CD137 Costimulation Enhances the Antitumor Activity of Vγ9Vδ2-T Cells in IL-10-Mediated Immunosuppressive Tumor Microenvironment

Yujun Pei1,2, Zheng Xiang1, Kun Wen1, Chloe Ran Tu3, Xiwei Wang1, Yanmei Zhang1, Xiaofeng Mu1, Yinping Liu1 and Wenwei Tu1*

1 Department of Paediatrics and Adolescent Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, Hong Kong SAR, China, 2 Bioland Laboratory (Guangzhou Regenerative Medicine and Health Guangdong Laboratory), Guangzhou, China, 3 Computational and Systems Biology Interdepartmental Program, University of California, Los Angeles, Los Angeles, CA, United States

Although γδ-T cell-based tumor immunotherapy using phosphoantigens to boost γδ-T cell immunity has shown success in some cancer patients, the clinical application is limited due to the rapid exhaustion of Vγ9Vδ2-T cells caused by repetitive stimulation from phosphoantigens and the profoundly immunosuppressive tumor microenvironment (TME). In this study, using a cell culture medium containing human and viral interleukin-10 (hIL-10 and vIL-10) secreted from EBV-transformed lymphoblastoid B cell lines (EBV-LCL) to mimic the immunosuppressive TEM, we found that the antitumor activity of Vγ9Vδ2-T cells was highly suppressed by endogenous hIL-10 and vIL-10 within the TME. CD137 costimulation could provide an anti-exhaustion signal to mitigate the suppressive effects of IL-10 in TME by suppressing IL-10R1 expression on Vγ9Vδ2-T cells. CD137 costimulation also improved the compromised antitumor activity of Vγ9Vδ2-T cells in TME with high levels of IL-10 in Rag2−/−γc−/− mice. In humanized mice, CD137 costimulation boosted the therapeutic effects of aminobisphosphonate pamidronate against EBV-induced lymphoma. Our study offers a novel approach to overcoming the obstacle of the hIL-10 and vIL-10-mediated immunosuppressive microenvironment by costimulating CD137 and enhancing the efficacy of γδ-T cell-based tumor therapy.

Keywords: CD137, γδ-T cells, antitumor activity, IL-10, immunotherapy

INTRODUCTION

Epstein-Barr virus (EBV) is a predominant type of human herpesviruses. It infects over 95% of the population by adulthood (1, 2). EBV infection is highly correlated with several human malignancies (1–3). As the first known human tumor virus, the carcinogenesis of EBV has been identified in various hematopoietic and epithelial cell cancers, including EBV-associated tumors and lymphoproliferative disorder (1, 2, 4). Current therapeutic approaches for EBV-associated tumors are restricted by undesirable side effects and ineffectiveness for refractory or relapsed
It was reported that EBV-specific CTL-based therapy is effective in the control of EBV-associated malignancy (5, 6). However, its clinical application is hampered due to insufficient quantity of EBV-specific CTL generated ex vivo (7).

As a major subset of human γδ-T cells, Vγ9Vδ2-T cells have been extensively demonstrated to have promising anti-tumor effects (8–13). Vγ9Vδ2-T cells can be activated specifically by phosphoantigens from isoprenoid biosynthesis in an MHC-unrestricted manner. Aminobisphosphonates pamidronate (PAM) and zoledronate (ZOL) are commonly used pharmacological phosphoantigens for osteoporosis and Paget's disease treatment (11, 14). Previously, we demonstrated that direct administration of PAM could expand Vγ9Vδ2-T cells in vivo and thus control EBV-induced lymphoma in humanized mice, suggesting that Vγ9Vδ2-T cell-based immunotherapy is promising for treating EBV-associated tumors (15). A recent meta-analysis of about 18,000 human cancers revealed that tumor-infiltrating γδ T cells are the most favorable cancer-wide prognostic marker (16). However, the clinical application was limited by the rapid exhaustion of Vγ9Vδ2-T cells caused by the repetitive stimulation from phosphoantigens in vivo (17) and the profoundly immunosuppressive tumor microenvironment (TME) (18–20).

Interleukin (IL)-10, as a major immunosuppressive cytokine in TME secreted by tumor cells, can help tumor cells escape immunological recognition and destruction (21–24). Current evidence indicates that EBV codes a homologue of human IL-10 (vIL-10) with immunosuppressive properties to evade immunity and establish persistent/latent infections (25–28). EBV-LCL also express and release various amounts of human IL-10 (hIL-10) (29, 30). hIL-10 and vIL-10 are crucial for B cell transformation of B cell (31, 32) and oncogenesis of EBV-associated tumors (33). However, whether the antitumor activity of Vγ9Vδ2-T cells was suppressed by IL-10 in TME remained largely unknown.

CD137 (4-1BB), a membrane-bound receptor, is a costimulatory molecule expressed in many lymphocytes (34–36). Recently, we demonstrated that CD137 costimulation enhanced the activation and cytolytic activity of Vγ9Vδ2-T cells against virus-infected cells (37). Importantly, boosting cancer immunotherapy with agonistic CD137 antibodies has been demonstrated to be a promising therapeutic strategy for different tumors (38, 39). However, the roles of CD137 signaling for human Vγ9Vδ2-T cells in the immunosuppressive TME remained to be determined.

In this study, we aim to clarify whether IL-10 in the TME is responsible for the exhaustion of Vγ9Vδ2-T cells and determine whether targeting CD137 can enhance the antitumor activity of Vγ9Vδ2-T cells compromised by the immunosuppressive TME.

