CD138-directed adoptive immunotherapy of chimeric antigen receptor (CAR)-modified T cells for multiple myeloma

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Abstract
Adoptive immunotherapy with T cells expressing a tumor-associated chimeric antigen receptor (CAR) provides a promising approach for tumor therapy. We designed a clinical trial for multiple myeloma (MM) treatment with CAR-modified T cells recognizing CD138 (CART-138). Five patients diagnosed with chemotherapy-refractory MM were enrolled into this trial, although one later advanced to plasma cell leukemia. By intravenous infusions, these patients received CD3\textsuperscript{+} CART-138 cells in an escalating dose. No intolerable toxicity was observed during this process. CART-138 cells were expanded to a level 1000 times higher than the initial engraftment level and were maintained in the peripheral blood. In addition, increased CART-138 cells were also detected in the bone marrow. Four of the five patients had stable disease (SD) longer than three months, and one patient with advanced plasma cell leukemia had a reduction of the myeloma cells in her peripheral blood (from 10.5\% to <3\%). This study suggests that the treatment of CART-138 is safe, feasible, and tolerable and has potential antitumor activity in vivo, warranting further research in MM treatment using CART-138.

Keywords: Chimeric antigen receptor-modified T cells; Multiple myeloma; CD138

1. Introduction
Multiple myeloma (MM) is a fatal plasma cell neoplasm affecting one to five per 100,000 individuals and constitutes the second most common hematological malignancy [1]. Despite the application of auto/allo-hematopoietic stem cell transplantation and the development of many new agents, such as bortezomib, thalidomide, and lenalidomide, MM yet currently remains incurable. The vast majority of MM patients continue to suffer from relapses that are refractory to chemotherapy based on immunomodulatory drugs (IMiD) and proteasome inhibitors. The prognosis of such patients is very poor, with a median overall survival of nine months and an event-free survival of just five months [2]. Therefore, it is urgent to find novel approaches for patients with chemotherapy-resistant and advanced MM.

Ongoing studies with monoclonal antibodies could potentially expand the armamentarium against myeloma and could foster significant advances in the field. CD138 is highly expressed on MM cells and is involved in their development and/or proliferation [3], making CD138 an attractive therapeutic target [4–8]; however, the clinical benefit of anti-CD138 therapy without immunonjugate is very limited. Clinical trial reports of labeled radioimmunotherapies, such as iodine-131 anti-CD138 monoclonal antibody (mAb), have suggested that CD138-directed cytotoxicity therapy may be a
clinically effective approach and is at least an alternative therapeutic strategy, especially for those patients refractory to chemotherapy and with progressive MM [9].

The adoptive transfer of genetically engineered immune effector cells aims to rapidly establish T cell-mediated tumor immunity. In this approach, the patient’s own T cells are targeted to tumor cells through a transgene-encoded antigen receptor consisting of either chains or a chimeric antigen receptor (CAR) [10]. When expressed in T cells, CARs efficiently redirect T cell specificity and cytotoxicity to cells expressing the targeted antigen in an HLA-independent manner. The CARs used in these experiments contained T cell activation domains from molecules such as CD3ζ and a variety of costimulatory domains, such as those from CD28 and 4-1BB. Recently accumulated clinical trial data have demonstrated that genetically modified T cells with CAR against CD19 or CD20 provide promising therapeutic strategies for leukemias and lymphomas [11–14]. These reports prompted us to initiate a pilot clinical trial on the treatment with autologous T cells expressing an anti-CD138 chimeric antigen receptor (CART138) for MM patients. This study is registered at clinicaltrials.gov as #NCT01886976. To date, five patients have been treated. Here, we report the feasibility and clinical response of in vivo T cell treatment with CD138-directed CAR in MM patients.

2. Materials and methods

2.1. Patient

Five patients were enrolled in this study (Table 1), the first patient was enrolled into our clinical trial in July 2013 and the last patient was enrolled in January 2014. The primary endpoint was the time that the patients received complete response or withdrew from the trial. All of these patients were diagnosed as refractory and developed relapsed MM; one patient was first diagnosed with plasma cell leukemia because of the percentage of myeloma cells in the blood was above 10.5%. Before enrollment into our protocol, each patient had received 5–18 standard chemotherapy, and one had been treated with auto-PBSCT. We defined refractory as a 1-month progression after the end of the most recent chemotherapy. Before treatment, flow cytometry showed bone marrow aspirates expressing a monoclonal phenotype and CD138 in all five cases. Large numbers of cells with strong staining for CD138 were detected in the bone marrow biopsy tissue by immunohistochemistry before treatment. The clinical efficacy was evaluated according to the international myeloma working group (IMWG) (2009) [15]. Briefly, SD refers to stable disease that is described by providing the time to progression estimates. PD is progressive disease in which patients with a twenty-five percent increase of any of the response indicators (M-protein, FLC, and bone marrow plasma cell percentages) concurrently have new bone lesions or soft tissue plasmacytomas or increase in the size of pre-existing tumor.

