PD-1 blockade potentially enhances adoptive cytotoxic T cell potency in a human acute myeloid leukaemia animal model

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ABSTRACT

Objectives: Acute myeloid leukaemia (AML) is a malignant haematological disease that remains difficult to cure. Cytotoxic T cell (CTL) adoptive infusion therapy may be conducive to tumour remission by boosting physical immunity. Furthermore, programmed death receptor-1 (PD-1) blockade immunotherapy has shown tremendous success in many cancer therapies.

Method: We attempted to combine these two immunotherapy strategies to intervene in AML by generating AML cell-specific cytotoxic T lymphocytes in vitro and in vivo with an AML cell strain expressing specific antigens.

Results: First, we observed that peripheral blood mononuclear cells (PBMCs) could be induced to generate large numbers of CD8+ CTL cells through immune stimulation. In addition, these CD8+ cells could effectively recognize a human AML cell line and exert cytotoxicity. In animal tests, PD-1 blockade combined with CTL infusion could induce significantly more AML tumour reduction than either treatment alone. This synergistic effect was thought to be connected to immune modulation enhancement, as regulatory T cells (Tregs) in the peripheral blood (PB) were found to be suppressed.

Conclusions: This finding suggested the potential application of PD-1 blockade in AML. The present work demonstrated an excellent synergistic tumour therapeutic effect of PD-1 blockade and CTL therapy compared with either treatment alone.

KEYWORDS

Programmed death receptor-1; adoptive cytotoxic T cell; acute myeloid leukaemia

Introduction

Because of the standard ‘3 + 7’ intensive chemotherapy regimen, most adult acute myeloid leukaemia (AML) patients can achieve complete remission. However, only 20–40% of patients experience more than 5 years of disease-free survival [1]. The outcomes of older patients who cannot tolerate intensive chemotherapy without serious side effects remain less optimistic; the median survival ranges from 5 to 10 months. No other induction regimen is superior, except for the addition of gemtuzumab, a humanized anti-CD33 monoclonal antibody. Meta-analysis showed that adding gemtuzumab ozogamicin to induction therapy could reduce the risk of relapse and improve survival among younger and older patients with favourable and intermediate-risk cytogenetic findings but did not increase response rates [2].

Post-remission therapy with allogeneic haematopoietic stem cell transplantation (HSCT) may provide a powerful antineoplastic strategy because of pretransplantation cytoreductive conditioning and the graft-versus-leukaemia (GVL) effect [3]. Nevertheless, because of high rates of treatment-related mortality and morbidity, whether to use an HSCT strategy during the first remission remains controversial [4].

There is a growing need for additional or innovative treatment options.

Cancer immunotherapy has shown a strong potential for blood cancer therapy. In addition, programmed death receptor-1 (PD-1) blockade-mediated immunotherapy has attracted tremendous attention recent years in cancer immunotherapy. The PD-1 molecule was recognized as an important marker for cell exhaustion, and PD-1-expressing antigen-specific T cells are incapable of secreting cytokines and proliferating even after antigen restimulation [5,6]. In addition, expression of the homologous ligand of PD-1, PD-L1, induces T cell apoptosis via interaction with PD-L1, CD8+ cytotoxic T cell immunological function and incidence of tumours and blood cancers [7]. Additionally, high expression of PD-L1 and elevation after interferon-γ (IFN-γ) exposure were found in AML [8]. PD-L1 and PD-1 play vital roles in regulating immune responses, immune tolerance, autoimmunity, and antitumour immunity [9]. PD-1 expression on T cells suppresses CD8+ cytotoxic T cell immunological function and induces T cell apoptosis via interaction with PD-L1, which has expedited the increase in anti-PD-1 and anti-PD-L1 immunotherapy for cancer. Inhibition of the interaction between PD-1 and PD-L1 enhances T cell responses in vitro and promotes preclinical antitumour activity [10,11]. Subsequently, good clinical trial
results led to approval of the PD-1 antibody for use in many types of cancer, such as melanoma and non-small-cell lung cancer (NSCLC) [12–14]. At the same time, the GVL effect after allogeneic HSCT [15], donor lymphocyte infusions [16], and the success of PD-1 blockade in Hodgkin’s lymphoma (HL) [17], all present us with the prospect of blood cancer immunotherapy. Therefore, we designed this experiment, which aims to exploit the possible effect of combining PD-1 blockade and adoptive AML cytotoxic T cell (CTL) therapy in an animal model.