**MATERIALS AND METHODS**

**Vγ9Vδ2-T Cell Cultures**

hPBMC were isolated fromuffy coats by Ficoll-Hypaque gradient centrifugation of EBV⁺ healthy donors after informed consents were obtained. PAM-expanded Vγ9Vδ2-T cells were prepared according to the protocol we established before (40). Briefly, PBMC were cultured in RPMI1640 medium with 10% fetal bovine serum (FBS) in the presence of PAM from day 0 to day 3 at a concentration of 9μg/ml. Recombinant human IL-2 was added to medium from day 3 to day 14 at a concentration of 500 IU/ml. After 2 weeks, the γδ-T cells were purified by positive selection with α-TCRγδ MicroBead (Miltenyi Biotec).

**Cytotoxic Assay**

Purified Vγ9Vδ2-T cells were cultured with IL-10(low) or IL-10(high) conditioned medium for 24 h, RPMI 1640 with 10% FBS medium (plain medium, PM) as a control. The pretreated Vγ9Vδ2-T cells (effector cells, E) were cocultured with autologous EBV-LCL (target cells, T) at an E: T ratio of 10:1 for 4 to 6 h in the IL-10(low/high) CM or PM, and then the death of target cells was analyzed with flow cytometry. Cells were stained with anti-CD3 to identify Vγ9Vδ2-T cells and propidium iodide (PI) was used to identify dead cells. The death of EBV-LCL was shown as the percentage of PI⁺ cells in the CD3⁺ population (40). In some experiments, neutralizing antibody against IL-10 (abcam) was added to block IL-10 mediated pathways. To confirm the suppressive role of IL-10 in the CM, recombinant hIL-10 (Peprotech) or recombinant vIL-10 (R&D systems) was added to culture medium at the indicated concentration.

**Establishment of EBV-LCL**

EBV-secreting cell lines B95-8 and B95.8EBfaV-GFP were cultured and EBV-containing supernatants were collected for the following infection. hPBMC were incubated with EBV-containing supernatants, and then cultured in the RPMI 1640 medium containing 15% FBS with the addition of cyclosporine-A (1μg/ml) as we describe before (15).

**Collection of EBV-LCL Conditioned Medium**

EBV-LCL were cultured in RPMI1640 medium for 24 h. The conditioned medium (CM) was collected, centrifuged at 5000 rpm at 4°C for 10 min to remove cell debris and then frozen at −80°C in aliquots. Stored CM was passed through a 0.22-μm syringe filter (Millipore) before use. Plain medium (PM) collected from complete medium without cell incubation under the same experimental conditions served as the control for CM.

**Determination of hIL-10 and vIL-10 Levels**

For hIL-10, the concentrations in conditioned medium were measured by ELISA. The procedures for human IL-10 ELISA kits (Biolegend, San Diego, CA, USA) were performed based on the manufacturer’s instructions. For vIL-10, the concentrations in conditioned medium were measured according to the method described before (41). The conditioned medium was concentrated by Amicon-Ultra centrifugation filters (Millipore) following the manufacturer’s instructions. Then, the concentrated conditioned medium was used for performing Western blot assay. Mouse monoclonal antibody against vIL-10 (R&D) was used as primary antibody for incubating transferred membranes at 4°C overnight. Horseradish peroxide...
conjugated goat anti-mouse secondary antibody (R&D) was used as secondary antibody for detecting vIL-10 levels. The bands of Western blot were quantified by “Gels” analysis tool of ImageJ. Recombinant vIL-10 was used as a standard to quantify the vIL-10 level in conditioned medium.

Establishment and Treatment of EBV-Associated Lymphoma in Mice

All animal studies were approved and performed in compliance with the guidelines for the use of experimental animals by the Committee on the Use of Live Animals in the Teaching and Research, the University of Hong Kong. Rag2<sup>−/−</sup>γc<sup>−/−</sup> mice were bred in Centre for Comparative Medicine Research of the University of Hong Kong. Humanized mice were generated according to the protocol we established before (14, 15). Rag2<sup>−/−</sup>γc<sup>−/−</sup> or humanized mice were inoculated with EBV-LCL expressing high or low level of IL-10 (0.1×10<sup>6</sup>/mouse) by subcutaneous injection to establish the EBV-associated lymphoma model. For Rag2<sup>−/−</sup>γc<sup>−/−</sup> mice, PAM-expanded Vγ9Vδ2-T cells (5×10<sup>6</sup>/mouse) with or without the addition of SA-hCD137L (5μg/mouse) were adoptively transferred intravenously into EBV-associated lymphoma murine model at indicated time. For humanized mice, PAM (5mg/kg body weight) and SA-hCD137L (15μg/mouse) were injected intraperitoneally at the indicated time. The mice treated with an equivalent volume of PBS or SA were used as controls. The tumor volume and mice survival were monitored every day and calculated at the indicated time. Mice were counted as dying when their subcutaneous tumor diameter was larger than 17 mm and thus sacrificed according to the regulation of Centre for Comparative Medicine Research of the University of Hong Kong. Otherwise, mice were monitored for 100 days before being sacrificed. The tumor tissues were reserved for immunohistochemical evaluation.

Preparation of the Recombinant SA-hCD137L Protein

Recombinant SA-hCD137L proteins were generated as described before (37). Briefly, the DNA sequences were synthesized encoding the extracellular domain of human CD137L (a.a. 58-254) and the core streptavidin (SA; a.a. 16-133) with an N-terminal 6xHis tag. The recombinant SA-hCD137L protein was expressed in E. coli by inserting the SA-hCD137L DNA fragments into the pETH expression vector and transforming into competent cells. After purifying with Ni-nitrilotriacetic acid fragments into the pETH expression vector and transforming into competent cells. After purifying with Ni-nitrilotriacetic acid, the recombinant SA-hCD137L protein was filtered a and quantitated by BCA Protein Assay Kit (Pierce, USA).

