Membrane-anchored and tumor-targeted IL-12(attIL12) PBMC therapy for osteosarcoma

Qing Yang

The University of Texas MD Anderson Cancer Center

Jiemiao Hu

The University of Texas MD Anderson Cancer Center

Zhiliang Jia

The University of Texas MD Anderson Cancer Center

Qi Wang

The University of Texas MD Anderson Cancer Center

Jing Wang

The University of Texas MD Anderson Cancer Center

LongHoang Dao

The University of Texas MD Anderson Cancer Center

Wendong Zhang

The University of Texas MD Anderson Cancer Center

Xueqing Xia

The University of Texas MD Anderson Cancer Center

Richard Gorlick

The University of Texas MD Anderson Cancer Center

Shulin Li (✉ Sli4@mdanderson.org)

The University of Texas MD Anderson Cancer Center

Research Article

Keywords: attIL12-PBMC, osteosarcoma, PDx, osteogenic differentiation

Posted Date: March 2nd, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1392663/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background

Chimeric antigen receptor (CAR) T cell therapy has shown great promise for hematologic malignancies but requires a long duration of T-cell expansion, is associated with severe toxicity, and has limited efficacy for treating solid tumor.

Methods

To address these challenges, we generated a cell membrane-anchored and tumor-targeted interleukin-12 (attIL12) to arm peripheral blood mononuclear cells (PBMCs) instead of T cells to omit the expansion phase for required CAR-T cells.

Results

This attIL12-PBMC therapy showed significant antitumor efficacy in both heterogeneous osteosarcoma patient-derived xenograft (PDx) tumors and metastatic osteosarcoma tumors with no observable toxic effects. Mechanistically, attIL12-PBMC treatment resulted in tumor-restricted antitumor cytokine release and accumulation of attIL12-PBMCs. It also induces terminal differentiation of osteosarcoma cells into bone like cells to impede tumor growth.

Conclusion

In summary, attIL12-PBMC therapy is safe and effective against osteosarcoma. Our goal is to move this treatment into a clinical trial. Owing to the convenience of the attIL12-PBMC production process, we believe it will be feasible.

Introduction

Osteosarcoma is a rare malignant tumor that arises primarily in children and adolescents[1]. Even with the combination of surgery and adjuvant chemotherapy, the overall 5-year survival rate is about 20% for patients with advanced osteosarcoma, a rate that has remained virtually unchanged over the past 30 years[2]. Therefore, novel therapeutic strategies are urgently needed to treat advanced osteosarcoma.

In light of the emerging in-depth understanding of the molecular mechanisms of osteosarcoma, numerous molecularly targeted inhibitors and chimeric antigen receptor (CAR) T-cell therapies have been investigated in clinical trials[3, 4]. However, the outcomes of antigen-targeted therapy for osteosarcoma have not been impressive. For example, no objective response was observed in a phase II clinical trial of cixutumumab, an inhibitor of insulin-like growth factor (IGF)[3], in osteosarcoma patient. Similarly, ERBB2

(HER2) inhibitor treatment did not lead to significant different outcomes in patients with ERBB2-positive and ERBB2-negative osteosarcoma[3]. In fact, ERBB2-targeted CART-cell treatment induced objective responses in only 3 of 16 patients with ERBB2-positive osteosarcoma[4]. The main reason for these unsatisfactory responses to targeted treatments is the heterogeneity of osteosarcoma tumors, which consist of multiple cell lineages and therefore cannot be cured by single-target-based approaches. To address the challenges posed by tumor heterogeneity, CART-cell approaches targeting multiple antigens have been investigated[5–7], but these approaches were frequently associated with toxic effects[8–11]. Finally, the long duration of T-cell expansion in vitro not only is expensive but also may miss the optimal time window for intervention. Thus, alternative approaches are urgently needed to make progress in treating osteosarcoma.

In this study, we determined the extent to which a cell-membrane-anchored tumor-targeted interleukin-12 (attIL12) could arm unexpanded peripheral blood mononuclear cells (PBMCs) to treat mice bearing patient-derived-xenograft (PDX) tumors or metastatic osteosarcoma tumors in the lungs. This approach does not require the costly and time-consuming steps of T-cell isolation and expansion[12, 13]. Moreover, the attIL12-modified PBMCs treatment yielded no observable toxic-side-effect. Notably, we discovered that attIL12-modified PBMCs impeded osteosarcoma tumor growth by triggering terminal differentiation of cancer into bone-like cells instead of by direct killing of the tumor cells. Single cells RNA sequencing analysis supported this finding of osteosarcoma cell differentiation into bone-like phenotype at the molecular level and uncovered a bone-cells gene signature.

Materials And Methods

Animal studies and tumor models

Six- to eight-week-old NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (NSG) mice of both sexes were purchased from The Jackson Laboratory. The mouse care and handling procedures were approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center, the IACUC protocol number 00001046-RN02.

Patient-derived OS1, OS2, and OS17 osteosarcoma tumor cells (generously provided by Dr. Richard Gorlick, the Pediatric Preclinical Testing Consortium, the University of Texas MD Anderson Cancer Center) were implanted subcutaneously into C.B-Igh-1^b/IcrTac-Prkdc scid mice. Tumors were measured with calipers twice weekly after implantation. Tumor volume was calculated by the formula: $V = (\pi / 8) \times (a \times b^2)$, where V = tumor volume in cubic centimeters, a = maximum tumor diameter, and b = diameter at 90° to a . Survival curves were generated by the Kaplan-Meier method. attIL12 PBMCs (2.5×10^6 cells/mouse) or control PBMCs (2.5×10^6 cells/mouse) were i.v. injected into the mice 3 times at intervals of 2 weeks..

To establish the osteosarcoma lung metastasis model, human OSD cells (5×10^5 cells/ 10 μ L/mouse) were injected into the proximal tibia of 6-week-old NSG mice. Tumor growth was monitored by measuring

tumor volume as described. Lung metastases were allowed to develop for 4 weeks after OSD cell injection. The leg into which OSD cells were was amputated 4 weeks after injection. attIL12 PBMCs (2.5×10^6 cells/mouse) or control PBMCs (2.5×10^6 cells/mouse) were i.v. injected into the 3 times at intervals of 2 weeks.

Cell culture

OSD, OSO, SAOS2 and U2OS human osteosarcoma cells were kindly provided by Dr. Dennis PM Hughes (The University of Texas M.D. Anderson Cancer Center, Houston, TX). OSD, OSO, SAOS2 and U2OS cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum [FBS] and supplemented with antibiotics, non-essential amino acid solution, and minimum essential medium (MEM) vitamin mixture and cultured in an incubator maintained at 5% CO₂ and 37°C. Patient-derived OS1 and OS2 osteosarcoma cells were dissociated from osteosarcoma xenograft tumors and cultured in high-glucose DMEM supplemented with 15% FBS and 1% penicillin/streptomycin. All the tumor cell lines were characterized by DNA fingerprinting within 6 months of initiating the experiments at MD Anderson Cancer Center's Characterized Cell Line Core Facility and were treated with a mycoplasma removal agent from Bio-Rad to ensure that cells were free of mycoplasma before inoculation.