2.2. Study design

The trial (Clinical Trials number, NCT01886976) was designed to assess the safety and efficacy of infusing autologous CART138 T cells in patients with relapsed or refractory multiple myeloma. The trial was approved by the institutional review board at Chinese PLA General Hospital. No commercial sponsor was involved in the study.

2.3. Vector production

The initial DNA sequence of the scFv domain targeting the CD138 antigen was derived from NK-92-scFv (CD138)-ζ, which had been identified to possess both ex vivo and in vivo anti-myeloma effects when expressed on nature killer cells [16]. According to the gene sequence in GenBank for the fusion gene, anti-CD138-ScFV-CD8ζ-CD137-CD3ζ was designed, synthesized, and inserted into the lentivirus packaging plasmid vector pWPT (Fig. 1A). Then, the fusion gene, together with plasmid psPAX2 and pMD2g transfected 293T cells, were cultured with high glucose DMEM, including 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. The supernatant was collected, and the lentivirus was concentrated in DMEM without FBS.

2.4. Generation and expansion of CAR T cells

PB was collected in cell preparation tubes (BD Biosciences), and PBMCs were isolated through Ficoll-Hypaque density gradient centrifugation. Next, the PBMCs were washed three times and cultured in a 25 cm² flask that had been coated with 5 μg/ml anti-human CD3 monoclonal antibody (Takara, Japan) at 4 °C overnight for 2 × 10⁶ cells/ml final concentration with GT-T551 medium (Takara, Japan)

Table 1
Baseline demographic and clinical patient characteristics.

<table>
<thead>
<tr>
<th>UPN</th>
<th>Age, y</th>
<th>Gender</th>
<th>Diagnosis/stage</th>
<th>The no. of prior therapies</th>
<th>Disease status at study entry</th>
<th>Conditioning regimen before T-cell infusions</th>
<th>Times of infusion of CIK before CART-138</th>
<th>Response and time since treatment (mon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>57</td>
<td>Female</td>
<td>MM (IgG-LAM, IIIB)</td>
<td>15</td>
<td>PD</td>
<td>PCD</td>
<td>2</td>
<td>SD (3)</td>
</tr>
<tr>
<td>2</td>
<td>62</td>
<td>Male</td>
<td>MM (IgA-LAM, IIIA)</td>
<td>8</td>
<td>PD</td>
<td>CP</td>
<td>4</td>
<td>SD (3), PD</td>
</tr>
<tr>
<td>3</td>
<td>55</td>
<td>Female</td>
<td>MM (IgA-KAP, IIIA)</td>
<td>18</td>
<td>PD</td>
<td>CP</td>
<td>2</td>
<td>SD (7)</td>
</tr>
<tr>
<td>4</td>
<td>68</td>
<td>Female</td>
<td>MM (IgA-LAM, IIIB)</td>
<td>5</td>
<td>PD</td>
<td>CP</td>
<td>3</td>
<td>PD</td>
</tr>
<tr>
<td>5</td>
<td>48</td>
<td>Female</td>
<td>MM (KAP, IIIA)</td>
<td>7 + auto-PBSCT</td>
<td>PD</td>
<td>VAD</td>
<td>3</td>
<td>SD (6)</td>
</tr>
</tbody>
</table>

SD is Stable disease, PD is Progressive disease.
containing the patient serum. Moreover, 1000 μ/ml recombi-
nation human IFN-γ (rhIL-2; Peprotech) and 1000 μ/ml recombi-
nation human IL-2 (rhIL-2; Peprotech) were added on
day 0 of culture. On day 2 of culture, the lentivirus plus 6 μg/ 
ml protamine sulfate salt from salmon were added into the
culture flask, and most of the cells adhered to flask. The cell
culturing supernatant was refreshed with IFN-γ and IL-2 after
12 h. The second lentivirus transfection was performed on day
3 of culture. In addition, all of the cells were moved into a new
flask without precoated with GT-T551 medium supplement
IL-2. Fresh GT-T551 medium and IL-2 were added every three
days. The cytokine-induced killer (CIK) cells were harvested
after approximately 11 days of culturing (Fig. 1B).

