Bi-specific Antibody Y111, Targeting PD-L1 and CD3 Considerably Enhances the Therapeutic Efficacy of Adoptive Transferred Vy2Vδ2 T Cells

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Research

Keywords: [CD3xPD-L1], Vγ2Vδ2 T cells, adoptive transfer, immunotherapy

Posted Date: December 15th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-125326/v1>

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Abstract

Background:

Anti-cancer immunotherapy based on the adoptive transfer of V γ 2V δ 2 T cells has benefited to some patients in clinical trials, but the overall responses are inconsistent. Therefore, new strategies are urgently needed to improve the current therapy.

Methods:

In this study, a designed bispecific antibody Y111, which binds to both CD3 and PD-L1, is applied to optimally potentiate V γ 2V δ 2 T cell-based killing of cancer cells. The binding activities of Y111 was determined by Flow cytometry. CFSE/PI-based flow cytometry was applied to check the re-directed killing ability induced by Y111 in the condition of using T cell subsets, or expanded- and purified- V γ 2V δ 2 T cells as effector cells. Moreover, expanded- and purified- V γ 2V δ 2 T cells were co-cultured with tumor cells in the presence/absence of Y111 to assess the activation, degranulation, and cytokine production by intracellular cytokine staining, and CBA method. Finally, NPG-based subcutaneous tumor mouse models were used to check the *in vivo* therapeutic efficacy of the combination of transfused V γ 2V δ 2 T cells and Y111.

Results:

Due to its binding activities, Y111 apparently prompts fresh $\alpha\beta$ -mediated lysis of tumor cell line H358 cells, but spare the effect on the fresh enriched V γ 2V δ 2 T cells from the same donors. However, Y111 increases cytotoxicity of expanded and purified V γ 2V δ 2 T cells against various NSCLC-derived tumor cell lines in a tumor cell dependent fashion. Y111 also prompted the releases of granzyme B, IFN γ and TNF α . Supporting to these observations *in vitro*, a combination of adoptive transferring V γ 2V δ 2 T cell and Y111 into the tumor-bearing NPG mice inhibited the growth of the established tumors in the mice.

Conclusions:

Taken together, our data suggest clinical potential for adoptive transferring the bispecific antibody-armed V γ 2V δ 2 T cells to treat solid tumors, such as NSCLC.

Background

The V γ 2V δ 2 T cells, accounted for about 90% of total $\gamma\delta$ T in healthy adults' peripheral blood, appear to be a fast-acting non-conventional T-cell population that contribute to both innate and adaptive immune responses against microbial infections and cancers [1]. Due to their unique biological features, V γ 2V δ 2 T cells have been widely used for adoptive cell immunotherapy in clinical trials to treat a broad range of cancers that display resistance to standard therapies [2]. However, the current V γ 2V δ 2 T cells-based therapies have showed limited efficacy against solid tumors due to poor infiltration and impaired anti-tumor functions of transfused V γ 2V δ 2 T cells induced by tumor environment [3].

Various preclinical strategies are currently developed to enhance the therapeutic potential of novel optimized V γ 2V δ 2 T cell-based immunotherapies targeting various pathways. Over three decades ago, Ferrini et al initially verified the concept that bispecific antibodies (bsAbs) targeting the $\gamma\delta$ TCR and a folate binding protein enhanced the cytotoxicity of $\gamma\delta$ T-cells to human ovarian carcinoma cells [4]. Several studies to exploit the synergic effects of the combination of a bispecific antibody with V γ 2V δ 2 T cells on fighting tumors were reported in recent years. The combination of designed bispecific antibodies targeting to Her2 and CD3 or V γ 2 [5, 6], together with the transferred V γ 2V δ 2 T cells in presence of IL2, has achieved a significant delay in growth of pancreatic ductal adenocarcinoma tumor in murine model [7]. Another bispecific VHH construct, namely 7D12-5GS-6H4, targeting to epidermal growth factor receptor (EGFR) and V δ 2 has also been developed to activate V γ 2V δ 2 T cells [8], and the BsAb significantly prompted the survival time of xenograft bearing mice in the presence of transfused V γ 2V δ 2 T cells pulsing the repeatedly injections of IL2 [9]. Moreover, a recent study demonstrated that the combination of checkpoint blockade and immune cell redirection could further enhance the anti-tumor effects of the adoptively transfused- and IL2 stimulated-V γ 2V δ 2 T cells [10]. However, these mentioned bispecific antibodies are either originally from mice, which raised the risks including the occurrence of the immunogenicity and cytokine release syndrome, or in a form of VHH structure, which has a short half-life time in the blood [11]. Also, the TCR-based bispecific antibodies showed modest activities of tumor growth inhibitions with the co-administration of IL2, yet, this approach attracted less efforts than the exploring on CD3-targeting bsAbs [12].