Human PBMCs

Buffy coats from de-identified healthy blood donors were purchased from the Gulf Coast Regional Blood Center, and their acquisition was approved by the MD Anderson Institutional Review Board. PBMCs were isolated from buffy coat samples via centrifugation over Ficoll-Paque. PBMCs were cultured in medium containing 10% FBS, supplemented with recombinant human IL-2 (rhIL-2) (50 U/mL), rhIL-7 (10 ng/mL), and rhIL-15 (5 ng/mL).

Generation and transfection of lentivirus

High-titer replication-defective lentiviral vectors were produced and concentrated by the MD Anderson Functional Genomics Core Facility. Briefly, HEK293T human embryonic kidney cells were transfected with pVSV-G (a VSV glycoprotein expression plasmid), pCMV-Gag/Pol/Rev, and a transfer plasmid by using Lipofectamine 2000 (Thermo Fisher Scientific). The viral supernatant was harvested at 48 h after transfection. Viral particles were concentrated using Lenti-X Concentrator (Takara Bio, Inc.).

PBMC lentiviral transduction

Human PBMCs were stimulated by CD3/CD28 Dynabeads (Thermo Fisher Scientific) according to the manufacturer's instructions. On day 2, PBMCs were plated in non-tissue culture-coated 24-well plates. Lentiviral supernatant (Vector Builder) was first spun at 1500g for 2h on retronectin (Takara)-coated non-tissue culture-treated plates. PBMCs were then plated and centrifuged at 1000g for 20 minutes and incubated at 37 °C. After 24h, the medium was changed to 45% RPMI-1640 and 45% Click's medium containing 10% FBS, supplemented with rhIL-2 (50 U/mL), rhIL-7 (10 ng/mL), and rhIL-15 (5 ng/mL).

Immunohistochemistry and immunofluorescence staining

Frozen tumor sections and organs were sequentially fixed with cold acetone, acetone plus chloroform (1:1), and acetone. Paraffin-embedded sections were deparaffinized and heated in antigen retrieval buffer. Tissue sections were blocked with 3% H₂O₂ in distilled water for 20 minutes and then in blocking buffer (5% normal horse serum and 1% normal goat serum in phosphate-buffered saline (PBS)). Slides were incubated with primary antibodies overnight at 4 °C and secondary antibodies for 1 h at room temperature. For immunohistochemistry staining, the secondary antibody was biotin conjugated, the sections were treated with ABC reagent (Vector Labs), and the nuclei were counterstained with hematoxylin (Sigma-Aldrich). Tumor sections were mounted with Cytoseal mounting medium (Life Technologies). Quantifications of immunohistochemistry images were assessed by examining 3 randomly selected low-power fields per slide. For immunofluorescence staining, tumor sections were mounted in an antifade fluorescence mounting medium with 4-6-diamidino-2-phenylindole (DAPI). Slides were visualized under a Nikon Eclipse Ti fluorescence microscope.

ELISA

Tumor lysates were collected from treated and control tumor-bearing mice. Tumor lysates were collected from tumors that were lysed with RIPA buffer as described before[40]. Serum was collected from the supernatant of centrifuged mouse whole blood. Culture medium was collected at 1 mL medium/10⁶ cells. The levels of IL-12, TNF α and IFN γ were measured by using ELISA Ready-SET-Go! kits (eBioscience).

Flow cytometry

Cells were sequentially incubated with primary and secondary antibodies for 30 minutes each at 4 °C. Stained cells were analyzed using an Attune acoustic focusing cytometer (Applied Biosystems) or a BD LSR-Fortessa cell analyzer (BD Biosciences). Flow cytometry data were analyzed using the FlowJo software program (FlowJo, LLC).

Tumor cell dissociation

Tumors were minced into 2-mm fragments, placed in 5 mL of dissociation buffer (RPMI/1640 medium with 100 U/mL collagenase type IV and 100 U/mL DNase I), and incubated at 37 °C while shaking at 120 rpm for 30 minutes to 1 hour. The released cells were filtered with 70- μ m strainers and centrifuged at 600g for 5 minutes, followed by red blood cell lysis. Cells were then resuspended in FACS solution containing 2% FBS. Single-tumor-cell suspensions were obtained after CD45 depletion using an EasySep Human CD45 Depletion Kit (Stem Cell Technologies).

Cytolytic activity assay

Tumor cells (1×10^5) were cocultured with transduced PBMCs in 24-well plates at tumor-to-PBMC ratios of 1:5 for 48h. PBMCs and tumor cells were identified by staining with anti-human CD45 and green fluorescent protein (GFP), respectively.

Microfluidic chip binding assay

A PDMS microfluidics slide chip (Abnova) was coated with 1 mg/mL streptavidin (Agilent). The chip was then washed and coated with 0.4 mg/mL biotin-vimentin (MD Anderson) for 1 h. Next, 1.5×10^6 attIL12-PBMCs (labeled with Calcein Green AM [ThermoFisher]) and 1.5×10^6 control-PBMCs (labeled with CMTPIX red cell tracker [ThermoFisher]) were mixed at a ratio of 1:1, loaded into a spiral chamber (Abnova), and passed through the slide chip at a flow rate of 1.8 mL per hour using the CytoQuest microfluidics pump (Abnova). The slide chip was then imaged on a fluorescent microscope (Keyence). Cells were counted using Keyence BZ-X700 analysis software.

Sample preparation and cell isolation for single-cell RNA-sequencing.

Fresh tumor lesions were stored in RPMI medium and processed on ice within 30 minutes after the surgical removal. The specimens were washed with Hanks Balanced Salt Solution (HBSS) 3 times and minced into 1 to 2 mm pieces. Then, mixed enzymes were added according to the protocol provided with the Miltenyi Biotec tumor dissociation Kit (Miltenyi Biotec:130-095-929) and the dissociation was performed in a gentleMACS™ dissociator (Miltenyi Biotec:130-093-235). After digestion, the samples were filtered through 40-μm sterile strainers and centrifuged at 800g for 5 minutes. Subsequently, the supernatants were discarded, and the cell pellets were suspended in 1 mL PBS (HyClone). To remove red blood cells, 2 mL of red blood cell lysis buffer (eBioscience™:00-4333-57) was added, and cells were incubated at 25 °C for 10 minutes. The solution was then centrifuged at 500g for 5 minutes and re-suspended in PBS[25]. The samples were stained with trypan blue (Sigma) and cell viability was evaluated under phase-contrast light microscope (Nikon).

single-cell RNA-sequencing data analysis

The raw Unique molecular identifier (UMI) count data was loaded into the R Seurat (version 4.0.0)[41] package for downstream analysis. Cells with UMI numbers <200 or with over 25% mitochondrial-derived UMI counts were considered low-quality cells and were filtered out. The count matrix was first log-normalized with the scale factor set to be 10,000 total genes per cell. The 6 samples were then integrated into a new matrix using the IntegrateData function with the top 2,000 highly variable genes selected by the SEURAT function FindVariableGenes with default parameters. Principle component analysis (PCA) was performed on the integrated data matrix. The top PCs were selected based on the Elbowplot, representing at least 80% of the total variances. The main cell clusters were identified with the FindClusters function offered by Seurat, with resolution set to be 0.5. And then they were visualized with UMAP plots.