Flow Cytometric Analysis

Cells were stained for surface molecules with the following antibodies: αIL10R (Miltenyi Biotec, clone REA239), αCD3 (Biolegend, clone HIT3a), αTCRγ (Biolegend, clone B3), αTCRδ (Biolegend, clone B6), and αCD137 (Biolegend, clone 4B4-1). All samples were performed with a FACS LSR II (BD). The results were analyzed with FlowJo software.

Histological Staining and Immunohistochemical Assays

The tumor tissues were fixed with 10% formalin for 24 h and maintained in 70% ethanol. Fixed tumor tissues were embedded in paraffin and sectioned. The tumor sections were performed immunohistochemistry staining with αIL-10 antibody (abcam) (42).

Statistics

Data are shown in the form of mean ± standard error of the mean (SEM). All data were tested by Shapiro-Wilk test to verify the normality. For data that did not meet normal distribution, Mann-Whitney U test was used for analysis. For data that met normal distribution, one-way analysis of variance (ANOVA) with Bonferroni correction was used for analysis. For multiple variables, two-way ANOVA was used. Kaplan-Meier log-rank test was used for comparing survival among different groups. Two-tailed test was used for all analyses. P < 0.05 was regarded as significant.

RESULTS

Antitumor Activity of Vγ9Vδ2-T Cells Was Inhibited by IL-10 Secreted From EBV-LCL In Vitro

To investigate the effects of IL-10 in TME on the antitumor activity of Vγ9Vδ2-T cells, conditioned medium (CM) was obtained by collecting the supernatant of EBV-LCL culture for modeling TME in vitro. As shown in Figure 1A, CM from EBV-LCL culture established from different donors contained distinct levels of IL-10, and vIL-10 accounted for about 9.56 ± 5.74% of total IL-10. CM collected from EBV-LCL1 and EBV-LCL6, which contained the lowest and highest concentrations of IL-10, was used as IL-10<sub>low</sub> CM and IL-10<sub>high</sub> CM, respectively, in the following experiments. Importantly, the cytotoxic activity of IL-10<sub>high</sub> CM-treated Vγ9Vδ2-T cells against EBV-LCL was significantly lower than IL-10<sub>low</sub> CM- or PM-treated Vγ9Vδ2-T cells (Figure 1B). To verify the immunosuppressive role of IL-10 in the CM, an IL-10 neutralizing mAb was applied to block IL-10 signaling during Vγ9Vδ2-T cells exposed to IL-10<sub>high</sub> CM. The reduced cytotoxicity of Vγ9Vδ2-T cells against EBV-LCL was significantly abrogated when blocked with the IL-10 neutralizing mAb (Figure 1C). Furthermore, both hIL-10 and vIL-10 recombinant proteins showed dose-dependent inhibitions in the cytotoxicity of Vγ9Vδ2-T cells against EBV-LCL in the PM (Figures 1D, E). Taken together, our data indicate that the antitumor activity of Vγ9Vδ2-T cells against EBV-LCL was suppressed by both the hIL-10 and vIL-10 in the CM from EBV-LCL in vitro.

Antitumor Activity of Vγ9Vδ2-T Cells Against EBV-Induced Lymphoma Was Decreased Under IL-10<sub>high</sub> TME In Vivo

To determine whether the therapeutic effects of Vγ9Vδ2-T cells on EBV-induced B cell lymphoma were inhibited by IL-10 within the TME, EBV-LCL1 expressing low levels of IL-10 (IL-10<sub>low</sub> LCL) and EBV-LCL6 expressing high levels of IL-10 (IL-10<sub>high</sub> LCL) were inoculated into Rag2<sup>−/−</sup>γc<sup>−/−</sup> mice, respectively.
Figure 2A). After 21 days, large subcutaneous tumors developed in all the mice as detected by in vivo imaging (Figures 2B, C). The expressions of IL-10 in the tumor tissues generated from IL-10low LCL and IL-10high LCL were detected by immunohistochemistry (Figure 2D). Consistent with our previous results (15), Vγ9Vδ2-T cell treatment constrained tumor growth and prolonged the survival of tumor-bearing mice in contrast with the mice treated with PBS as the control (Figures 2E, F). Importantly, Vγ9Vδ2-T cells showed less efficacy in controlling EBV-induced lymphoma developed from IL-10high LCL compared with that developed from IL-10low LCL, along with larger tumor volume and lower survival rates (Figures 2E, F). These results suggest that the decreased antitumor activity of Vγ9Vδ2-T cells against EBV-induced lymphoma may be associated with IL-10high TME in vivo.

CD137 Costimulation Suppressed IL-10R1 Expression and Restored the Antitumor Activity of Vγ9Vδ2-T Cells

IL-10 mediates its biological effects mainly through a heterodimeric membrane receptor composed of IL-10R1 and IL-10R2 (43). Since IL-10R2 is shared by more than five IL-10 family cytokines (44), we investigated the expression of IL-10R1 on Vγ9Vδ2-T cells exposed to the IL-10high CM upon gd-TCR activation in vitro. Importantly, we found that following activation, IL-10R1+ Vγ9Vδ2-T cells expressed high levels of CD137 compared with IL-10R1−/lo Vγ9Vδ2-T cell subset in the IL-10high CM, indicating that CD137 could be an effective costimulatory signaling to restore the antitumor activity of Vγ9Vδ2-T cells compromised by the IL-10 in TME (Figures 3A, B).