Lung cancer is still the leading cause of cancer patients' deaths worldwide (<https://canceratlas.cancer.org/the-burden/lung-cancer/>). The clinical response rates of non-small cell lung cancer (NSCLC) patients, which accounts for approximately 85% of lung cancers, to the current first or second line treatment are still unsatisfying [13, 14]. Although the adoptive transfer of V γ 2V δ 2 T cells could reduce the growth of lung cancer cell and prolong the survival of NSCLC-tumor bearing mice [15, 16], this immunotherapy has been proved to be safe but only effective in stabilizing the cancer progress in clinical trials during the past decades [17–19]. Also, the landscape-changing “Magacurve” for advanced NSCLC has shown the therapeutic successes of PD1/PD-L1 blockade [20], the monotherapy of receiving anti-PD1/PD-L1 agents results in response only in a small proportion of NSCLC patients [21]. Hence, to underline and illustrate a new strategy regarding the combination of the V γ 2V δ 2 T cells-based transfer therapy and PD-L1 targeting therapy, is to be explored for the treatment of NSCLC.

In this study, we designed a novel bispecific antibody Y111, targeting CD3 and PD-L1, on the format of patented Y-body®. Our data showed that the binding affinity of Y111 to PD-L1 was similar to that of Tecentriq, while Y111 displays reduced affinity to CD3 expressed on T cells, comparing to parental monoclonal antibody. As Y111 efficiently bridged T cells and tumor cells, it could selectively trigger the enhanced expressions of IFN γ and TNF α , and up-regulate CD107a on V γ 2V δ 2 T cells co-cultured with tumor cells. Furthermore, Y111 redirected the killing ability of V γ 2V δ 2 T cells against various NSCLC cell lines at a 1:1 E: T ratio with the cytotoxicity ability (EC₅₀) at pM scale, through secretion of IFN γ , TNF α , and Granzyme B. Finally, the combination of Y111 and transfused V γ 2V δ 2 T cells displayed therapeutic

effects by delaying the growth of the established xenograft in immunodeficient NPG mice. Taken together, our data revealed a new strategy for developing more efficient Vy2Vδ2 T cells-based immunotherapy, and this approach for NSCLC could be applied to other types of cancers with the advantages of the easy replacement of versatile tumor associated targets in the Y-body® platform and the potential anti-tumor nature of Vy2Vδ2 T cells.

Methods

Generation, production and purification of bispecific Antibody

The Y111 is a recombinant anti-PD-L1 and anti-CD3 (PD-L1 x CD3) bispecific antibody generated from CHO cell expression system. The anti-PD-L1 monovalent unit was from Tecentriq. The protein sequence of Tecentriq was obtained from the drug bank website (<https://go.drugbank.com/drugs/DB11595>), and then was reversely translated into the DNA sequence for synthesizing. The sequence encoding the anti-CD3 single chain unit was reversely translated from the protein sequences of 2A5 (W02020168555), a CD3 monoclonal antibody. These coding sequences were inserted into pEASY-T1 vector (Transgene, China) and verified by sequencing these vectors (Huada Gene, China). Then these expression cassettes were introduced into expression vectors respectively. The alternative control molecule, CD3 Isotype, targeting to CD3 and fluorescein (derived from Clone 4-4-20 [22]) was similarly prepared. Subsequently, these expression plasmids were transfected into CHO cells (Invitrogen) using the Fecto PRO Reagent (Ployplus, 116 – 010) according to the manufacturer's protocol. After 7-day culturing, the cell culture supernatant was collected and the serially purified by Sepharose Fast Flow protein A affinity chromatography column (GE), Fab Affinity KBP Agarose High Flow Resin (ACROBio systems), and SP cation exchanged chromatography column (GE). The purified proteins were analyzed by SDS-PAGE and coomassie blue staining.

Cancer cell lines

The four human NSCLC cell lines, NCI-H1975 (human adenocarcinoma epithelial cell line, CRL-5908) and NCI-H358 (human lung bronchioalveolar carcinoma cell line, CRL-5807), A549 (human adenocarcinoma epithelial cell line, CRL-185), and NCI-H1299 (human NSCLC metastatic cell line, CRL-5803) were purchased from ATCC. Cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS (Excell) except for A549, which was cultured in F-12K medium (Gibco) supplemented with 10% FBS (Excell). Prior to use, the viability and density of cells were determined by Vi-Cell counter (Beckman Coulter). All cell lines in use were routinely tested for Mycoplasma infection using a 16 s-based PCR kit (Vazyme, Nanjing, China), and new cultures were established monthly from frozen stocks as described previously[23].

Ex vivo expansion and purification of Vy2Vδ2 T cells in PBMC cultures

The protocols for human blood samples for *in vitro* experimental procedures were evaluated and approved by the institutional review boards for human subjects' research and institutional biosafety committees at Huazhong University of Science and Technology (Wuhan, China). All subjects are adults who signed on the written informed consents.