For each cluster, the marker genes were identified by comparing the cluster with the other clusters using the FindConservedMarkers function. The Seurat FindAllMarker function was performed to identify preferentially expressed genes in clusters and differentially expressed genes between control and treatment cells. The enriched pathways and hallmarks were identified by pre-ranked GSEA using the gene

list ranked by log-transformed P values with signs set to positive/negative for a fold change of >1 or <1, respectively.

Statistical analysis

The directly measured outcomes were analyzed using a 2-sided Student t-test to compare 2 treatment groups or 1-way analysis of variance to compare more than 2 treatment groups. The statistical analyses were conducted using GraphPad Prism 8 software. All data values represent 3 replicates and are shown as median $\pm$ SEM. Significance was defined as $P < 0.05$.

Results

1. Biological functions of attIL12-transduced PBMCs *in vitro* and *in vivo*;

In previous studies we reported our discovery of a linear peptide (VNTANST) that can target VNTANST-IL12 (ttIL12) protein to tumor cell surface vimentin (CSV)[14-17], which is expressed on the surfaces of almost all highly malignant tumor cells[15, 18]. Our previous studies confirmed accumulation of IL-12 in tumors and inhibition of tumor progression in numerous murine tumor models after ttIL12 gene therapy, in which ttIL12 encoding plasmid DNA was administrated directly into mice[14, 16, 17]. However ttIL12 gene therapy via intramuscular injection is only able to eliminate microscopic tumor[19].

In current study, we used these attIL-12 armed PBMCs, which express on the surface of cell membranes without releasing out of cells in tumor targeting and treatment. Briefly, the attIL12 construct was generated by first including a trans-membrane domain before the stop codon of the human IL-12 p35 subunit to anchor IL-12 to the cell surface, and then inserting the tumor-targeted peptide-encoding sequence right before the stop codon of the human IL-12p40-encoding subunit (Figure 1A). This attIL12 was packed into a lentivirus that could transduce PBMCs stimulated with anti-CD3/28 beads and was expressed on the PBMCs' surfaces as determined by flow cytometry (Figure-1B, left panel) and immunofluorescence staining (Figure-1B, right panel).

To validate the biological function of attIL12-PBMCs, we monitored IL-12 and interferon gamma (IFN γ) secretion in the medium supernatant over time by ELISA (Figure 1C-D). We also transduced the PBMCs with a wild-type IL12 (wtIL12)-bearing lentivirus to serve as a positive control. Compared to medium conditioned with wtIL12-PBMCs, the detectable level of secretion of IL12 and IFN γ in medium conditioned with attIL12 transduced PBMCs was 5-10 fold lower (Figure 1C). To investigate whether these low IFN γ -inducing attIL12 PBMCs were still active *in vivo*, mice bearing OS1 osteosarcoma PDx were infused with attIL12-PBMCs or control-PBMCs. attIL12-PBMCs notably suppressed tumor growth after 3 infusions at an interval of every 2 weeks (Figure 1D-E). Most of the tumors from mice treated with attIL12-PBMC became smaller and maintained their small size.

2. attIL12-armed-PBMCs derived from different donors showed similar efficacy across tumor and model types;

The significant tumor growth inhibition we observed could have been a coincidence since the PBMCs were derived from an individual donor. To exclude this possibility, PBMCs from different donors were armed with attIL12 (Figure 2A). The attIL12 transduction process and the validation of attIL12 expression on the PBMCs was consistent among different donors, with expression on, ~60% (Figure1B, Figure2A). This showed that attIL12 transduction efficacy was consistent in PBMCs from different donors. Next, we assessed the antitumor efficacy of attIL12-PBMC in two osteosarcoma PDX models, OS2 and OS17. In order to eliminate the influence of sex, each group included 3 female and 3 male mice. The results demonstrated that attIL12-PBMCs significantly impeded both OS2 and OS17 tumor growth and notably prolonged the survival time of treated mice (Figure 2B-E). To boost the clinical relevance of our experiments, we also tested the efficacy of attIL12-PBMCs in the OSD model of osteosarcoma (OSD) lung metastasis (Figure 2F). As in subcutaneous PDX tumor model, attIL12-PBMCs dramatically extended the mice's overall survival time over control PBMC treatment (Figure 2G). On day 65 after tumor-cell inoculation, we randomly selected 3 mice from each group to detect lung metastasis with hematoxylin and eosin staining (H&E). We found that the area containing lung metastases in the attIL12-PBMC treatment group was significantly smaller than in the control group (Figure 2H). This result showed that attIL12-PBMCs also significantly inhibited the progression of OSD lung metastasis. Remarkably, these efficacy results were obtained with PBMCs from 3 independent donors.

3. Safety of attIL12 modified PBMCs in PDX model;

Toxicity is the key challenge for clinical application of IL12-based therapies because systemic infusion of IL12 protein has yielded lethal consequences[20]. Both wtIL12 gene therapy and wtIL12-armed-T cell (including CAR-T cell) therapy is safer than IL12 protein therapy, but the risky of toxicity remains high[21, 22].

As we have discovered that attIL12-modified PBMCs did not induce toxic cytokine production *in vitro*, it was plausible to hypothesize that infusion of attIL12-PBMCs would yield only minor toxicity, if so, this would be a crucial finding for the entire field of IL12-based cell therapy. To test this hypothesis, we measured levels of toxic cytokines in peripheral tissue and conducted histologic examination of major organ on day 5 after infusion of attIL12-, wtIL12- or control-PBMCs (Figure 3A). As expected, inflammatory cells were identified in the livers of mice treated with wtIL12-PBMCs, but not in the hearts, lungs, kidney, or brains (Figure 3B). Of note, inflammatory cells were not found in any organs of mice treated with attIL12- or control-PBMCs (Figure 3B). Consistent with the accumulation of inflammatory cells in the liver, substantial accumulation of IL12, IFN γ and tumor necrosis factor alpha (TNF α) were detected only in the livers of wtIL12-PBMC-treated mice, not in any other organs (Figure 3C). Likewise, neither attIL12 nor control-PBMCs infusion caused any toxic cytokine induction in the liver or other organs. In agreement with this wtIL12 induced, liver-specific inflammation, a high level of IL12 was detected in the serum of wtIL12-PBMC-treated mice (Figure 3D). In contrast to the finding in the livers and blood, a significantly higher level of IL12, IFN γ and TNF α were detected in tumors of mice that received either wtIL12- or attIL12-PBMC infusion (Figure 3E). To test our hypothesis further, we examined liver and kidney function by comparing blood chemistry. The results showed that both the alanine aminotransferase (ALT) and

aspartate aminotransferase (AST) levels in the blood were significantly higher in mice treated with wtIL12-PBMCs than in either control- or attIL12-PBMCs treated mice (Figure 3F). These results suggested that toxic effects in the liver can follow wtIL12-PBMC infusion, and that attIL12-PBMC infusion does not induce liver toxicity. Therefore, attIL12-PBMC infusion with in the therapeutic window is safe.