To determine whether CD137 costimulation could provide an anti-exhaustion signal to mitigate the inhibiting effects mediated by IL-10 in TME, a recombinant SA-hCD137L protein containing a core streptavidin (SA) molecule with the extracellular domains of human CD137L (hCD137L) was generated as we reported previously (37). We found that the addition of the recombinant SA-hCD137L protein significantly inhibited the surface expression of IL-10R1 in total and CD137+ Vγ9Vδ2-T cells in IL-10high CM in terms of both percentage and expression level (MFI) changes (Figures 3C–E). These results indicate that CD137 costimulation suppressed IL-10R1
expression in CD1377 γδVδ2-T cells, and thereby was able to reduce their sensitivity to endogenous IL-10 in the immunosuppressive TME.

To determine whether CD137 costimulation could rescue the impaired antitumor efficacy of γδVδ2-T cells in suppressive TME, the recombinant SA-hCD137L protein was added to the coculture of γδVδ2-T cells with EBV-LCL in IL-10high CM for mimicking the tumor milieu. As shown in Figure 3F, the SA-hCD137L protein significantly increased the cytotoxicity of γδVδ2-T cells against EBV-LCL under both the immunosuppressive and normal microenvironments mimicked by the IL-10high CM and the PM. Importantly, CD137 costimulation not only completely restored the reduced cytotoxicity of γδVδ2-T cells in the IL-10high CM to normal levels, but also had a better effect to enhance the cytotoxic activity of γδVδ2-T cells in IL-10low CM than that in PM (Figure 3F). These data demonstrate that CD137 engagement enables γδVδ2-T cells to withstand the hostile environment mediated by endogenous IL-10, resulting in the increase of the antitumor activity of γδVδ2-T cells in vitro.

**CD137 Costimulation Enhanced the Compromised Antitumor Activity of γδVδ2-T Cells With IL-10high TME in Rag2−/− γc−/− Mice**

Previously we have demonstrated that γδVδ2-T cells could control EBV-inducing lymphoma (15), and their antitumor activity in controlling EBV-induced lymphoma developed from IL-10low LCL was lower than that developed from IL-10high LCL (Figures 2E, F). To further elucidate the roles of CD137 costimulation in the compromised antitumor activity of γδVδ2-T cells in IL-10high TME in vivo, EGFP-expressing IL-10high LCL was inoculated in Rag2−/− γc−/− mice. (Figure 4A). Twenty-one days later, mice bearing subcutaneous tumors were randomly divided into three groups with the recombinant SA-hCD137L protein weekly from day 21 to day 42. The other two groups of mice were adoptively transferred to one group of the tumor-bearing mice with the recombinant SA-hCD137L protein weekly from day 21 to day 42.

---

**FIGURE 2** | Antitumor activity of γδVδ2-T cells against EBV-induced lymphoma was decreased under IL-10high TME in vivo. (A) IL-10low LCL and IL-10high LCL were injected s.c. in Rag2−/− γc−/− mice separately. After 21 days, mice that had developed subcutaneous tumor were randomly divided into two groups respectively followed by the treatment with allogeneic γδVδ2-T cells or PBS at indicated time (six mice per group). (B, C) Whole-body fluorescence images (B) and total radiant efficiency (C) of mice before treatment with γδVδ2-T cells or PBS. (D) Representative histology of IL-10 in tumor sections that developed from IL-10low LCL and IL-10high LCL. (E, F) The tumor volume (E) and mouse survival (F) were determined at the indicated time. The tumor volume was compared using two-way ANOVA analysis, and mouse survival was compared using Kaplan-Meier log-rank test. Data are representative for three independent experiments. *p < 0.05; **p < 0.01; ns, no significant difference.

---

Pei et al. CD137 Enhances γδ-T-Cell Antitumor Activity

---

Frontiers in Immunology | www.frontiersin.org June 2022 | Volume 13 | Article 872122

---

June 2022 | Volume 13 | Article 872122

---

---
transferred with Vγ9Vδ2-T cells in the presence of PBS or SA as the controls. Importantly, Vγ9Vδ2-T cells in combination with SA-hCD137L treatment significantly limited tumor growth (Figure 4D) and improved mouse survival (Figure 4E) compared to treatments of Vγ9Vδ2-T cells with PBS or SA. These data indicate that the costimulation of CD137 efficiently enhanced the antitumor activity of Vγ9Vδ2-T cells in the highly immunosuppressive microenvironment mediated by IL-10 in vivo.

CD137 Costimulation Improved the Therapeutic Effect of PAM in Controlling EBV-Induced Lymphoma With IL-10high TME in Humanized Mice

Previously we had demonstrated that PAM could expand Vγ9Vδ2-T cells in vivo to control EBV-induced lymphoma in humanized mice with functional hPBMC (15). We then investigated the role of CD137 costimulation on the therapeutic effect of PAM in controlling EBV-induced lymphoma with IL-10high TME in humanized mice. EBV-induced lymphoma with IL-10high TME model was generated by inoculation s.c. of IL-10high EBV-LCL in humanized mice (Figure 5A) (15). All humanized mice developed subcutaneous tumors after IL-10high EBV-LCL inoculation for 28 days with similar fluorescent density from tumor cells as detected by in vivo imaging (Figures 5B, C). PAM, SA-hCD137L, or the combination of these two agents were injected intraperitoneally (i.p.) at days 28, 35, 42, and 49 after IL-10high EBV-LCL inoculation (Figure 5A). PBS- and SA-treated mice were controls. As a result, PAM administration alone decreased the tumor volume significantly and extended the survival of the tumor-bearing humanized mice compared with the treatment with PBS, SA, or SA-hCD137L protein alone, respectively (Figures 5D, E). Importantly, the combination treatment of PAM with SA-hCD137L was more potent than PAM alone to control the development of EBV-induced lymphoma with IL-10high TME in humanized mice, in terms of tumor growth and survival (Figures 5D, E).