This expansion protocol was performed as previously described [24]. Human peripheral blood mononuclear cells (PBMCs) were isolated from the fresh blood collected from random healthy adult donors (LDEBIO, Guangzhou) and purified by density gradient centrifugation using Ficoll-Hypaque PLUS (GE). These PBMCs were frozen in liquid nitrogen prior to use, as clinic trials evaluating the anti-cancer efficiency of the V γ 2V δ 2 T cells usually utilized frozen PBMCs as starting cell population for further culture. After thawing quickly and counting cell numbers using AO/PI staining with Cellometer K2 Fluorescent Cell Viability Counter (Nexcelom Bioscience), PBMCs were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS (Excell), 2.5 μ M Zoledronic Acid (Sigma Aldrich), and 1000 IU/mL IL2 (Sihuan Pharm, Beijing) at 2×10^6 cells/mL seeded in 6-well-plate. Every 3 days, half volume of the culture media was removed and replaced with fresh culture media containing 1000 IU/mL IL2. Depending on the media color and cell density, cultured cells were pooled and transferred into T75 flasks. During days 12–14, V γ 2V δ 2 T cells were purified by negative selection from these cultures using the Miltenyi TCR γ/δ + T Cell Isolation Kit (130-092-892). Cell purity was > 96% assessed by flow cytometry, and these cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS (Excell) overnight for rest. Then, these cells were used for either killing and functional assays *in vitro* or assessing anti-tumor activity *in vivo* (Sup. Figure 1)

Cell binding and co-binding assay

To determine the affinity of Y111 at its anti-CD3 and anti-PD-L1 moieties to T cells and tumor cells, a flow cytometry-based method was applied to measure the cell binding activities of Y111 as described in a previous reprot [25]. T cells or tumor cells were incubated in the presence of serially diluted antibodies for 1 hour at room temperature. Subsequently, the cells were washed twice in FACS buffer (PBS + 2%FBS + 2 mM EDTA) and stained with PE-conjugated anti-human IgG Fc antibody (Clone HP6017, Biolegend) diluted in 1:100 into FACS buffer for 25 minutes. Bound antibodies were detected using flow cytometry.

To determine the cell-to-cell association mediated by Y111, CFSE-stained H1975 cells were co-cultured with PKH26-labelled Jurkat cells at a ratio of 1:1 with range concentrations of the Y111 or CD3 Isotype for 1 hour in a 96-well-plate. Samples were measured by flow cytometry on a FACSelectra instrument (BD) and analyzed with FlowJo software (BD). Co-binding% of two cells was indicated as the percentages of the CFSE⁺PKH26⁺ double positive cells among the total cells.

In vitro tumor cell killing assay

All tumor cell lines were cultured and maintained in T75 flasks prior to use. These cell lines were selected as target cells for the cytotoxicity assay due to their high expression levels of PD-L1 (Sup. Figure 4). On the first day of the cytotoxicity assay, 2×10^4 CFSE-labelled target cells were seeded and co-cultured with

the enriched- and expanded- Vy2Vδ2 T cells at a 1:1 ratio, or with the purified T cell subsets from fresh PBMC at 1:10 ratios (using the respective negative isolation kits (Miltenyi Biotech) according to the manufacturer's instructions, and Sup. Figure 6), with a various doses of indicated antibodies. The cells were incubated at 37 °C for 12 h in a humidified CO₂ incubator. Flow cytometry was used to determine antibody-induced cytotoxic activity-mediated by Vy2Vδ2 T cells as described previously [25]. The percentages of CFSE⁺PI⁺ cells among the total of target cells (CFSE⁺) were defined as "Cytotoxicity %".

Intracellular cytokine staining (ICS) for T cell activation evaluation

Flow cytometry was performed as described in the other reports[26, 27]. 0.1 Million H1975 cells was firstly plated in 24-well-plate. On the next day, 0.25 million expanded and negatively enriched Vy2Vδ2 T cells were added into the wells with doses of Y111 or CD3 Isotype with BV510-anti-CD107a (H4A3, Biolegend) and BFA (Golgi Plug, BD). After co-cultured for 6 hours, the cells were stained with Zombie Fixable Viability Kit (Biolegend), incubated with APC-anti-CD3 (SP34-2, BD), PE-anti-Vδ2 (B6, Biolegend) for 20 min at room temperature in dark. Cells were permeabilized for 30 min at 4 degrees (Cytofix/Cytoperm, BD). After wash, cells were incubated with BV650-anti-IFNγ (4S.B3; Biolegend), BV421-anti-TNFα (Mab11, Biolegend) for 30 min at room temperature in dark. Then cells were washed and analyzed by a BD FACSelesta flow cytometry. Flow data was analyzed by FlowJo (BD).

CBA for measuring released cytokines

The supernatant was collected from T cell and tumor cell co-culture system before adding propidium iodide (PI) for measuring cytotoxicity, and stored at -80 degree until measurement. The human IFNγ (560111, BD), TNFα (560112, BD) and Granzyme B (560304, BD) Flex Set kit were used for determining the concentrations of these cytokines by the cytometric bead array (CBA) method according to manufacturer's instructions. In order to display the increased production of cytokines induced by the antibodies, raw values were subtracted from the values of E + T groups.

Mice tumor generation and treatment with purified Vy2Vδ2 T cells pulse Y111

All handling and care procedures of animal were performed in accordance to guidelines issued from the relevant Ethics Committee. The animal study protocols were approved by the Institutional Animal Care and Use Committee at Huazhong University of Science and Technology (Wuhan, China).