In our present study, we examined the effect of PD-1 blockade and adoptively transferred AML-CTLs in a murine model and explored potential mechanisms by studying variations in the expression of PD-L1 in organs and circulating immune cells. The objective is to explore whether the cytotoxicity of CTLs can be potentiated via combination therapy with PD-1 inhibitors or how CTLs may maximize the efficacy of PD-1 inhibitors compared to that mediated by T cells.

**Materials and methods**

**Cell lines and reagents**

The Kasumi-6 human AML cell line expressing CD33 and CD13 was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were traditionally cultured in RPMI-1640 (Sigma-Aldrich, Shanghai, Trading Co. Ltd.) with 2 mM L-glutamine and 2 ng/ml human recombinant granulocyte macrophage colony-stimulating factor (GM-CSF, R&D Systems China Co. Ltd.) containing 20% foetal bovine serum (Sigma Chemical) under conditions of 5% CO₂ in an incubator at 37°C. Immune cell specialized culture medium was purchased from Sino-Biocan (Beijing) Biology Technology Ltd. The Ficoll lymphocyte separation liquid was from General Electric Company. Antibodies for flow cytometry were purchased from Becton Dickinson. The mouse PDL-1 monoclonal antibody was from Abcam.

**AML-CTL culture and characterization**

Monocyte-depleted peripheral blood lymphocytes (PBLs) from healthy donors were stimulated with Kasumi-6 cells and incubated overnight at 37°C. Before they were added to the PBLs, the 1 × 10⁶ tumour cells were treated with mitomycin C (40 μg/ml) for 30 min or irradiated with 8,000 rad rays. Then, the treated Kasumi-6 cells were added to the PBLs and co-cultured for 4 days. Half of the medium was then discarded. The PBLs were collected and added to 2 × 10⁵ fresh Kasumi-6 cells for continuous stimulation. In addition, 10 U/ml human recombinant IL-2 (BioDee, Beijing, China) was added to the PBL medium on day 3, half of the medium was discarded every 3 days and the rIL-2 concentration was kept at 10 U/ml. The stimulation procedure was repeated every week. After the third restimulation, the cells were enriched for CD8⁺ cells by using CD8⁺ MoAb-coated immune magnetic beads (StemCell Biotec). Immune phenotype characterization analysis of AML-CTLs was performed using fluorescein isothiocyanate (FITC)-conjugated antibodies against human CD3 and phycoerythrin (PE)-conjugated antibodies against human CD8 (Beckton Dickinson, CA) for flow cytometry.

**CTL cytotoxicity assay**

The CTL cytotoxicity assay was performed with a standard 4-hour chromium (51Cr)-release assay (CRA) [18]. In brief, the T cell effectors were harvested 3 weeks after stimulation with Kasumi-6 cells, washed, and plated in 96 well plates at 37°C for 4 hours in triplicate at 2.5 × 10⁴, 5 × 10⁴, 10 × 10⁴, and 20.0 × 10⁴ cells/well with 1 × 10⁴ target cells. Three identical wells were set at the same ratio of effector to target cells. After centrifugation and incubation, 100 μl aliquots of cell-free supernatant were harvested and counted. The percentage of specific cytolysis was calculated.

**CTL adoptive transfer and PD-1 blockade test in vivo**

Balb/c nude female mice at 5–7 weeks of age were purchased from HFK Bioscience Co., Ltd., China. The mice were raised according to the institutional guidelines approved by the Cheng Du Military General Hospital in line with the current regulations and standards of the Ministry of Labour and Welfare. Then, 1 × 10⁶ logarithmic phase Kasumi-6 cells in 100 μl of serum-free medium were injected into the tail veins of the mice. Blood was extracted from the eye angular veins of the animals to quantify CD33 antigen expression with a flow cytometer every week. Three weeks later, 15% CD33 expression in the whole blood was deemed as an established AML model. The mice were then randomly divided into four groups. For the single PD-1 blockade immunotherapy group, a 1 mg/kg concentration of the PD-1 antibody (MERCK) diluted in normal saline (NS) was infused into the animal’s body by intraperitoneal injection every week for a total of 4 weeks. Each mouse in the single-cell immunotherapy group was given 1 × 10⁶ CTLs/kg per administration through an intravenous injection every week for a total of 4 weeks. These two drugs were both administered simultaneously in the combined group. The control group was injected with NS. All groups received 2 × 10³ IU of IL-2 via peritoneal injection every 3 days. At the end of the experiment, blood was extracted through the eye angular vein. Visceral organs and the
shins from the hind legs were also removed and fixed in 4% paraformaldehyde.