Humanized mice reconstituted with Vγ9Vδ2-T-cell-depleted hPBMC were also used to confirm whether the effect of SA-hCD137L costimulation on the control of EBV-induced
FIGURE 5 | CD137 costimulation improved the therapeutic effect of PAM in controlling EBV-induced lymphoma with IL-10^{high} TME in humanized mice. (A) The evaluation protocol of the synergistic therapeutic effect of PAM and SA-hCD137L on EBV-induced lymphoma in humanized mice (five mice per group). (B, C) Whole-body fluorescence images (B) and total radiant efficiency (C) of mice before treatment with PAM, SA-hCD137L, SA, and PBS. (D, E) The tumor volume (D) and mouse survival (E) were determined at the indicated time. The tumor volume was analyzed by two-way ANOVA test, and mice survival was analyzed by Kaplan-Meier log-rank test. Data are representative for three independent experiments. *p < 0.05; **p < 0.01; ns, no significant difference.

FIGURE 4 | CD137 costimulation enhanced the compromised antitumor activity of V_{g}9V_{d}2-T cells with IL-10^{high} TME in Rag2^{-/-}bgc^{-/-} mice. (A) Protocol for evaluation of the synergistic therapeutic effect of V_{g}9V_{d}2-T cells and SA-hCD137L on EBV-induced lymphoma in Rag2^{-/-}bgc^{-/-} mice (five mice per group). (B, C) Whole-body fluorescence images (B) and total radiant efficiency (C) of mice before treatment with PAM, SA-hCD137L, SA, and PBS. (D, E) The tumor volume (D) and mouse survival (E) were determined at the indicated time. The tumor volume was analyzed by two-way ANOVA test, and mice survival was analyzed by Kaplan-Meier log-rank test. Data are representative for three independent experiments. *p < 0.05; **p < 0.01; ns, no significant difference.
Elevated IL-10 levels are correlated with shorter survival and adverse disease features in patients with EBV-associated tumors (33, 57). Thus, we reasoned that hIL-10 and vIL-10 may be associated with the suppression of Vγ9Vδ2-T cells' antitumor activity. Such an interaction would provide a suitable microenvironment for viruses to evade immunity and cause tumorigenesis. Here, our in vitro data revealed that vIL-10 derived from EBV and hIL-10 derived from EBV-LCL were the dominant factors for inhibiting the antitumor activity of Vγ9Vδ2-T cells in TME. Our in vivo data also suggested that the reduced antitumor activity of Vγ9Vδ2-T cells against EBV-induced lymphoma may be associated with IL-10 high TME. Further study using IL-10 neutralizing mAb or IL-10 knockout mice is required to determine the exact role of IL-10 in antitumor activity of Vγ9Vδ2-T cells in vivo. Of note, additional factors in the CM might also contribute to suppressing Vγ9Vδ2-T cells activity because a smaller extent of cytotoxicity reduction after treatment with recombinant hIL-10 and vIL-10 proteins was observed when compared with IL-10 high CM (Figure 1).

Recently, clinical trials utilizing bisphosphonates, such as PAM and ZOL, to expand γδ-T cell in vivo in combination with IL-2 therapy or adoptive transfer of ex vivo cultured γδ-T cells were performed in patients with tumors and virus infections (11, 15, 58, 59). Administration of bisphosphonates with IL-2 and the transfer of expanded autologous Vγ9Vδ2 T-cells have been demonstrated to be safe with limited adverse events (60). However, there is only a modest efficacy in the treatment of some tumors (61, 62). One drawback of γδ-T cell-based immunotherapy is the rapid exhaustion of proliferation and effector responses due to repeated phosphoantigen treatments (17). Another drawback of this therapy is the impaired antitumor activity of γδ-T cells caused by the tumour immunosuppressive microenvironment (18, 63).

CD137 is a promising costimulatory immunologic target for enhancing antitumor immune responses (39). CD137 costimulation, known as “stepping on the accelerator,” is believed to be a compelling complement for “removing the brakes” via blocking inhibitory signaling. Importantly, it is now appreciated that CD137 signaling not only works as an “accelerator” to provide costimulation, but also breaks and reverses the established anergy in cytotoxic T lymphocytes (CTLs) (64, 65). However, the role of CD137 signaling in Vγ9Vδ2-T cells within the context of IL-10-mediated TME is not clear. Here, we revealed that IL-10R1 Vγ9Vδ2 T-cell subset expressed high levels of CD137. Moreover, CD137 costimulation suppressed IL-10R1 in Vγ9Vδ2-T cells, suggesting that CD137 engagement possessed the potential to ameliorate the exhaustion and dysfunction of Vγ9Vδ2-T cells.