Female NOD.Cg-Prkdc^{scid} IL2rg^{tm1Vst/Vst} (NPG) mice were obtained from the VITALSTAR (Beijing, China) at age of 6–8 weeks and housed in the central laboratory in Hubei Province Food and Drug Safety Evaluation Center. 5 million H1975 cells were injected *s.c.* into NPG mice for tumor xenotransplantation on Day 0. Until to Day 15 post tumor cell inoculation, tumor volumes had reached to about 220 mm³, mice were randomly divided into four groups (n = 7 per group) (Sup. Figure 6A). On Day 17, mice were injected *i.v.* with 1 million purified Vy2Vδ2 T cells with/without 1 mg/kg or 4 mg/kg Y111. This treatment

was followed on Days 20, 24 and 27 (twice a week for 2 weeks). Mice treated with PBS were used as negative control. For each treatment, purified V γ 2V δ 2 T cells were negatively enriched from frozen PBMC from the same donor treated with 2.5 μ M Zol + 1000 IU/mL IL2 for 10–14 days. Tumor volumes were measured with a digital caliper three times a week and calculated using the formula: Tumor Volume (mm^3) = $(a \times b^2) / 2$, where a is the longitudinal length and b is the transverse width.

IHC analysis

To assess the infiltration and accumulation of transferred V γ 2V δ 2 T cells *in vivo*, mice were sacrificed at the end of the study. The tumor tissues were immediately removed, and cut into small pieces embedded in 4% paraformaldehyde for fixation. Then these tumor pieces were sectioned and examined by IHC staining using a rabbit-anti-human CD3 antibody (Clone SP7). Positive cells were counted in five random selected microscopic fields (magnification 20X) and supplied for further quantification analysis.

Statistical analysis

Statistical analyses were performed with Prism 6.0 (GraphPad software). Non-linear regression methods were used to analyze data derived from cell binding, co-binding, activation, and killing assays to plot Dose-Response curves. *P* values were assessed by student's t-test, nonparametric Mann–Whitney U test, and one-way or two-way ANOVA, followed by Dunnett test or Tukey multiple comparison as appropriate. *P* values < 0.05 was considered significant.

Results

Design and Generation of Y111 based on patented Y-body® platform

Y111 (PD-L1 x CD3), aCD3-targeting bispecific antibody, that redirected T cells to attack PD-L1-expressing cancer cells, was designed with the Y-body® format in structure[25, 28]. Y111 consisted of a Fab structure targeting PD-L1 derived from Tecentriq (Atezolizumab), a single chain variable fragment (scFv) for activating CD3 originated from a monoclonal antibody 2A5 (WO2020168555), and a modified Fc region (Fig. 1A). The Fc region of Y111 is emanated from human IgG1, but engineered with “Knob-into-Hole” mutations for favorable formation of the heterodimer, and with deficiency in ADCC activity as previously described [25]. The purity and molecular weight of Y111 was verified by size-exclusion chromatograms SEC and SDS-PAGE. As expected, under reducing conditions the bands in the gel demonstrated the three chains of Y111, i.e. heavy chain (Y111H), light chain (Y111L), and single chain (scFv), while a monoclonal antibody displayed two bands consisting of the heavy (NH) and light (NL) chains derived from Nivolumab (Fig. 1B). Moreover, the SEC analysis indicated that the purity of this Y111 sample was more than 99% (Fig. 1C).

Binding properties of Y111

The cell binding activity of Y111 was measured using flow cytometry. We initially assessed the affinity of Y111 at the anti-CD3 moiety on Jurkat cells. It was not surprising to find that the affinity of Y111 was 360-folds weaker than that of 2A5 (the parental CD3-targeting mAb of Y111) to Jurkat cells, as the dissociation constants (K_d) for Y111 and 2A5 to Jurkat were 711.4 nM and 1.96 nM, respectively (Fig. 2A), which were consistent with previous reports [11, 25]. Then, H1975 cells were chosen for measuring the binding affinity of the anti-PD-L1 moiety of Y111 to PD-L1. The K_d for Y111 binding to H1975 was 0.84 nM, and the K_d of Tecentriq binding to the cell was 0.21 nM (Fig. 2B). The cell affinity of Y111 was equivalent to that of Tecentriq, as both had the same variable regions of anti-PD-L1.

Bispecific antibody mediating T cells recruitment to cancer cells is thought to be a critical working mechanism [11]. We therefore investigate whether Y111 could induce the formation of an in-tans bridge between T cells and tumors cells through its dual binding functions. To this end, Jurkat cells stained with CFSE were incubated with H1975 cells labelled with PKH26 for 1 hour, then the proportion value of double positive cells was measured to represent the bridging activity of Y111[25]. In the presence of CD3 Isotype at 10 $\mu\text{g/mL}$, the double positive cell population was 1.79% (Fig. 2C). Y111 at the concentration of 10 $\mu\text{g/mL}$ significantly induced the formation of bridge between T cells and tumor cells as indicated by the increased up to 34.6% of double positive population, (Fig. 2C). Interestingly, while Y111 displayed a dose-dependent manner in enhancing the levels of cell-to-cell association ($\text{EC}_{50} = 72.1 \text{ pM}$), CD3 Isotype control was unable to increase the levels of cell-to-cell association (Fig. 2D). Taken together, these data demonstrated the unique binding activities of Y111 by the anti-PD-L1 moiety to the tumor cells and by the anti-CD3 moiety to the T cells.