4. attIL12 promotes the tumor-targeted migration of modified PBMCs *in vitro* and *in vivo*;

To identify the mechanism by which attIL12-PBMCs fail to induce systemic toxicity, we tested the migration and accumulation of CSV-targeted attIL12-PBMCs. We hypothesized that attIL12-PBMCs not only abolished toxic cytokine induction but also promptly accumulated in tumors, thereby avoiding toxic effects. To test this hypothesis *in vitro*, we developed a cytoquest microfluidic chip assay by coating vimentin on the chip to show specific capture of attIL12-PBMC but not the control PBMC because attIL12 contains vimentin binding ligand peptide (Figure 1A). In brief, equal number of control-PBMCs (red CMTPIX labeled) and attIL12-PBMCs (green calcein AM labeled) were mixed and loaded onto the vimentin-coated microfluidic chip, and the chip was washed under an automatic microfluidic flow. After binding, unbound cells were washed away, and bound cells were scanned with an automated fluorescence microscope to determine which cells types were bound to vimentin on the chip (Figure 4A). We found that most of the vimentin-bound cells were attIL12-PBMCs (green), and only rarely control-PBMCs (red). This result showed that attIL12-PBMCs could bind vimentin protein on the chip. Next, instead of coating the chip with vimentin, we coated the chip with osteosarcoma tumor cell lines with different percentages of CSV positive cells. As showed in Figure 4B that CSV expression of osteosarcoma cell lines ranged from 49.9% to 19.4%. The binding activity was higher in tumor cells with higher CSV expression (Figure 4D).

In light of these *in vitro* data, we also tested tumor targeted migration of attIL12-PBMCs *in vivo*. We collected tumors from PDx-bearing mice treated with attIL12-PBMC or control-PBMC and quantified the infiltrated PBMCs using IHC and flow cytometry. In line with our *in vitro* findings, the population of CD45+ cells was significantly higher in tumors from attIL12-PBMC treated mice than in those from either wtIL12-PBMC or control-PBMC treated mice (Figure 4E-F). Significantly, the IHC staining of several organs revealed that PBMCs were only found in tumors, not in any other organs in attIL12-PBMCs treated mice. However, wtIL12-PBMC accumulated in the liver. Thus, we found that attIL12 promotes the tumor-target migration of modified PBMCs both *in vitro* and *in vivo*. This property may serve as the key mechanism for avoiding toxicity following attIL12-PBMC cell infusion.

5. attIL12-PBMCs infusion induces osteogenic differentiation of osteosarcoma PDx cells;

To uncover the specific mechanism by which attIL12-PBMCs mediated PDx tumor growth inhibition, we first measured tumor-cell apoptosis in attIL12-PBMC treated and control-PBMC treated PDx-bearing mice. Results of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays showed that the percentage of apoptotic cells was very low, at 3-5%, in both the attIL12-PBMC-treated and control PBMC-treated groups; no significant differences were detected between the groups (Supplementary Figure 1A-B).

This result suggested that apoptosis may not be the primary mechanism by which attIL12-PBMC treatment inhibits PDX tumor growth.

To validate this *in vivo* observation, we have also performed *in vitro* CTL assay to confirm that tumor-cell killing is not the primary mechanism by which CAR-T cells inhibit tumor growth[18, 23, 24]. To assess the CSV-targeted cell-killing activity of attIL12-PBMCs, we co-cultured the CSV-high-expressing metastatic osteosarcoma cell line-OSD with attIL12-PBMCs or control-PBMCs at 1:5 ratio (tumor cells to PBMCs) for 48 hours. After coculture, we determined the number of PBMCs and tumor cells by flow cytometry (Figure 5A). attIL12-PBMCs did not kill osteosarcoma cells more effectively than did control- PBMCs. This result confirmed the *in vivo* observation.

The lack of direct cell killing promotes us checking other underlying mechanism for tumor shrinking and stabilization following attIL12-PBMCs infusion. Unexpectedly, hematoxylin and eosin staining revealed apparent osteogenic differentiation in 3 independent osteosarcoma PDx tumor models following attIL12-PBMC infusion (Figure 5B-C). This result suggested that attIL12-PBMCs may influence the terminal differentiation of osteoblasts.

To further explore the molecular changes induced by attIL12-PBMCs in OS tumor cells, we used single-cell RNA sequencing to analyze differences in gene expression. After initial quality control assessment and doublet removal, we captured between 3589 and 5074 cells from the tumor samples, which included 3 OS1 tumor samples of the control-PBMC infusion group and 3 OS1 tumor samples of the attIL12-PBMC infusion group. In all samples, more than 90% of tumor cells were viable (Supplementary Figure 1C-D). Unbiased clustering of the cells with uniform manifold approximation and projection (UMAP) analyses[25] identified 16 main gene profile clusters in parallel (Figure 5D-E). Using canonical markers, we identified chondroblastic cells, Endothelial cell, Myeloid cells, Pericytes, osteoclast and 12 types of osteoblast. We called out these 12 types of osteoblast on the basis of expression of 2 early osteogenic differentiation markers, Alkaline Phosphatase (ALPL) and Integrin Binding Sialoprotein (IBSP), and 2 late osteogenic differentiation markers, osteopontin (SPP1) and osteocalcin (BGLAP)[26, 27]. As shown in Figure 5F, only 2 clusters differed significantly between the attIL12-PBMC treated and control groups, cluster 7 and cluster 10. The percentage of cells in cluster 7 in the attIL12-PBMC samples was 9.6% whereas it was 3.3% in the control samples. The percentage of cluster 10 cells in the attIL12 group was 2.3%, whereas it was 0.9% in the control group. Cluster 7 was the cluster with the largest difference between the attIL12-PBMC and control groups.

To provide additional line of evidence that an increase in the cluster 7 cell population was associated with osteogenic differentiation in tumors from attIL12 group-PBMC-treated mice, we performed a volcano plot analysis of the differentially expressed genes between cluster 7 and other clusters. The results confirmed induction of osteogenic differentiation genes in cluster 7 (Figure 5F). This finding demonstrated that cluster 7 was the most differentiated osteoblast cluster. We therefore speculated that programming cells toward a bone like, terminally differentiated cluster 7 phenotype may be one of the molecular mechanisms by which attIL12-PBMC infusion slow tumor growth. To further validate this

working hypothesis, we determined the expression of the markers of late osteogenic differentiation osteocalcin (BGLAP) and osteopontin (SPP1) [26, 27]. Both were notably increased in OS1, OS2 and OS17 PDx tumors following attIL12-PBMC infusion. These results support the notion that attIL12-PBMC infusion induced terminal differentiation of osteosarcoma cells.