Ligation of CD137 is correlated with effective antitumor responses; however, the application of anti-CD137 agonistic antibodies in patients is limited by a variety of side effects (66). The natural CD137 ligand is an alternative to the CD137-specific antibodies to stimulate antitumor T cell responses. Shirwan lab reported that a streptavidin-conjugated murine CD137L (SA-mCD137L) complex could induce effective antitumor immune responses (67, 68). SA-mCD137L induces less pathological side effects than anti-CD137 agonistic antibody therapy, suggesting a
higher therapeutic index of SA-mCD137L. Previously, we demonstrated that recombinant SA-hCD137L enhanced the cytotoxic effect of Vγ9Vδ2- T cells against influenza virus infection (37). Here, we further found that SA-hCD137L restored the antitumor activity of Vγ9Vδ2-T cells compromised by the IL-10-mediated TME. These data indicate that SA-hCD137L can provide an alternative to anti-CD137 agonistic for anti-tumor therapy.

There are no Vγ9Vδ2-T cells in mice, thus it is impossible to study these cells in mouse models (69). Previously, we successfully established humanized mice with a similar proportion of Vγ9Vδ2-T cells in murine peripheral blood to that in humans (12, 14, 70, 71). Importantly, here the synergistic effect of PAM and recombinant SA-hCD137L was verified in humanized mice.

In conclusion, our study further elucidates the role of CD137 in the antitumor activity of human Vγ9Vδ2-T cells in the IL-10-mediated immunosuppressive TME. The combination of a phosphoantigen and CD137 agonist also provides a novel strategy for treating EBV-induced tumors by avoiding Vγ9Vδ2-T cell exhaustion and enhancing the efficacy of Vγ9Vδ2-T cell-based therapy.

AUTHOR CONTRIBUTORS

YP, WT, and YL conceived and designed the study, interpreted the results, wrote and edited the manuscript; YP, KW, ZX, CT, XW, YZ, and XM designed and performed the experiments, analyzed the results. WT supervised this study. All authors contributed to the article and approved the submitted version.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional review board of The University of Hong Kong/Hospital Authority Hong Kong West Cluster. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong.

FUNDING

This study was supported partially by GRF, RGC (17122519, 17126317); Health and Medical Research Fund, Food and Health Bureau, Hong Kong SAR Government (18192021); Seed Funding for Strategic Interdisciplinary Research Scheme, the University of Hong Kong; Hong Kong SAR, China.

REFERENCES

1. Cohen JI, Fauci AS, Varmus H, Nabel GJ. Epstein-Barr Virus: An Important Vaccine Target for Cancer Prevention. Sci Transl Med (2011) 3(107):10767. doi: 10.1126/scitranslmed.3002878
2. Young LS, Yap LF, Murray PG. Epstein-Barr Virus: More Than 50 Years Old and Still Providing Surprises. Nat Rev Cancer (2016) 16(12):789–802. doi: 10.1038/nrc.2016.92
3. Zhu QY, Zhao GX, Li Y, Talakatta G, Mai HQ, Le QT, et al. Advances in Pathogenesis and Precision Medicine for Nasopharyngeal Carcinoma. MedComm (2020) (2021) 2(2):175–206. doi: 10.1002/mco2.32
4. Taylor GS, Long HM, Brooks JM, Richardson AB, Hislop AD. The Immunology of Epstein-Barr Virus-Induced Disease. Annu Rev Immunol (2015) 33:787–821. doi: 10.1146/annurev-immunol-032414-112326
5. Israel BF, Kenney SC. Virally Targeted Therapies for EBV-Associated Malignancies. Oncogene (2003) 22(33):5122–30. doi: 10.1038/sj.onc.1205648
6. Dharmidharka VR, Mohanakumar T. New Approaches to Treating B-Cell Cancers Induced by Epstein-Barr Virus. N Engl J Med (2015) 372(6):569–71. doi: 10.1056/NEJMci1411517
7. Bollard CM, Rooney CM, Heslop HE. T-Cell Therapy in the Treatment of Post-Transplant Lymphoproliferative Disease. Nat Rev Clin Oncol (2012) 9(9):510–9. doi: 10.1038/nrclinonc.2012.111
8. Hayday AC. Gammadelta T Cell Update: Adaptate Orchestrators of Immune Surveillance. J Immunol (2019) 203(2):311–20. doi: 10.4049/jimmunol.1800934
9. Silva-Santos B, Mensurado S, Collell SB. Gammadelta T Cells: Pleiotropic Immune Effectors With Therapeutic Potential in Cancer. Nat Rev Cancer (2019) 19(7):392–404. doi: 10.1038/s41568-019-0153-5
10. Xiang Z, Tu W. Dual Face of Vγ9Vδ2Δ-T Cells in Tumor Immunology: Anti- Versus Pro-Tumoral Activities. Front Immunol (2017) 8:1041. doi: 10.3389/fimmu.2017.01041
11. Tanaka Y, Murata-Hirai K, Iwasaki M, Matsumoto K, Hayashi K, Kumagai A, et al. Expansion of Human Gammadelta T Cells for Adoptive Immunotherapy Using a Bisphosphonate Prodrug. Cancer Sci (2018) 109(3):587–99. doi: 10.1111/cas.13491
12. Wang X, Xiang Z, Liu Y, Huang C, Pei Y, Wang X, et al. Exosomes Derived From Vδ2-T Cells Control Epstein-Barr Virus-Associated Tumors and Induce T Cell Antitumor Immunity. Sci Transl Med (2020) 12(563):eaaz3426. doi: 10.1126/scitranslmed.aaz3426
13. Wang X, Zhang Y, Mu X, Tu CR, Chung Y, Tsao SW, et al. Exosomes Derived From Gammadelta-T Cells Synergize With Radiotherapy and Preserve Antitumor Activities Against Nasopharyngeal Carcinoma in Immunosuppressive Microenvironment. J Immunother Cancer (2022) 10(2):e003832. doi: 10.1126/jitc-2021-003832
14. Tu W, Zheng J, Liu Y, Xia SP, Liu M, Qin G, et al. The Aminobisphosphonate Pamidronate Controls Influenza Pathogenesis by Expanding a Gammadelta T Cell Population in Humanized Mice. J Exp Med (2011) 208(7):1511–22. doi: 10.1084/jem.20110226
15. Xiang Z, Liu Y, Zheng J, Liu M, Lv A, Gao Y, et al. Targeted Activation of Human Vγ9Vδ2Δ-T Cells Controls Epstein-Barr Virus-Induced B Cell Lymphoproliferative Disease. Cancer Cell (2014) 26(4):565–76. doi: 10.1016/j.ccr.2014.07.026
16. Gentles AJ, Newman AM, Liu CL, Bratman SV, Feng W, Kim D, et al. The Prognostic Landscape of Genes and Infiltrating Immune Cells Across Human Cancers. Nat Med (2015) 21(8):938–45. doi: 10.1038/nm.3909
17. Sicard H, Ingoure S, Luciani B, Serraz C, Fournier JL, Bonneville M, et al. In Vivo Immunomanipulation of V Gamma 9 Delta 2 T Cells With a Synthetic