Y111 induced activation of Vy2V δ 2 T cells was selectively depended on the presence of tumor cells

Vy2V δ 2 T cells exerted direct killing the tumor cells through the releasing of cytokines, such as IFN γ and TNF α , and cytotoxicity [2]. We then sought to investigate the effect of Y111 on the activation of Vy2V δ 2 T cells to determine whether the Y111-mediated activation was depended on the presence of a tumor cell. To this end, the purified Vy2V δ 2 T cells (the purity and quality of Vy2V δ 2 T cells was shown in Sup. Figure 1) were cultured with/without H1975 cells under a serial diluted concentration of the Y111 antibody for 6 hours. Then, we measured the cell surface expression of CD107a to assess the degranulation of cytotoxic molecules for killing activity of the Y111[24, 29], and the intracellular expression of IFN γ and TNF α for evaluating its anti-tumor effector function [30]. With the stimulation of both Y111 and tumor cells, a higher proportion of Vy2V δ 2 T cells displayed potent effector function and degranulation at 1 $\mu\text{g/mL}$, which was not the case when the addition of CD3 Isotype instead of Y111 was used (Fig. 3A, Sup. Figure 2). Furthermore, the considerably unregulated expression of TNF α , IFN γ , and CD107a was aborted in the absence of tumor cells even under the stimulation by Y111 (Fig. 3A). These data indicated that the activation of Vy2V δ 2 T cells was controlled jointly by both Y111 and tumor cells. Moreover, this specific activation was in a Y111 dose-dependent manner (Fig. 3B). Multifunctional Vy2V δ 2 T cells have been reported playing central roles in controlling the intracellular bacterial infection and killing transformed

tumor cells [1, 31]. Indeed, we found the co-stimulation of Y111 and H1975 cells induced larger percentages of effector cells to produce three- and two- cytokines simultaneously (Fig. 3C). At last, we also observed a dose-dependent increase of these multifunctional V γ 2V δ 2 T cells after co-incubation of both the Y111 and tumor cells (Sup. Figure 3). Taken together, these data demonstrated that the efficient activation of V γ 2V δ 2 T cells was dependent on the simultaneous binding of the Y111 to both V γ 2V δ 2 T cells and PD-L1 positive tumor cells.

The killing various PD-L1-positive NSCLC cell lines by V γ 2V δ 2 T cells was significantly increased by Y111

As the crosslinking of PD-L1 on the target cells with CD3 on the T cells mediated by the bispecific antibody was expected to cause effector T-cell-dependent lysis of target cells [11], we then checked whether Y111 could redirect V γ 2V δ 2 T cells to kill PD-L1 positive tumor cells. Although the expression levels of PD-L1 was used as a favorable predictive biomarker, approximately only 20–30% NSCLC patients responded to the current PD1/PD-L1 blocking therapy [32]. Thus, we initially chose four NSCLC cell lines including A549, H1299, H1975 and H358 in this proof-of-concept study. These four types of cancer cells exhibiting in PD-L1 high expression demonstrated by flow cytometry, were used for the investigation of cytotoxicity of V γ 2V δ 2 T cells mediated by Y111 *in vitro* (Sup. Figure 4). When CFSE-stained tumor cells were co-cultured with purified V γ 2V δ 2 T cells at a ratio of 1:1 with a range of serial diluted antibodies for 12 hours, tumor cells were killed efficiently in a dose-dependent manner of Y111, but this was not the case for CD3 Isotype or Tecentriq at any tested concentrations (Fig. 4). As Y111 alone did not alter the viability of tumor cells (Sup. Figure 5), it was believed that the observed high cytotoxicity was directly elicited by Y111. Although the anti-PD-L1 activity of Y111 may block the PD1/PD-L1 interaction and act as a checkpoint blockade, we could not find the increase of cytotoxicity in Tecentriq group (Fig. 4).

We further tested whether Y111 redirected fresh V γ 2V δ 2 T cells to kill PD-L1 positive tumor cells. Three T cell subsets, including CD4 $^{+}$, CD8 $^{+}$, V δ 2 T cells, were negatively isolated from the same donor, and co-cultured with H358 cells in a ratio of 10:1 (E: T) in the presence of diluted Y111. Indeed, we could not observe an elevated trend of cytotoxicity for V δ 2 T cells, but Y111 could redirect $\alpha\beta$ T cells (CD4 $^{+}$, CD8 $^{+}$ T cells) to lyse H358 cells in a dose-dependent fashion (Sup. Figure 6A-B). In contrast, when H358 cells were co-cultured with established V γ 2V δ 2 T cells purified from the same donor's PBMC treated with Zol + IL2 for 14 days in the presence of various antibodies (Y111, CD3 Isotype, and Tecentriq), serial diluted Y111 enhanced the cytotoxicity of V γ 2V δ 2 T cells, whereas CD3 Isotype and Tecentriq did not (Sup. Figure 6C). This spurious finding was consistent with a previous study using a bispecific antibody targeting Her2 and CD3 [33]. The observed induced cytotoxicity ability of Y111 was PD-L1 dependent, as Y111 plus V γ 2V δ 2 T cells had no effect on normal cells, such as PBMC from unrelated donors (Sup. Figure 7). Taken together, these results demonstrated that Y111 could redirect V γ 2V δ 2 T cells to kill PD-L1 positive tumor cells, but spared this ability to fresh V δ 2 T cells.