6. IFN γ promotes terminal differentiation of OS1 and OS2 cells;

To understand how attIL12-PBMCs promoted the cluster7 osteoblast phenotype in vivo, we assessed the results of our analysis of Kyoto Encyclopedia of Genes and Genomes data, which identified an association between expression of bone differentiation genes and protein levels of IFN γ (Figure 6A). To validate this expected increase in IFN γ level after attIL12-PBMC infusion, we tested IFN γ and CD45 double-positive cells in tumors of both control PBMC- and attIL12-PBMC-treated mice using flow cytometry. As expected, attIL12-PBMCs dramatically enhanced the proportion of IFN γ and CD45 double positive cells in both OS1 and OS2 tumor models (Figure 6B-C). These data implied that attIL12-PBMC treatment substantially increased IFN γ protein secretion in tumors. In order to determine whether attIL12-PBMC treatment directly induced terminal differentiation of tumor cells, we co-cultured OS1 or OS2 tumor cells with attIL12-PBMCs or control PBMCs in the presence or absence of a neutralizing anti-IFN γ antibody for 6 days. Indeed, attIL12-PBMC co-culture induced a significantly higher percentage of SPP1 and BGLAP double-positive OS1 and OS2 tumor cells compared to coculture with control PBMCs, and tumor cells cocultured with attIL12-PBMCs show evidence of induction of terminal differentiation (Figure 6D-E). More importantly, neutralizing IFN γ completely prevented this terminal differentiation.

Discussion

To avoid the toxicity and boost tumor specific antitumor immune response of IL12, we discovered a tumor cell-surface-vimentin targeted IL12(ttIL12) which displayed an effective inhibitory effect on a variety of tumors with small size (> 5mm in diameter). To eliminate larger tumors than ttIL12 can, we generated a second generation of ttIL12, -attIL12, and used it to modify PBMCs such that ttIL12 is anchored to the PBMC membrane, preventing cytokine release in the peripheral blood and healthy tissues. As shown from our results (Fig. 2), we found that infusion of attIL12-PBMCs inhibited PDx osteosarcoma growth and osteosarcoma lung metastasis. Interestingly, this inhibition was associated not with direct cell killing or apoptosis induction but with osteogenic differentiation. In other words, attIL12-PBMC treatment transforms tumor cells into bone-like terminally differentiated cells, which slows tumor growth.

In contrast with classic CAR-T cell therapy, we employed PBMCs in this study. We used this strategy because T cell expansion for standard CAR T-cell therapy requires multiple weeks from T-cell retrieval to infusion to the patient[12, 13]. Our procedure allows attIL12-PBMCs infusion within a week after blood collection. The long expansion time for standard CAR-T cells may not only delay timely treatment and thereby reduce efficacy, but also increase medical costs and patient's stress. attIL12-PBMC therapy is promising way to address these issues. As far as we know, this is the first application of genetically modified PBMCs for directly adoptive therapy of in cancer.

Inclusion of a co-stimulatory signal in second generation CARs has dramatically enhanced the antitumor efficacy of CAR-T cell therapy. Nonetheless, in clinical trials these improvements have not been successful in treating large, heterogeneous solid tumors. [28–31]. To eliminate these tumors, researchers have included cytokines such as IL12 or double co-stimulatory signals in the newest generation of CAR-T cell therapies[32–35]. Whether these new CART vector improve therapeutic efficacy requires further clinical investigation, but it is clear that the addition of cytokines and other co-stimulatory signals increases the risk of toxic effects. In contrast, attIL12-based cell modification diminished the toxicity risk because the membrane-anchored IL12 does not induce any of the cytokines that may cause cytokine release syndrome. The toxicity study shown here demonstrated that attIL12-PBMCs were safer than wtIL12-PBMCs not only because attIL12-PBMCs inhibited cytokine release but also because they did not accumulate in the peripheral blood and healthy organs (Fig. 3C-E, Fig. 4E,F). In addition, attIL12-PBMCs bound CSV on tumor cells to boost the prompt tumor-specific distribution. In previous studies we discovered that CSV is a universal marker exclusively expressed on the surface of highly malignant tumor cell, such as sarcoma, breast cancer, and colon cancer cells[15, 18]. More importantly, the VNTANST peptide specifically binds CSV on the surface of cancer cells but not healthy cells[36]. We also demonstrated that IL12 genetically modified with VNTANST (ttIL12) binds to CSV on tumor cells and delivers IL-12 to tumor sites[14]. Those prior works were essential to the design of this safe and effective attIL12 vector for PBMC modification.

The concept of differentiation therapy arose from the observation that cytokines and hormones can promote cell differentiation, thereby irreversibly changing the phenotype of cancer cells[37]. It is believed that this terminal differentiation in cancer cells leads to tumor remission[26]. The rapid clearance of acute promyelocytic leukemia (APL) in response to retinoic acid (RA) was a hallmark success of differentiation therapy[38, 39]. Interestingly, our results showed that attIL12-PBMCs significantly enhanced osteogenic differentiation in osteosarcoma PDx (Fig. 5A-B). Our single cell sequencing data supports the notion that attIL12-PBMCs significantly boosted terminal osteogenic differentiation (indicated by an increase in the cluster 7 cell population) (Fig. 5E). In addition, KEGG analysis of genes that are differential expressed between cluster 7 and other clusters indicated that genes differentially expressed in osteoblasts are associated with induction of IFN γ and cytokines pathway. *In vitro*, IFN γ productin induced by attIL12-PBMCs treatment was the key factor inducing terminal differentiation of primary osteosarcom cell (Fig. 6D-E). Research on differentiation therapy in osteosarcoma is still relatively rare, so this study opens up new directions and perspectives for future osteosarcoma therapy research.

Limitations of our study should be noticed when interpreting and extrapolating our data. attIL12-PBMC should be tested in other solid cancer entities. Additionally, the detailed mechanism of attIL12-PBMC promoting terminal differentiation of osteosarcoma cells into bone like cells needs to be further revealed.

Conclusions

In summary, attIL12-PBMC therapy is safe and effective against osteosarcoma. Our goal is to move this treatment into a clinical trial. Owing to the convenience of the attIL12-PBMC production process, we

believe it will be feasible.