Frontiers in Immunology | www.frontiersin.org 9 June 2022 | Volume 13 | Article 872122
Phosphoantigen in a Preclinical Nonhuman Primate Model. J Immunol (2005) 175(8):5471–80. doi: 10.4049/jimmunol.175.8.5471

18. Yi, Y., He, HW., Wu, XJ., Cai, XY., Li, YW., Zhou, J., et al. The Functional Impairment of HCC-Inflicting γδ T Cells Partially Mediated by Regulatory T Cells in a γδT- and IL-10-Dependent Manner. J Hepatol (2013) 58(5):977–83. doi: 10.1016/j.jhep.2012.02.015

19. Park, JH., Lee, HK. Function of γδ T Cells in Tumor Immunology and Their Application to Cancer Therapy. Exp Mol Med (2021) 53(3):318–27. doi: 10.1038/s12276-021-00576-0

20. Pitt, JM., Marabelle, A., Eggermont, A., Soria, JC., Kroemer, G., Zitvogel, L. Targeting the Tumor Microenvironment: Removing Obstruction to Anticancer Immune Responses and Immunotherapy. Ann Oncol (2016) 27(8):1482–92. doi: 10.1093/annonc/mdw168

21. Dennis, KL., Blatner, NR., Gounari, F., Kharazia, K. Current Status of Interleukin-10 and Regulatory T-Cells in Cancer. Curr Opin Oncol (2013) 25(6):637–45. doi: 10.1097/CCO.0b013e32835d337b

22. Kajino, K., Nakamura, I., Bamba, H., Sawai, T., Ogasawara, K. Involvement of IL-10 in Exhaustion of Myeloid Dendritic Cells and Rescue by CD40 Stimulation. Immunology (2007) 120(1):28–37. doi: 10.1111/j.1365-2672.2006.02474.x

23. Gasa, A., Jian, F., Ferkalhan, D., Van, H., Honke, N., Shaabani, N., et al. IL-10 Induces T Cell Exhaustion During Transplantation of Virus Infected Hearts. Cell Physiol Biochem (2016) 38(3):1711–81. doi: 10.1159/000443067

24. Shi, R., Tang, YQ., Miao, H. Metabolism in Tumor Microenvironment: Implications for Cancer Immunotherapy. MedComm (2020) 1(1):47–68. doi: 10.1002/mco.2

25. Stuart, AD., Stewart, JP., Arrand, JR., Mackett, M. The Epstein-Barr Virus Encoded Cytokine Viral Interleukin-10 Enhances Transformation of Human B Lymphocytes. Oncogene (1995) 11(9):1711–9.

26. Brooks, DG., Trifilo, MJ., Edelmann, KH., Teyton, L., McGavern, DB., Oldstone MB. Interleukin-10 Determines Viral Clearance or Persistence In Vivo. Nat Med (2006) 12(11):1301–9. doi: 10.1038/nm1492

27. Ejrnaes, M., Filippi, CM., Martinic, MM., Ling, EM., Togher, LM., Crotty, S., et al. Resolution of a Chronic Viral Infection After Interleukin-10 Receptor Blockade. J Exp Med (2006) 203(11):2461–72. doi: 10.1084/jem.20061462

28. Johcm, S., Moosmann, A., Lang, S., Hammerschmidt, W., Zeidler, K. The EBV Phosphoantigen in a Preclinical Nonhuman Primate Model. J Immunol (2005) 179(12):8225–34. doi: 10.4049/jimmunol.179.12.8225

29. Miyazaki, K., Mochida, K., Matsuda, K., Koyama, K., Nakanishi, M., et al. CD137 (4-1BBL–CD137L) Blockade Enhances Antitumor Activity of Vγ9Vδ2-T Cells Against EBV and Human Papillomavirus Infection. J Immunother (2016) 40(1):22–30. doi: 10.1097/CIN.0000000000000871

30. Stoll, A., Bruns, H., Fuchs, M., Volki, S., Nimmerjahn, F., Kunz, M., et al. CD137 (4-1BB) Stimulation Leads to Metabolic and Functional Reprogramming of Human Monocytes/Macrophages Enhancing Their Tumoricidal Activity. Leukemia (2021) 35(12):2482–96. doi: 10.1038/s41373-021-01287-1