**Flow cytometry assays**

White blood cells were separated from whole blood with Ficoll Paque Plus (Sigma) before assays according to the instructions for use. The cells were suspended in 5 ml of phosphate-buffered saline (PBS) (pH = 7.4) and counted by a cell counting instrument (Sigma-Aldrich, China). The cell concentration was modulated to $1 \times 10^5/100 \mu l$. Subsequently, a 100 μl cell suspension was removed and incubated with antibodies for flow cytometry. The cell surface phenotype of the T cells was determined by staining with the following markers: CD4-PE and CD25-FITC. The antibodies were incubated with the cells for 30 min at 4°C. Isotype controls were set as the negative controls. The specimens were rewashed with PBS to remove redundant antibodies. Then, the cells were fixed with 4% paraformaldehyde for 30 min. Intracellular staining with a PerCP-Cy-conjugated FOXP3 mAb (BD Biosciences) was then performed on T cells. The cells were resuspended in 200 μl of PBS for evaluation by flow cytometry (Becton Dickinson). The total cells to be harvested were set at $1 \times 10^4$, and the speed of cell collection was controlled at 200–300 cells/sec. The data analysis was completed by FlowJo software, version 7.6 (FlowJo, Ashland, OR, USA).

**Immunohistochemistry (IHC) test of PDL-1 expression in the viscera**

Before they were embedded in paraffin, shins from the hind legs were decalcified with a hydrochloric acid and formaldehyde decalcification-fixation fluid until the tissues were very soft. In addition, consecutive 3- to 5-μm tissue sections were cut and baked for 1 h at 60°C before dewaxing. These sections were dewaxed by successively treating the samples with dimethylbenzene, an alcohol gradient, and distilled water. Then, the samples were treated in the dark for 10 min with 3% H$_2$O$_2$ to block endogenous peroxides. Antigen retrieval was necessary to accurately detect PDL-1 expression. Briefly, the specimens were submerged in folic acid sodium buffer (10 mM, pH 6.0), loaded into an electric pressure cooker, and boiled for 3 min. When the slides were cooled to room temperature, the sections were rinsed with PBS (pH 7.4) twice. To prevent nonspecific antigen binding, the tissues were treated with rabbit serum (Abcam) for 15 min at 37°C. Then, the sections were rinsed and incubated overnight with an anti-PDL-1 monoclonal antibody (Abcam). The next steps were reaction with a biotinylated secondary antibody and SAB complex for 40 min at 37°C and 3, 3′-diaminobenzidine (DAB) staining for visualization. After dehydration with alcohol and vitrification with dimethylbenzene, the slices were sealed with neutral balsam.

**Statistical analysis**

The data were shown as the means ± SD. Intergroup analysis of variables was performed with the one-way ANOVA method employing SPSS16.0 software. Survival analysis was performed using Kaplan-Meier survival curve analysis. Values of $p < 0.05$ were considered to be statistically significant.

**Results**

**Cytotoxicity of AML-CTLs**

CTL differentiation and proliferation require continuous antigen stimulation and cytokine induction. More than 70% of the cells in the whole PBLs were converted to the CD3$^+$CD8$^+$ phenotype through stimulation and induction (Figure 1(a)). In Cr-51 release assays, the lysis ratio increased with a varying ratio of effectors to targets (E:T). The results showed that the lysis ratios were $4.8 \pm 0.21\%$, $9.8 \pm 0.05\%$, $14.8 \pm 0.21\%$, $19.8 \pm 0.21\%$, and $24.8 \pm 0.21\%$ at E:T ratios of 2.5:1, 5:1, 10:1, 20:1, and 25:1, respectively. This indicated that the AML-CTLs were highly cytotoxic to AML cells.