Abbreviations

CART
Chimeric antigen receptor T
attIL12
a cell membrane-anchored and tumor-targeted interleukin-12
PBMCs
peripheral blood mononuclear cells
PDx
patient-derived xenograft
attIL12-PBMC
a cell membrane-anchored and tumor-targeted interleukin-12 armed peripheral blood mononuclear cells
IGF
an inhibitor of insulin-like growth factor
CSV
cell surface vimentin
IFN γ
interferon gamma
H&E
hematoxylin and eosin staining
ALT
alanine aminotransferase
AST
aspartate aminotransferase
TUNEL
terminal deoxynucleotidyl transferase dUTP nick end labeling
ALPL
Alkaline Phosphatase
IBSP
Integrin Binding Sialoprotein
SPP1
osteopontin
BGLAP
osteocalcin
TNF α :tumor necrosis factor alpha.

Declarations

Ethical Approval

The ethics approval statements for animal work were provided by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center, the IACUC protocol number 00001046-RN02.

Consent for publication

Not applicable.

Availability of supporting data

Not applicable.

Competing financial interests

No conflicts of interest were declared.

Funding

This study was supported by the National Institutes of Health through grant 2R01CA120895.

Authors' Contributions

Qing Yang: designed and conducted most of the experiments, data analysis, and interpretation and wrote and edited the manuscript; Jiemiao. Hu: designed the experiments, data analysis; Wendong. Zhang: established PDX animal models; Zhiliang. Jia: perform animal studies and T cell culture; Qi Wang and Jing Wang analyze single-cell sequencing data; Longhoang. Dao: conducted microfluidic chip binding assay; Xueqing. Xia: prepared viral constructs; Richard. Gorlick: provided osteosarcoma PDX animal models; Shulin. Li: directed experimental design, oversaw development of the study concept, and reviewed and edited the manuscript.

Acknowledgements

Not applicable

Authors' information

1.Department of Pediatrics–Research, Division of Pediatrics, The University of Texas MD Anderson Cancer Center, Houston, TX, USA. 2.Department of Orthopedic Surgery, The First People's Hospital of Xiangtan City, 411101, Xiangtan, China; 3. Department of Bioinformatics and Computational Biology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA.

References

1. Jo VY, Fletcher CD: **WHO classification of soft tissue tumours: an update based on the 2013 (4th) edition.** Pathology 2014, **46**:95–104.
2. Link MP, Goorin AM, Miser AW, Green AA, Pratt CB, Belasco JB, Pritchard J, Malpas JS, Baker AR, Kirkpatrick JA, et al.: **The effect of adjuvant chemotherapy on relapse-free survival in patients with osteosarcoma of the extremity.** N Engl J Med 1986, **314**:1600–1606.
3. Weigel B, Malempati S, Reid JM, Voss SD, Cho SY, Chen HX, Krailo M, Villaluna D, Adamson PC, Blaney SM: **Phase 2 trial of cixutumumab in children, adolescents, and young adults with refractory solid tumors: a report from the Children's Oncology Group.** Pediatr Blood Cancer 2014, **61**:452–456.
4. Ahmed N, Brawley VS, Hegde M, Robertson C, Ghazi A, Gerken C, Liu E, Dakhova O, Ashoori A, Corder A, et al: **Human Epidermal Growth Factor Receptor 2 (HER2) -Specific Chimeric Antigen Receptor-Modified T Cells for the Immunotherapy of HER2-Positive Sarcoma.** J Clin Oncol 2015, **33**:1688–1696.
5. Hegde M, Corder A, Chow KK, Mukherjee M, Ashoori A, Kew Y, Zhang YJ, Baskin DS, Merchant FA, Brawley VS, et al: **Combinational targeting offsets antigen escape and enhances effector functions of adoptively transferred T cells in glioblastoma.** Mol Ther 2013, **21**:2087–2101.
6. Ruella M, Barrett DM, Kenderian SS, Shestova O, Hofmann TJ, Perazzelli J, Klichinsky M, Aikawa V, Nazimuddin F, Kozlowski M, et al: **Dual CD19 and CD123 targeting prevents antigen-loss relapses after CD19-directed immunotherapies.** J Clin Invest 2016, **126**:3814–3826.
7. Shah NN, Maatman T, Hari P, Johnson B: **Multi Targeted CAR-T Cell Therapies for B-Cell Malignancies.** Front Oncol 2019, **9**:146.
8. Han X, Wang Y, Wei J, Han W: **Multi-antigen-targeted chimeric antigen receptor T cells for cancer therapy.** J Hematol Oncol 2019, **12**:128.
9. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA: **Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2.** Mol Ther 2010, **18**:843–851.
10. Lamers CH, Sleijfer S, Vulto AG, Kruit WH, Kliffen M, Debets R, Gratama JW, Stoter G, Oosterwijk E: **Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience.** J Clin Oncol 2006, **24**:e20-22.
11. Tran E, Chinnasamy D, Yu Z, Morgan RA, Lee CC, Restifo NP, Rosenberg SA: **Immune targeting of fibroblast activation protein triggers recognition of multipotent bone marrow stromal cells and cachexia.** J Exp Med 2013, **210**:1125–1135.
12. Kohl U, Arsenieva S, Holzinger A, Abken H: **CAR T Cells in Trials: Recent Achievements and Challenges that Remain in the Production of Modified T Cells for Clinical Applications.** Hum Gene Ther 2018, **29**:559–568.
13. Depil S, Duchateau P, Grupp SA, Mufti G, Poirot L: **'Off-the-shelf' allogeneic CAR T cells: development and challenges.** Nat Rev Drug Discov 2020, **19**:185–199.
14. Cutrera J, Dibra D, Xia X, Hasan A, Reed S, Li S: **Discovery of a linear peptide for improving tumor targeting of gene products and treatment of distal tumors by IL-12 gene therapy.** Mol Ther 2011,

19:1468–1477.