2.5. Cytotoxicity assays

Cytolytic activity of CART138 to HL-60 or HL-60/CD138
(HL-60 cells transfected to express CD138) was detected
using the standard 6-h carboxyfluorescein succinimidyl ester
(CSFE) cytotoxicity test [17]. Briefly, HL-60 or HL-60/CD138

![Fig. 1. Generation and expansion of CART-138 cells (A) Schematic representation of the αCD138-8a-137-ζ chimeric T-cell receptor cDNA plasmid, not to scale. (B) The median expansion (-fold) of control (NT; not transfected) and CART-138 cells generated from all patients after being cultured for approximately 11 days. Means and SDs are shown for seven different T-cell lines. (C) Phenotypic analyses of PBMCs, NT and CART-138 cells. Phenotypic comparisons were performed in samples from seven patients, and the results are expressed as the means ± SD. (D) Cytotoxic activity of control NT and CART138 cells obtained from three patients (NPN 1, 3, and 5) using the HL-60 cell line (acute myeloid leukemia, CD138-). Cytotoxic activity was evaluated in a 6-h CFSE-staining assay, and the results are shown as effector:target (E:T) ratios of 5:1, 10:1, 20:1, and 40:1. The data are represented as the means of triplicate values, and the error bars represent SEM. (E) The verified percentage of CART-138 in the NT cells by FACS (left). The CART cells are shown in the optical microscope (middle), and the verified cells were detected by fluorescence in situ hybridization (FISH) (right).]
was labeled with CFSE, and CART138 were subsequently incubated with CFSE-labeled target cells for 6 h at the ratios of 40:1, 20:1, 20:1, 5:1, and 1:1. The cells were then collected and further stained with 7-aminoactinomycin D (7-AAD); 7-AAD-positive cells were identified as dead cells.

2.6. Real-time qPCR

The PBMCs collected serially after T-cell infusions were isolated by Ficoll density gradient centrifugation, and genomic DNA was extracted using a QIAamp DNA Blood Mini Kit (Qiagen). The standard consisted of 10-fold serial dilutions of purified scFvFc: plasmid DNA starting at 10^4 copies/UL, with each sample containing 1 μg of preinfusion PBMC DNA to control for background signals. The negative control was preinfusion PBMC genomic DNA. A 153-bp (base pair) fragment containing portions of the CD8a chain and adjacent CD137 chain was amplified using forward primer 5'-GATCTCTCTCTGCACCTTGT-3' and reverse primer 5'-TCTTCTCTTCGGAATCGCAG-3'. The beta-actin gene was used as an internal control. Quantitative real-time PCR was performed in triplicate with 1 μg DNA in each reaction, using SYBR qPCR Mix in a 7900HT Sequence Detection System (all Applied Biosystems).

2.7. Statistics

The results are shown as the means ± standard error of the mean (SEM). Data were plotted using GraphPad Prism version 5.0. Two way analysis of variance (ANOVA) was used to determine the significance of the differences between the means in all experiments. A P value <0.05 was considered to be statistically significant.

3. Results

3.1. Characterization and specific cytotoxicity in vitro of patient-derived CART-138 cells

After 10- to 12-days of culture, the total numbers of cultured cells reached to a 26- to 84-fold expansion and were collected for infusions (Fig. 1B). Generally, the time needed to achieve a clinical dose of CART-138 cells was 11 days. We detected the subsets of cultured cells using FACS. As shown in Fig. 1C, a mean of 97.08 ± 6.09% of the infused cells were CD3+ cells and were principally composed by the CD8+ subset (average 71.89%), and a mean of 31.89% (range 11.73-41.33%) of the cells expressed CAR. We also defined cytotoxicity of CART-138 cells against target cells. CART-138 cells and target cells were incubated at different E/T ratios. We found that the cytolytic activity of CART-138 cells against HL-60/CD138 was markedly increased compared with that against HL-60 (Fig. 1D).

3.2. Cell infusions

PB (fifty-ml) was collected to generating CIK cells with the transduction of a chimeric antigen receptor recognizing CD138. A split-dose cell infusion schedule was used to address potential safety concerns related to the evaluation of a previously untested CAR molecule. This protocol represents a standard phase one dose-escalation trial with 3—6 planned CD3+ T-cell doses, average of 0.756 × 10^7 CIK cells per kilogram (the average percentage of verified CART-138 cells in CD3+ cells was 32%) (Fig. 1E, Table 3). Treatment was tolerated during the immediate process of CAR cell infusion, including provisions for patients who developed a transient infusion-associated chill and fever, from which the patient automatically recovered overnight. The general clinicopathological characteristics, pre-conditioning regimens, and corresponding clinical responses of patients prior to CART-138 infusions are summarized in Tables 1 and 2.