The secretion of IFN γ , TNF α , and granzyme B from V γ 2V δ 2 T cells was enhanced by Y111 during killing

The killing ability of V γ 2V δ 2 T cells induced by Y111 prompted us to check the induced production of anti-tumor cytokines, including IFN γ and TNF α , and cytotoxic mediator granzyme B in the above co-culture system. We found Y111, but not CD3 Isotype or Tecentriq, could significantly prompted the secretion of IFN γ , TNF α , and granzyme B from V γ 2V δ 2 T cells in the presence of tumor cells (Fig. 5A). Moreover, the evaluated production of IFN γ and TNF α , and cytotoxicity mediator was consistent with the enhanced killing ability of V γ 2V δ 2 T cells mediated by Y111, as inferred from the significantly correlation coefficients between the induced secretion of IFN γ , TNF α , and granzyme B and the cytotoxicity rates (Fig. 5B). However, no obvious increase of IFN γ and TNF α in the co-culture of Y111 to redirect V γ 2V δ 2 T cells to kill the unrelated PBMCs was observed (Sup. Figure 7).

Adoptive transfer of purified- and expanded- V γ 2V δ 2 T cells with bispecific antibody Y111 displayed potent anti-tumor efficacy in NPG mice

To assess the therapeutic potential of transfusing V γ 2V δ 2 T cells with bispecific antibody Y111, we utilized H1975-NPG model to check whether this combination treatment could fight against advanced tumors *in vivo* (Fig. 6A). Tumors in experiments were allowed to grow for the formation of large established xenograft (about 230 mm³). As the cell expansion methodologies are not optimal at present for preparation of large-scale and clinic-grade V γ 2V δ 2 T cells used for clinic tumor therapy [34], and high numbers and repetitive administration of human V γ 2V δ 2 T cells with phosphoantigens and IL2 are required to get optimal results in murine model [35]. We expected that using a relatively small amount of V γ 2V δ 2 T cells for transfer without IL2 or aminobisphosphonates would generate a treatment window to assess the synergic effects of combination of V γ 2V δ 2 T cells and Y111. In fact, adoptive transfer of purified- and expanded- V γ 2V δ 2 T cells alone had no effects in preventing the growth of established H1975-derived xenograft, compared to the control group (Fig. 6B-C). In contrast, the supplementation of Y111 with V γ 2V δ 2 T cells purified from the same donor significantly delayed the malignant progression, comparing to the control or the T cells alone groups (Fig. 6B-C). This significant inhibition effects of tumor growth started at Day 27 after tumor cell inoculation in the mice received both V γ 2V δ 2 T cells and 4 mg/kg Y111 (Fig. 6B, Sup. Figure 8A). Moreover, this 4 mg/kg Y111 elicited superior therapeutic effects than 1 mg/kg Y111, with a greater extend of delaying tumor growth of this group than other groups (Fig. 6B-C). Ultimately, the observed inhibitory effects were associated with significant increases in the infiltration and accumulation of transfused V γ 2V δ 2 T cells induced by Y111 (Fig. 6D-E). During the study, the Y111 treatment resulted in no or little weight loss in mice, suggesting potential safety to clinical applications (Sup. Figure 8B). Taken together, these data demonstrated that Y111 could further enhance the anti-tumor efficacy of transfused V γ 2V δ 2 T cells.

Discussion

Since the discovery of V γ 2V δ 2 T cells in the late 1980s, a significant amount of knowledge has been accumulated concerning its vital roles in killing tumor cells and controlling tumor growth, raising the possibility of its therapeutic application for anti-cancers [31, 36, 37]. However, the results of various completed clinical trials using transferred V γ 2V δ 2 T cells against both hematological malignancies and solid tumors were proved to be ineffective with only a few complete response [31, 38]. This consistent-lacked results are related to the failure of transfused V γ 2V δ 2 T cells into tumor sites and suppressive tumor environment [3, 39]. Thus, we redirected the transfused V γ 2V δ 2 T cell to tumor sites to kill PD-L1 positive tumor cells by its combination with a CD3 and PD-L1 targeting bsAb, which prompted the its infiltration and persistence in tumor sites. This proof-of-concept study also verified the value of bsAb-based immunotherapeutic approach to leverage the potent anti-tumor capacity of V γ 2V δ 2 T cells, and suggested that this combination strategy could be used to other types of cancer.

With the obligatory ability in binding two specificities simultaneously, bispecific antibodies are progressing into clinical developments for a wide variety of tumors [11, 28]. In this study we generated an anti-PD-L1 X anti-CD3 bsAb Y111, based on Y-body® technological platform, which was characterized as an asymmetric format for easily purification, with the modified Fc fragment to abolish Fc-mediated effector functions [25, 28]. The observed MW of Y111 was larger than theoretical MW, as a result of the N-linked glycosylation, which prompted its stability [25]. Moreover, Y111 retained relatively weaker binding affinity to CD3 molecule, comparing to its parent monoclonal antibody 2A5, but displayed a similar affinity to PD-L1 as that of Tecentriq. The reduced affinity for CD3 of Y111 (711.4 nM) was desired for clinical applications since several studies had shown that a lower affinity of the anti-CD3 moiety of bsAbs contributes to the efficient tumor infiltration of T cells without rapid CD3-modulated plasma clearance [40–42], in addition of mitigating cytokine release syndrome (CRS) [25, 28]. Indeed, our data indicated that Y111 could prompt T cell infiltration into tumor sites *in vivo*, while it still induces highly potential cytotoxicity against tumor cells *in vitro*.

