV of gastric cancer cell line AGS-EBV and SNU-719 was verified by the surface expression of target antigen LMP2A.

Generation of hPD-1-sgRNA-disrupted T cells by electroporation

The hPD-1-sgRNA-disrupted T cells were prepared using PBMC from healthy donors or gastric cancer patients as described previously.21 Cells were transfected with the intended plasmids by Nucleofector II (Lonza) using the Amaxa Human T cells Nucleofector Kit, VPA-1002 (Lonza). 5 × 10^6 cells were washed with DPBS by centrifuging at 800 rpm for 5 min and suspended in a 100 µL transfection buffer and then transferred into the electroporation cuvette. Program T-007 was selected for both high transfection and high efficiency. After electroporation, cells were re-suspended in 500 µL pre-warmed AIM-V medium containing 10% FBS and transferred into six-well cell plate and incubated at 37°C in 5% CO2. The transfection efficiency was evaluated by fluorescent counts 24 h after electroporation, and cells were harvested on day 7 for T7EN1 cleavage assay and sequencing as described previously. The cells culture medium was half replaced by fresh complete medium containing IL-2 (100~300 U/mL) every 2~3 d.

Generation of LMP2A-specific CTL

DCs were generated from monocytes enriched by adherence for 1~2 h, and cultured in AIM-V medium containing human GM-CSF (500 U/mL, Peprotech) and IL-4 (500 U/ mL, Peprotech) to obtain immature DCs. To obtain mature DCs (mDCs), fresh complete medium containing TNF-α (500 U/mL, Peprotech), IFN-α (500 U/mL, Peprotech), and PGE2 (50 mg/mL, Peprotech) was added to the culture on day 5. The culture was continued for an additional 2 d. As described previously, these DCs possess the ability to present peptide antigen and express CD80, CD86, HLA-DR, and CD11c. Mature DCs were pulsed by peptide (10 µg/ mL) for 4~6 h at 37°C, washed with pre-warmed PBS and then incubated with control T cells or sgRNA PD-1 T-cells at a ratio of 1:10 in complete AIM-V medium supplemented with IL-7 (25 ng/mL) and IL-15(10ng/mL) in six-well-plates (4×8×10^6 cells/well) on day 3~4 post-electroporation. IL-2 (100 U/mL, Peprotech), IL-7 and IL-15 (10 ng/mL, Peprotech) were added with fresh medium on day 3 post-DCs stimulation. For re-stimulation, autologous DCs were pulsed with peptide (10 µg/mL) for 4 h and added to the cultured cells for another 7 d. Fresh complete medium was added containing cytokines every 2 to 3 d until use for experiments.

Flow cytometry

FACS Ariar (BD Bioscience) was used to perform fluorescent expression analysis. Cells were harvested the following days after transfection and stained with mouse anti-human antibody labeled by fluorescence for 30 min in 4 °C in darks as follows: CD3-PerCP-Cy5.5 (OKT-3, eBioscience) or CD3-FITC (HIT3a, BD Bioscience), CD4+ -APC (RPA-T4, BD Bioscience), CD8+ -PE (HIT8a, BD Bioscience), PD-1-PerCP-CY5.5 (EH12.1, BD Bioscience), PD-L1 (5H1, BD Bioscience), CD25-
PE (BC96, ebioscience), CD62L-FITC (DREG-56, ebioscience), CD27-PE (0323, ebioscience), CD28-PE (CD28.2, ebioscience), CD45RO-PE (UCHL1, BD Bioscience), CD69-PE (FN50, BD BioLegend), HLA-DR-PE (G46-6, BD Bioscience). For multiple cytokine detection, LEGENDplex human Th1/Th2 (7-plex) (Biolegend) kit was used.

**ELISPOT assay**

IFN-γ ELISPOT kit (Dakewe) was used to determine the frequency of cytokine-expressing T cells after overnight activation with peptides. Briefly, T cells (10^6 per well) and peptides (50 μg/ml) were added to duplicate wells and DCs were added at a ratio of 1:10 (DC:T) for 18–20 h. The plates were washed before the addition of the diluted antibody solution (1:100 dilution) and then incubated for 1 h at 37 °C. After washing the plates, streptavidin-AP (1:100 dilution) was added and incubated at 37°C for another 1 h. AEC solution mix was then added to each well, and the plates were left in the dark for about 15–25 min at room temperature before deionized water was added to stop development. Plates were scanned by ELISPOT Reader (Cell Technology Inc., Columbia, MD) and the results were analyzed with Elispot software (AID, Strassberg).

**Cytotoxicity assay**

Transduced T cells were tested for lytic activities by CFSE/PI labeling cytotoxicity assay. Target tumor cells were labeled with 4 μM CFSE (Carboxyfluorescein succinimidyl ester) (Invitrogen) for 10 min at 37 °C in PBS. Labeling was stopped by adding 10-fold volume of PBS and extensively washed in PBS before seeding into the 24-well plates. CFSE-labeled cells were then incubated with T cells by different effector to target ratio for 6 h. Propidium iodide (PI) (Sigma) was added to determine the ratio of cell death. Samples were analyzed by flow cytometry.

**Xenogenic mouse model**

BALB/c nude mice were inoculated subcutaneously with 1 × 10^7 SNU-719 cells. Irradiation was performed on day 21 and day 29 since the start of tumor inoculation (when tumors were at least 200 mm^3). 0.5~1 × 10^7 T cells were transferred by tail vein in 200 μL saline on day 21 and 24 h after each radiotherapy circle. 40,000 U human recombinant IL-2 were given intraperitoneally once a day for three consecutive days after adoptive transfer. Tumor volume was recorded every 2~3 d. Peripheral blood serum was collected to measure cytokine production 20 d after the start of treatment. Mice were sacrificed and tumors were excised for IHC or flow cytometry.

**Statistical analysis**

Graphpad Prism 5.0 (Graphpad software, San Diego, CA) was used for all statistical analysis. The mean ± SEM was determined for each treatment group in the individual experiments, and the Student’s t-test was used to determine the significances between treatment and control group(s). p-values < 0.05 were significant.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

The authors thank the entire Liu Lab and Huang Lab for their support and advice.

**Funding**

This work was supported by grants from the National Natural Science Foundation of China (Grant No. 81220108023, 81370064, 81401969) and by the grant from the State Key Laboratory of Oncogenes and Related Genes (Grant No. 901408).

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