**Figure 1.** Generation and characterization of AML-specific cytotoxic T lymphocytes. (a) Flow cytometry assay of the phenotype of monocyte-depleted PB lymphocytes stimulated with Kasumi-6 cells pretreated with mitomycin C or irradiation. The Q2 zone represents the ratio of CD3-positive to CD8-positive lymphocytes. (b) The target cell killing ratio after treatment with CTL at varying E:T ratios. E:T, effector vs. target cell. $^*p < 0.05$ vs. 2.5:1 and 10.0:1. $^{**}p < 0.05$ vs. the remaining E:T ratios.
1.22%, 17.4 ± 0.83%, and 25.8 ± 1.43% for E:T ratios of 2.5:1, 5.0:1, 10.0:1, and 20.0:1, respectively, with mitomycin induction. There was no obvious difference between mitomycin and irradiation induction at the same E:T ratio (Figure 1(b)).

**CTLs with a PD-1 inhibitor promote apoptosis in vitro**

We have previously described the cytotoxicity of CTLs in AML [19]. Similarly, CTLs exhibited a 50.3 ± 4.33% apoptosis induction ability for Kasumi-6 cells versus values of 15.24 ± 1.25% for anti-PD1 and 3.82 ± 1.57% for the controls at 8 h. This value was 64.2 ± 5.35% when the PD-1 inhibitor was added (Figure 2(a)). In addition, this cytotoxicity was enhanced with an increasing incubation time. Another 20% apoptosis induction ability was observed at 12 h. Compared with any single drug, CTLs with the PD-1 inhibitor were more sensitive to Kasumi-6 cells (Figure 2(b)).

**Figure 2.** AML cell apoptosis induced by CTLs and/or PD-1. (a) Target cells were treated with CTLs and/or a PD-1 inhibitor for 8 h at 37°C. Annexin-V and propidium iodide (PI) staining for flow cytometry was performed for the apoptosis assay. (b) Apoptosis rate statistics for AML cells following different treatments for 8 and 12 hours. *p < 0.05 vs. CTLs alone; **p < 0.01 vs. the remaining groups.

**Figure 3.** Synergistic in vivo anti-AML effects of a PD-1 inhibitor and CTLs. (a) CD33 and CD13 staining for flow cytometry was used to assess the tumour burden on the 7th and 14th days post-treatment. An enhanced anti-AML effect was observed in the PD-1 with CTL treatment group. (b) The survival curve after the in vivo test. *p < 0.05 vs. the anti-PD1 alone and CTL alone groups.

**CTLs with the PD-1 inhibitor promote anti-AML effects in vivo**

Although PD-1 inhibitors have been proved to be effective in many malignant tumours, its roles are not fully clear in AML. To better understand the effects of these drugs, we performed antitumour experiments in vivo. The CD33+ cells in the peripheral blood (PB) were tested. On the 7th day of treatment, the CD33+CD13+ cells in the PB were reduced after the anti-PD1 treatment, the CTL monotherapy treatment...
and the combined treatment compared to the control. On the 14th day, the tumour burden of CD33+ cells was further decreased. Compared to CTL monotherapy, CTLs combined with the PD-1 inhibitor showed superior anti-AML outcomes, with an effect of 20% (Figure 3(a)). In contrast, the counts showed little variation in anti-PD1 monotherapy. No survival was observed on day 50 after inoculation in the monotherapy group, whereas in the combined therapy group, the animals remained alive 75 days after inoculation. Cumulative survival increased significantly (Figure 3(b)).

Regulatory T cell proliferation and PDL-1 expression are inhibited in vivo

Immunity is thought to be enhanced during immunotherapy. However, it remains unclear how long this enhancing effect would last and if the infused cytotoxic T cells would be inhibited by the AML microenvironment. Therefore, immune cell alterations were next assessed in the PB. After gating on CD4+ cells, the CD25+Foxp3+ proportion was reduced to different extents (Figure 4(a)). The statistical results revealed percentages of 8.6 ± 0.67%, 7.5 ± 0.72%, and 2.3 ± 0.56% for the anti-PD1 treatment, the CTL treatment and the combined treatment, respectively, compared to 11.26 ± 0.74% for the control (Figure 4(b)). The infused CTLs were partially transformed to an inhibitory phenotype. In addition, based on the extensive expression of PDL-1 in organs, its expression was tested in paraffin-embedded tissue. Notable PDL-1 suppression was found in the bone marrow and spleen. Relative suppression was observed in the lungs and liver (Figure 5).