31. Wang, C., Lin, GH., McPherson, AJ., Watts, TH. Immune Regulation by 4-1BB and 4-1BBL: Complexities and Challenges. Immunol Rev (2009) 229(1):192–215. doi: 10.1111/j.1600-065X.2009.00765.x

32. Shuford, W., Klussman, K., Tretcher, DL., Loo, DT., Chalupny, J., Niidom, AW., et al. 4-1BB Costimulatory Signals Preferentially Induce CD4+ T Cell Proliferation and Lead to the Amplification In Vivo of Cytotoxic T Cell Responses. J Exp Med (1997) 186(1):47–55. doi: 10.1084/jem.186.1.47

33. Pei, Y., Wen, K., Xiang, Z., Huang, C., Wang, C., Xu, X., et al. CD137 Costimulation Enhances the Antiviral Activity of γδT-Cells Against Influenza Virus. Signal Transduct Target Ther (2020) 5(1):74. doi: 10.1038/s41392-020-0174-2

34. Slobedman, B., Barry, PA., Spencer, JV., Andric, A., Abendroth, A. Virus-Encoded Cytokine Viral Interleukin-10 Enhances Transformation of Expanded Human Gammadelta T Cells Displaying Potent Cytotoxicity Against Monocyte-Derived Macrophages Infected With Human and Avian Influenza Viruses. J Infect Dis (2009) 200(6):855–65. doi: 10.1086/650413

35. Hashimoto, K. CD137 as an Attractive T Cell Co-Stimulatory Target in the Human Monocytes. Front Immunol (2018) 9:2198. doi: 10.3389/fimmu.2018.02198

36. Qin, G., Mao, H., Zheng, J., Sia, SF., Liu, Y., Shan, PL., et al. Phosphoantigen-Expanded Human Gammaddelta T Cells Display Potent Cytotoxicity Against Monocyte-Derived Macrophages Infected With Human and Avian Influenza Viruses. J Infect Dis (2009) 200(6):855–65. doi: 10.1086/650413

37. Kajino, K., Nakamura, I., Bamba, H., Sawai, T., Ogasawara, K. Involvement of IL-10 in Exhaustion of Myeloid Dendritic Cells and Rescue by CD40 Stimulation. Immunology (2007) 120(1):28–37. doi: 10.1111/j.1365-2672.2006.02474.x

38. Shuford, W., Klussman, K., Tretcher, DL., Loo, DT., Chalupny, J., Niidom, AW., et al. 4-1BB Costimulatory Signals Preferentially Induce CD4+ T Cell Proliferation and Lead to the Amplification In Vivo of Cytotoxic T Cell Responses. J Exp Med (1997) 186(1):47–55. doi: 10.1084/jem.186.1.47
64. Wilcox RA, Tamada K, Flies DB, Zhu G, Chapoval AI, Blazar BR, et al. Ligation of CD137 Receptor Prevents and Reverses Established Anergy of CD8+ Cytolytic T Lymphocytes In Vivo. Blood (2004) 103(1):177–84. doi: 10.1182/blood-2003-06-2184

65. Long AH, Haso WM, Shern JF, Wanhainen KM, Murgai M, Ingaramo M, et al. 4-1BB Costimulation Ameliorates T Cell Exhaustion Induced by Tonic Signaling of Chimeric Antigen Receptors. Nat Med (2015) 21(6):581–90. doi: 10.1038/nm.3838

66. Asciano PA, Simeone E, Szoln M, Fu YX, Melero I. Clinical Experiences With Anti-CD137 and Anti-PD1 Therapeutic Antibodies. Semin Oncol (2010) 37 (5):508–16. doi: 10.1053/j.seminoncol.2010.09.008

67. Barsoumian HB, Batra L, Shrestha P, Bowen WS, Zhao H, Eglimzs NK, et al. A Novel Form of 4-1BBL Prevents Cancer Development via Non-specific Activation of CD4(+) T and Natural Killer Cells. Cancer Res (2019) 79 (4):783–94. doi: 10.1158/0008-5472.CAN-18-2401

68. Srivastava AK, Dinc G, Sharma RK, Yolcu ES, Zhao H, Shirwan H. SA-4-1BBL and Monophosphoryl Lipid A Constitute an Efficacious Combination Adjuvant for Cancer Vaccines. Cancer Res (2014) 74(22):6441–51. doi: 10.1158/0008-5472.CAN-14-1768-A

69. Born WK, Reardon CL, O’Brien RL. The Function of Gammadelta T Cells in Innate Immunity. Curr Opin Immunol (2006) 18(1):31–8. doi: 10.1016/j.coi.2005.11.007

70. Chen Q, Wen K, Lv A, Liu M, Ni K, Xiang Z, et al. Human Vgamma9Vdelta2-T Cells Synergize CD4(+) T Follicular Helper Cells to Produce Influenza Virus-Specific Antibody. Front Immunol (2018) 9:599. doi: 10.3389/fimmu.2018.00599

71. Ni K, Liu M, Zheng J, Wen L, Chen Q, Xiang Z, et al. PD-1/PD-L1 Pathway Mediates the Alleviation of Pulmonary Fibrosis by Human Mesenchymal Stem Cells in Humanized Mice. Am J Respir Cell Mol Biol (2018) 58(6):684–95. doi: 10.1165/rcmb.2017-0326OC

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher’s Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Pei, Xiang, Wen, Tu, Wang, Zhang, Mu, Liu and Tu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.