This different susceptibility of fresh $\alpha\beta$, fresh and expanded $\gamma\delta$ T cells-modulated killing of tumor cells induced by Y111 may be attributed to the various action mechanisms of TCR activation by these cells reported by several reports [24, 43, 44]. As bsAbs were expected to recruit and re-direct T cells into tumor sites directly from blood, the above finding indicated that this immunotherapy would miss out the power of V γ 2V δ 2 T cells. We therefore believed that the potential anti-tumor expanded V γ 2V δ 2 T cells would benefit to improve current therapy taking advantages of bsAbs. Indeed, our data demonstrated Y111 triggered strong expression of CD107a on the surfaces of V γ 2V δ 2 T cells in the presence of tumor cells, and selectively provoked the production of IFN γ and TNF α . However, the Y111-induced cytotoxicity of V γ 2V δ 2 T cells was abolished when normal cells, such as PBMC, were used for target, suggesting that the safety profiles remained even under the condition of combining V γ 2V δ 2 T cells with CD3-targeting bsAbs [10, 33]. Moreover, the observed killing of PD-L1-expressed NSCLC cell lines was not affected by gene mutation status in these cell lines, such as the mutation of KRAS (A549 and H358), of EGFR

(H1975), and the loss of P53 (H358 and H1299). This gene mutation-ignored killing mechanism of our approach further proved the potential anti-tumor nature exhibited by V γ 2V δ 2 T cells [31, 36, 45].

While expanding a large scale of autologous V γ 2V δ 2 T cells from cancer patients *ex vivo* still represents a major clinical challenge [37], we explored the antitumor activity of allogeneic V γ 2V δ 2 T cells in NPG mice bearing tumor cell line derived xenograft. Our approach seems particularly promising given the potential of controlling the growth of established tumors, while a single arm therapy of transfused V γ 2V δ 2 T cells alone was not effective. This better tumor control was consistent with the increased cytotoxicity of this treatment *in vitro*. Due to the limitation of immunodeficiency NPG mice used in this study, we could not test whether our strategy exert direct remittance to the suppressive tumor microenvironment. However, we found Y111 could increase the trafficking of transferred V γ 2V δ 2 T cells to the tumor site, which provided the proof of the concept for the efficiency of this combination therapy. Above all, comparing to other studies using various bispecific antibody with V γ 2V δ 2 T cells, our strategy provided effective and safety results even omitting the administration of IL2 with aminobisphosphonates or pyrophosphates for sensitizing tumor cells, or with other monoclonal antibody [10, 31].

Conclusions

In conclusion, our study demonstrates that bispecific antibody Y111, targeting CD3 and PD-L1, could harness the anti-tumor potential of V γ 2V δ 2 T cells to kill cancer cell lines *in vitro* and inhibit the growth of the established xenograft tumors *in vivo*. The current study provides new evidence to support the hypothesis that a CD3-targeting bispecific antibody has the potential to improve V γ 2V δ 2 T cells-based anti-tumor efficacy, and this novel immunotherapeutic approach need further clinical exploration for potential benefit to cancer patients.

Abbreviations

ADCC: antibody-dependent cell-mediated cytotoxicity;

ATCC: American Type Culture Collection;

BsAb: bispecific antibody;

CBA, cytometric bead array;

CFSE: Carboxyfluorescein succinimidyl ester;

EGFR: epidermal growth factor receptor;

Fab: antigen-binding fragment;

ICS: Intracellular cytokine staining;

IHC: Immunohistochemistry;

IFN γ : Interferon Gamma;

NPG: NOD.Cg-Prkdc^{scid} IL2rg^{tm1Vst}/Vst

NSCLC: non-small cell lung cancer

PBMCs: Peripheral blood mononuclear cells

PD1: Programmed cell death protein 1;

PD-L1: Programmed death-ligand 1;

PI: propidium iodide

TNF α : Tumor necrosis factor alpha

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

scFv: single chain variable fragment.

Declarations

Ethics approval and consent to participate

The protocols for human blood samples for *in vitro* experimental procedures were evaluated and approved by the institutional review boards for human subjects' research and institutional biosafety committees at Huazhong University of Science and Technology (Wuhan, China). All subjects are adults and signed written informed consents.

All handling and care procedures of animal were performed in accordance to guidelines issued from the relevant Ethics Committee. The animal study protocols were approved by the Institutional Animal Care and Use Committee at Huazhong University of Science and Technology (Wuhan, China).

Consent for publication

All authors read and approved the manuscript for publication.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

L.G declares no financial conflicts of interest. Others in the authorship are employees of Wuhan YZY Biopharma Co., Ltd.

Funding

This work was supported by National Natural Science Foundation of China (Grant No. 81901607).