Discussion

Enhancing pro-immunity factors is vital in cancer immunotherapy. Additionally, elimination mechanisms that negatively regulate immune capability are crucial. Combined immunotherapy may overcome the limitations and low efficiency of single strategies. In our present study, we focused on the two-factor effect in AML by applying PD-1 expression blockade and adoptive infusion of CTLs. We showed that T cells exhibited an outstanding proliferation ability after stimulation and cytokine induction by specific AML cell lines. CTLs displayed excellent cell toxicity in vitro, which could be enhanced by PD1 inhibitor application. For CTLs, the ability to lyse cells originates with the intense production of cytokines, such as IFN-γ, TNF-α and perforin, which releases granzymes [20]. In addition, this ability may be potentiated by chemotherapy [21]. However, CTLs are not effective in some cases. For CTL-mediated antitumour immunotherapy to be effective, the agents directed against resistant tumour cells must sensitize the cancer cells to CTL-mediated lysis. Histone deacetylase (HDAC) inhibitors, proteasome inhibitors, Bcl-2 family inhibitors, poly (ADP-ribose) polymerase (PARP), and antibodies are examples of such agents [22]. For melanoma [13], NSCLC [12] and Hodgkin’s lymphoma, PD-1 inhibitors were successfully approved for application in clinical treatment. Animal and clinical tests have been explored for leukaemia. PD-1/PD-L1 interactions could inhibit antitumour immune responses in a murine AML model [8]. For post-allogeneic stem cell transplantation patients with AML, high-PD-1-expressing T cells associate with and predict leukaemia relapse [23]. Collective data implied that PD-1 blockade may be a promising tool for AML. Additional data noted that increased PD-1 expression by T cells is seen only at the time of relapse, and T cell exhaustion does not play a major role in AML [24].

In addition, we observed synergistic anti-AML effects in vivo tests in our study. Overall survival was also markedly improved. However, PD-1 inhibitor monotherapy did not show apparent advantages. First, after adoptive T cell transfusion, the cells recognize specific antigen-expressing leukaemia cells and exert cytotoxicity. PD-1 blockade can eliminate the suppression of effector T cells that targeted leukaemia cells previously.
This effect may compensate for the cytotoxicity insufficiency of transfused T cells. Furthermore, based on the context of stem cell transplantation, the occurrence of CTLs specific for AML in patients supports the possibility of using expanded autologous antigen-specific CTLs to combat AML [25]. To generate sufficient numbers of T cells, sequencing the high-avidity T cell receptor (TCR) from T cell clones that recognize leukaemia antigen targets and inserting the TCR gene into T cells from the AML patient may be a promising strategy [26]. In addition, the purification of CTLs must also be further enhanced effectively. Second, abundant evidence showed that established AMLs can mutate to escape immune control. Weak expression of co-stimulatory molecules favours AML escape from T cell-mediated killing, and both CD80 and CD86 expression can promote sustained remission in AML patients [27]. Additionally, co-stimulatory molecules, such as 4-1BB shed by AML cells, may impair T cell abilities through the binding of soluble ligand to the T cell [28]. Therefore, blocking PD-1 alone may not efficiently eliminate the AML-related immune tolerance state to effectively achieve an anti-leukaemia effect. It is unlikely that a single immunotherapy strategy could support the successful treatment of AML. Effective AML treatments must coordinate an immunotherapy strategy with other treatments. Immunotherapy approaches combined with stem cell transplantation (SCT) can improve the curative potential of transplantation, which provides a great opportunity for leukaemia reduction with a myeloablative preparative regimen [29]. In addition, immune checkpoint inhibitors combined with chemotherapy and other immunotherapy strategies, including vaccines, cytokines, monoclonal antibodies, and engineered modified T cells, all provide future directions to study optimized treatments for AML.

Collectively, our study generated one effective AML-specific CTL, which can target AML cells lines and exert cytotoxicity in vitro and in vivo. PD-1 inhibitor introduction can produce synergistic effects in vitro and extend the AML immune tolerance state by suppressing regulatory T cell proliferation. To further improve these results, alternative or additional combined approaches are required to enhance all the components of the immune response to AML. Immune checkpoint inhibitors may be one point in the pathway to AML cell destruction that could be strengthened to improve therapeutic effects.

Acknowledgements
The present study was supported by the Cheng Du Military General Hospital Management Research Foundation (grant no. 2013YG-B045).

Disclosure statement
No potential conflict of interest was reported by the authors.
Funding

The present study was supported by the Cheng Du Military General Hospital Management Research Foundation [grant number 2013YG-B045].

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