15. Satelli A, Mitra A, Cutrera JJ, Devarie M, Xia X, Ingram DR, Dibra D, Somaiah N, Torres KE, Ravi V, et al: **Universal marker and detection tool for human sarcoma circulating tumor cells**. *Cancer Res* 2014, **74**:1645–1650.
16. Cutrera J, King G, Jones P, Kicenuik K, Gumpel E, Xia X, Li S: **Safety and efficacy of tumor-targeted interleukin 12 gene therapy in treated and non-treated, metastatic lesions**. *Curr Gene Ther* 2015, **15**:44–54.
17. Cutrera J, Dibra D, Satelli A, Xia X, Li S: **Intricacies for posttranslational tumor-targeted cytokine gene therapy**. *Mediators Inflamm* 2013, **2013**:378971.
18. Satelli A, Mitra A, Brownlee Z, Xia X, Bellister S, Overman MJ, Kopetz S, Ellis LM, Meng QH, Li S: **Epithelial-mesenchymal transitioned circulating tumor cells capture for detecting tumor progression**. *Clin Cancer Res* 2015, **21**:899–906.
19. Zhao Q, Hu J, Mitra A, Cutrera J, Zhang W, Zhang Z, Yan J, Xia X, Mahadeo KM, Livingston JA, et al: **Tumor-targeted IL-12 combined with tumor resection yields a survival-favorable immune profile**. *J Immunother Cancer* 2019, **7**:154.
20. Cohen J: **IL-12 deaths: explanation and a puzzle**. *Science* 1995, **270**:908.
21. Yeku OO, Purdon TJ, Koneru M, Spriggs D, Brentjens RJ: **Armored CAR T cells enhance antitumor efficacy and overcome the tumor microenvironment**. *Sci Rep* 2017, **7**:10541.
22. Zhang L, Morgan RA, Beane JD, Zheng Z, Dudley ME, Kassim SH, Nahvi AV, Ngo LT, Sherry RM, Phan GQ, et al: **Tumor-infiltrating lymphocytes genetically engineered with an inducible gene encoding interleukin-12 for the immunotherapy of metastatic melanoma**. *Clin Cancer Res* 2015, **21**:2278–2288.
23. Satelli A, Batth I, Brownlee Z, Mitra A, Zhou S, Noh H, Rojas CR, Li H, Meng QH, Li S: **EMT circulating tumor cells detected by cell-surface vimentin are associated with prostate cancer progression**. *Oncotarget* 2017, **8**:49329–49337.
24. Li H, Meng QH, Noh H, Somaiah N, Torres KE, Xia X, Batth IS, Joseph CP, Liu M, Wang R, Li S: **Cell-surface vimentin-positive macrophage-like circulating tumor cells as a novel biomarker of metastatic gastrointestinal stromal tumors**. *Oncoimmunology* 2018, **7**:e1420450.
25. Zhou Y, Yang D, Yang Q, Lv X, Huang W, Zhou Z, Wang Y, Zhang Z, Yuan T, Ding X, et al: **Single-cell RNA landscape of intratumoral heterogeneity and immunosuppressive microenvironment in advanced osteosarcoma**. *Nat Commun* 2020, **11**:6322.
26. Li W, Zhang X, Xi X, Li Y, Quan H, Liu S, Wu L, Wu P, Lan W, Shao Y, et al: **PLK2 modulation of enriched TAp73 affects osteogenic differentiation and prognosis in human osteosarcoma**. *Cancer Med* 2020, **9**:4371–4385.
27. Huang W, Yang S, Shao J, Li YP: **Signaling and transcriptional regulation in osteoblast commitment and differentiation**. *Front Biosci* 2007, **12**:3068–3092.
28. Zhang BL, Qin DY, Mo ZM, Li Y, Wei W, Wang YS, Wang W, Wei YQ: **Hurdles of CAR-T cell-based cancer immunotherapy directed against solid tumors**. *Sci China Life Sci* 2016, **59**:340–348.

29. Beatty GL, Moon EK: **Chimeric antigen receptor T cells are vulnerable to immunosuppressive mechanisms present within the tumor microenvironment.** *Oncoimmunology* 2014, **3**:e970027.
30. Mohammed S, Sukumaran S, Bajgain P, Watanabe N, Heslop HE, Rooney CM, Brenner MK, Fisher WE, Leen AM, Vera JF: **Improving Chimeric Antigen Receptor-Modified T Cell Function by Reversing the Immunosuppressive Tumor Microenvironment of Pancreatic Cancer.** *Mol Ther* 2017, **25**:249–258.
31. Ma S, Li X, Wang X, Cheng L, Li Z, Zhang C, Ye Z, Qian Q: **Current Progress in CAR-T Cell Therapy for Solid Tumors.** *Int J Biol Sci* 2019, **15**:2548–2560.
32. Pegram HJ, Lee JC, Hayman EG, Imperato GH, Tedder TF, Sadelain M, Brentjens RJ: **Tumor-targeted T cells modified to secrete IL-12 eradicate systemic tumors without need for prior conditioning.** *Blood* 2012, **119**:4133–4141.
33. Sadelain M, Brentjens R, Riviere I: **The basic principles of chimeric antigen receptor design.** *Cancer Discov* 2013, **3**:388–398.
34. Hou AJ, Chen LC, Chen YY: **Navigating CAR-T cells through the solid-tumour microenvironment.** *Nat Rev Drug Discov* 2021.
35. Carpenito C, Milone MC, Hassan R, Simonet JC, Lakhal M, Suhoski MM, Varela-Rohena A, Haines KM, Heitjan DF, Albelda SM, et al: **Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains.** *Proc Natl Acad Sci U S A* 2009, **106**:3360–3365.
36. Noh H, Yan J, Hong S, Kong LY, Gabrusiewicz K, Xia X, Heimberger AB, Li S: **Discovery of cell surface vimentin targeting mAb for direct disruption of GBM tumor initiating cells.** *Oncotarget* 2016, **7**:72021–72032.
37. de The H: **Differentiation therapy revisited.** *Nat Rev Cancer* 2018, **18**:117–127.
38. Degos L, Dombret H, Chomienne C, Daniel MT, Miclea JM, Chastang C, Castaigne S, Fenaux P: **All-trans-retinoic acid as a differentiating agent in the treatment of acute promyelocytic leukemia.** *Blood* 1995, **85**:2643–2653.
39. Burnett AK, Russell NH, Hills RK, Bowen D, Kell J, Knapper S, Morgan YG, Lok J, Grech A, Jones G, et al: **Arsenic trioxide and all-trans retinoic acid treatment for acute promyelocytic leukaemia in all risk groups (AML17): results of a randomised, controlled, phase 3 trial.** *Lancet Oncol* 2015, **16**:1295–1305.
40. Hu J, Bernatchez C, Zhang L, Xia X, Kleinerman ES, Hung MC, Hwu P, Li S: **Induction of NKG2D Ligands on Solid Tumors Requires Tumor-Specific CD8(+) T Cells and Histone Acetyltransferases.** *Cancer Immunol Res* 2017, **5**:300–311.
41. Hao Y, Hao S, Andersen-Nissen E, Mauck WM, 3rd, Zheng S, Butler A, Lee MJ, Wilk AJ, Darby C, Zager M, et al: **Integrated analysis of multimodal single-cell data.** *Cell* 2021, **184**:3573–3587 e3529.

Figures

Figure 1 The function of authored-attIL12 PBMCs *in vitro* and *in vivo*

Figure 1

The function of authored-aTTIL12 PBMCs *in vitro* and *in vivo*;

(A)Schematic showing aTTIL12 construct; (B) The simple production process of aTTIL12-PBMCs, and aTTIL12 expression after lentiviral transduction of human PBMCs demonstrated by flow cytometer and representative immunofluorescence.(C) IFN γ and IL12 levels in medium supernatant were tested by ELISA . The bar show the levels of the cytokines (pg/million cells) as median ($\pm$ SEM) (n =3). The results represent 3 repeated experiments. *P < 0.05; (D) OS1 tumor growth curve. Tumor size was measured twice weekly and recorded. (E)It showed tumor picture on 12 week time point.