Author Contribution

R.Y, G.L, J.Z.Y, and P.F.Z designed the project. Z.J, Y.X.Y, L.J. F and L.Z supervised the project. R.Y, S.S.S, Z.F, Y.X, C.G, X.W, F.L, Z.L.W, L.Y, and F.Y.L performed the experiments. R.Y, G.L, J.Z.Y and P.F.Z analyzed the data and jointly wrote the manuscript.

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Figures

Fig.1

Figure 1

Generation and purification of Y111, a bispecific antibody targeting both CD3 and PD-L1. (A) Schematic diagram of bispecific antibody Y111, which consists of a monovalent unit adapted from Atezolizumab, an antibody targeting to PD-L1, and a single chain variable fragment (scFv) from 2A5, an antibody for CD3 activation. The red asterisk indicates N297Q mutation for precluding Fc receptor-mediated cross-linking. A “Knob-into-hole” structure was introduced for purification. (B) SDS-PAGE analysis of purified Y111 under non-reducing (NR, left) and reducing (R, right) conditions. Nivolumab is a monoclonal antibody utilized as control. Molecular weight (MW) is indicated in KDa for protein marker. The MW of Y111 is a little smaller than monoclonal antibody Nivolumab as indicated from NR gel. There are 3 and 2 bands for Y111 and Nivolumab in reducing gel respectively as expected. Please note that the nominal MW of Y111H is 48.850 KDa, Y111L is 23.365 KDa, scFv is 52.057 KDa, and intact Y111 is 124.272 KDa as predicted by their protein sequences. While the predicted nominal MW of Nivolumab is 143.597 KDa, the predicted MW of heavy (NH) and light (NL) chain of Nivolumab is 48.422 KDa and 23.359 KDa, respectively. (C) Size exclusion chromatograms of Y111 purified by Protein-A and ion-exchange chromatography. The purity for this sample is 99.63%.

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Figure 2

Cell binding activities of Y111. (A-B) The binding affinity of Y111 and control antibodies (concentration range) to the CD3 expressed on Jurkat cells (A), the PD-L1 expressed on the H1975 cells (B). Flow cytometry was used to assess the geometric mean fluorescence (MFI) of PE channel, and data were analyzed using “One Site-Specific binding” method through least squares fitting. Plotted dots were means of triplicate wells in one performed assay, and the shown figures were representative of at least three experiments. (C-D) Y111 bridged the tumor cell and T cells in dose-dependent manner. CFSE-stained H1975 cells were co-cultured with PKH26-labelled Jurkat cells under dose of antibody Y111 or CD3 Isotype for 1hour. Co-binding% was indicated as percentages of the CFSE-PKH26-double positive cells (Q2) among cells. Representative co-binding dotplots were shown in C, a nonlinear regression depicting dose dependent-association modulated by Y111 was shown in D. Data in D were represented as means pooled from four experiments, and were analyzed using “log (agonist) vs. response (three parameters)” method through ordinary fitting.

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Figure 3

Y111 selectively triggered the cytokine production and degranulation of Vy2Vδ2 T cells in the presence of target tumor cells. (A-D) Y111 efficiently enhanced the activation of Vy2Vδ2 T cells to produce IFN γ , TNF α , and degranulation in tumor cell-dependent fashion. Vy2Vδ2 T cells were stimulated by Y111 or CD3 Isotype (1 μ g/mL of each) in (A), and indicated concentration ranges in (B-D) in the presence/absence of H1975 cells in a 1:1 ratio for 6 hours. The Y111-inuced anti-tumor effector function

was assessed by determining the percentages of Vy2Vδ2 T cells positive for IFN γ , TNF α , and CD107a. Bar graph data shown in A were represented as means $\pm$ SEM pooled from three experiments involving with 9 healthy donors, dots in B-D were means of these individual donors. The data in B were analyzed using “log (agonist) vs. response (three parameters)” method through ordinary fitting. (E) Part of whole graph shown the co-expression signatures of Vy2Vδ2 T cells treated by Y111 or CD3 Isotype (1 μ g/mL of each) in the presence or absence of H1975 cells. After gating cytokine positive population (Sup.Fig.5), the boolean analysis was utilized to determine the percentages of multi-functional effector subsets of Vy2Vδ2 T cells. Data shown were means pooled from 9 healthy donors.

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Fig.4

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Fig.6

Figure 6

The combination of transfused V γ 2V δ 2 T cells with Y111 significantly inhibited the tumor growth in vivo. (A) Experimental schema of protocols for establishing xenograft in NPG mice and evaluating the anti-tumor therapeutic efficacy of different treatments. Immunodeficient NPG mice were s.c. inoculated with H1975 NSCLC cells on day 0. After seventeen days, mice were treated with i.v. transfused V γ 2V δ 2 T cells w/wo 1 or 4 mg/kg Y111. These treatments were repeated twice a week for 2 weeks. Mice treated PBS were used as control. (B) Tumor growth curve in NPG mice treated with purified V γ 2V δ 2 T cells w/wo 1 or 4 mg/kg Y111. Mice received PBS or V γ 2V δ 2 T cells were served as control. The black arrows indicated treatment time point. Data are mean $\pm$ SEM with 7 mice per group, **** p < 0.0001, ** p < 0.01, * p < 0.05

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