Figure 2 PBMCs derived from different donors showed similar efficacy across tumor and model types when armed with attIL12

Figure 2

PBMCs derived from different donors showed similar efficacy across tumor and model types when armed with aTTIL12;

(A) aTTIL12 expression after lentiviral transduction of PBMCs demonstrated by flow cytometer from 3 different donors; (B-C) OS2 tumor growth curve and survival curve; (D-E) OS17 tumor growth curve and survival curve; (F) Schematic diagram of OSD cells lung metastases model; (G) OSD cells lung metastases mice survival curve; (H) Lung metastases lesion H&E staining.

Figure 3 Safety validation of attIL12 modified PBMCs in PDX model.

Figure 3

Safety validation of aTTIL12 modified PBMCs in PDX model;

(A)Schematic diagram of toxicity detection in PDX OS1 model; (B) Organs were collected at day 5 after indicated treatments, fixed in 10% formalin, and subjected to H&E staining. Inflammatory cells were detected in major organs; the results represent 3 repeated experiments; *P < 0.05; (C) Organs were

collected 5 days after the PBMCs treatment from mice. Lysis of organs was tested for cytokines, such as IFN γ , TNF α and IL12; the bar show the concentration of each cytokine as the median ($\pm$ SEM) (n=3); the results represent 3 repeated experiments; *P < 0.05; (D) Serum was collected at day 5 after treatment from mice for cytokines testing. The bar show the concentration of each cytokine as the median (pg/ml serum) ($\pm$ SEM) (n=3-4); the results represent 3 repeated experiments; *P < 0.05; (E) Tumor was collected at day 5 after treatment from mice for cytokines testing. The bar show the concentration of each cytokine as the median (pg/g lysis tumor) ($\pm$ SEM) (n=3-4); the results represent 3 repeated experiments; *P < 0.05; (F) Serum was collected at day 5 from mice. Blood chemistry testing, including albumin, AP, ALT, AST, BUN, creatinine, globulin and total proteins, was conducted in MD Anderson's clinical pathology core facility. The bar show the concentration of each chemical as the median ($\pm$ SEM) (n=3-4); the results represent 3 repeated experiments; *P < 0.05.

Figure 4 attIL12 promote the tumor-targeted migration of PBMCs *in vitro* and *in vivo*

Figure 4

ATTIL12 promotes the tumor-targeted migration of PBMCs *in vitro* and *in vivo*;

(A-B) ATTIL12-PBMCs (1.5×10^6 ; labeled with Calcein Green AM [ThermoFisher]) and control-PBMCs (1.5×10^6 ; labeled with CMTPIX red cell tracker [ThermoFisher]) were mixed at a ratio of 1:1 and loaded onto vimentin-coated microfluidic slide chips and washed using a Cytoquest microfluidics pump. The slide

chips were then imaged on a fluorescent microscope. Bar graphs show the ratios of green/red fluorescent cells as the median ($\pm$ SEM) per low-power field (LPF) before and after washing the chips. * $P < 0.05$. (C) CSV expression was detected in the osteosarcoma cell lines by cytometer flow; (D) ATTL12-PBMCs (1.5×10^6 ; labeled with Calcein Green AM [ThermoFisher]) and control-PBMCs (1.5×10^6 ; labeled with CMTPIX red cell tracker [ThermoFisher]) were mixed at a ratio of 1:1 and loaded onto OS cell line-coated microfluidic slide chips and washed using a Cytoquest microfluidics pump. The slide chips were then imaged on a fluorescent microscope. Bar graphs show the ratios of green/red fluorescent cells as the median ($\pm$ SEM) per low-power field (LPF) before and after washing the chips. * $P < 0.05$; (E) Representative images of organs and tumor sections at day 5 after treatments. Sections were stained with anti-human CD45 antibody to assess human PBMCs infiltration into the lungs. 20 $\times$ scale bar: 100 μ m. 40 $\times$ scale bar: 50 μ m. Bar graph shows the median ($\pm$ SEM) PBMCs numbers in organ and tumor per high-power field. * $P < 0.05$ (F) OS1 tumors were dissociated and stained with anti-human CD45 for flow cytometer. The graph show the frequency of CD45+ cells in tumors (n = 3). * $P < 0.05$.

Figure 5 attIL12 PBMCs induces terminal differentiation from PDX OS cells

Figure 5

ATTIL12 PBMCs induces terminal differentiation from PDX OS Cell;

(A) OSD cells were labeled with GFP and cocultured with Ctrl PBMCs or ATTIL12 PBMCs at E(PBMCs):T(tumor cells) = 1:5 for 48 hours. The residual cells were identified using flow cytometer. Tumor cells were GFP positive, and PBMCs cells were hCD45 positive. Bar graphs show the frequencies of residual tumor cells as the median ($\pm$ SEM) (n=3); (B) Representative H&E-stained sections of OS1, OS2 and OS17 from indicated mice groups (200 $\times$ magnification; 400 $\times$ magnification). And Histomorphometric analysis of osteoblast terminal differentiation areas in the tumor of indicated groups. Mean $\pm$ SEM; *, P < 0.05, and N.S. indicates no significance by unpaired t test. (C) The Un-form manifold approximation and projection (UMAP) plot of the 16 identified main cell types in OS1 tumor single cell sequencing data from ATTIL12 and Ctrl groups. (D) Dot plots showing the 30 signature gene expressions across the 16 cellular clusters. The size of dots represents the proportion of cells expressing the particular marker, and the spectrum of color indicates the mean expression levels of the markers. (E) Relative percentage of each cell cluster in ATTIL12 and Ctrl group. Bar graphs show the median ($\pm$ SEM) percentage (n = 3).*, P < 0.05, indicates significant by unpaired t test.(F) Volcano Plot indicated up-regulated and down-regulated genes between Cluster7 and other clusters. (G-H) Immunohistochemical (IHC) staining of SPP1 and BGLAP expression in representative samples of OS1, OS2 and OS17 of were shown(n $\geq$ 5) (200 $\times$ magnification); Mean $\pm$ SEM; *, P < 0.05, and N.S. indicates no significant by unpaired t test.

Figure 6 IFN γ promotes OS1 and OS2 cells terminal differentiation

Figure 6

IFN γ promotes OS1 and OS2 cells terminal differentiation;

(A)KEGG pathway analysis based differentially expressed genes between Cluster7 and other clusters. (B-C) IFN γ and CD45 double positive cells were detected from dissociated OS1 and OS2 tumor by flow cytometer. Bar graphs show the median ($\pm$ SEM) percentage (n = 3).*, P < 0.05, indicates significant by

unpaired t test; (D-E) Representative images of OS1 or OS2 cells after treated with Ctrl-PBMCs, ATTIL12-PBMCs, ATTIL12-PBMCs plus neutralizing anti-IFN γ antibody (10 μ g/mL) for 6 days. Cells (400 $\times$ magnification) were stained with anti-human SPP1 and BGLAP antibodies to determine terminal differentiation tumor cells. Bar graphs show the median ($\pm$ SEM) percentage (n = 3).*, P < 0.05, indicates significant by unpaired t test.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryFigure1.tif](#)