3.3. CART-138 cells in peripheral blood and bone marrow

We measured the copy numbers of the CAR molecule to determine the persistence of CART-138 cells in the peripheral blood at serial time points (Fig. 2A). A high copy number of the CAR gene (>1000/gDNA) was persistently maintained for at least four weeks in all patients. Among these patients, UPN2 had the highest number of copies of the CAR gene, which was continuously sustained for more than five months. Based on Q-PCR assays, high copy numbers of the CAR gene were also detected within one week after infusion. The peak level of the cells was noted approximately by day 10 after infusion in UPN1, 3, and 4; interestingly, there was a delayed peak in UPN2 eight weeks after infusion (Fig. 2B). However, a comparably delayed level was detected in the bone marrow, which peaked six weeks or later.

Table 2

<table>
<thead>
<tr>
<th>UPN</th>
<th>Adverse events</th>
<th>Grade</th>
<th>Time of occurrence</th>
<th>Description</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fever</td>
<td>3</td>
<td>1 h after infusion</td>
<td>With chills, the peak temperature was 39.2 °C</td>
<td>30 min</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Fever</td>
<td>3</td>
<td>4 h after infusion</td>
<td>With chills, nausea, the peak temperature was 39.5 °C</td>
<td>90 min</td>
</tr>
<tr>
<td>4</td>
<td>Fever</td>
<td>3</td>
<td>2 h after infusion</td>
<td>With chills, nausea, the peak temperature was 39.5 °C</td>
<td>50 min</td>
</tr>
<tr>
<td>5</td>
<td>Fever</td>
<td>3</td>
<td>40 min after infusion</td>
<td>With chills, nausea, the peak temperature was 39.6 °C</td>
<td>2 h</td>
</tr>
</tbody>
</table>

The maximum grade experienced for the corresponding toxicity for a given patient.
3.4. Toxicity of the CART-138 cells

No intolerable toxicities were observed in this process. Only fever due to grade 3 toxicities appeared (Table 2). The patients had infusion fevers after the second T-cell infusion; the peak was 39.6 centigrade, which might have evolved into the macrophage activation syndrome. Although there was no direct evidence of tumor lysis syndrome, UPN1, UPN2, and UPN4 showed the development of grade 2 nausea and vomiting on days 13–to–18 with elevated CART-138 in the peripheral blood. In addition, UPN1 had a mildly elevated aspartate aminotransferase (AST) level, indicating grade 1 adverse events. The causes were probably multifactorial but could have included tumor lysis syndrome. For three months after the infusion of CART-138 in all five patients, blood urea nitrogen (BUN) and serum creatinine (Cr) fell within normal ranges, there were no abnormalities in the serum calcium or lactate dehydrogenase levels. Non-hematologic toxicity was noted during the observation, and other adverse reactions did not occur during treatment. (Adverse events were graded according to the Common Terminology Criteria for Adverse Events, version 3.0 (http://ctep.cancer.gov/)).

3.5. Overall clinical responses to CART-138 therapy

All five patients were available to evaluate the objective clinical responses after CART-138 therapy; four experienced myeloma regression during this trial (Table 1). Within two weeks from the beginning of the cell infusion, no obvious disease progression was noted. The serum content of M proteins continued to remain stable on day 14 after cell infusion, and the stabilization spanned approximately three to four weeks (UPN2, 3, 4, 5). However, a different tendency emerged in the peripheral blood of UPN1, with the increasing levels of CART-138 during two weeks after the cell infusions; myeloma cells in the peripheral blood reduced rapidly (from 10.5% to <3%) and were sustained for 12 weeks (Fig. 3A, B, C), coinciding with the constitutional symptoms of tumor lysis exhibited from day 16–to–18. The patient’s advanced disease was successfully intervened upon, and she reentered a stable status for 12 weeks. The conditions of the myeloma remained stable in UPN3 and UPN5 for eight months without another therapy among the two patients who submitted to follow up. Myeloma of UPN2 showed a progression after five months of infusion, and the IgG levels increased in the UPN4 after one month of infusion, such that other palliative care measures were administered.

4. Discussion

We studied five patients with advanced, progressive multiple myeloma on a clinical trial of chemotherapy followed by an infusion of CART-138 cells. This approach demonstrated that autologous PBMCs from MM patients could be reliably modified to express a CD138-specific transgene-encoded antigen, expanded in vitro, and reinfused with minimal toxicity. Moreover, the advanced disease was induced to reenter into a stable state after CART-138 infusions.

The treatment regimen used in this study is generally safe and well tolerated. The process of generating therapeutic numbers of CART-138 cells by limiting their dilutions proved
to be quite difficult; however, the target cell dose was reached with a toxicity of only one or two grades in all five patients. No evidence of obvious tumor lysis syndrome or an inflammatory storm was found under normal levels of serum calcium or lactate dehydrogenase. The development of tumor lysis syndrome after cellular immunotherapy has not been reported previously [18]. CART cells are capable of generating extensive cytotoxicity in vivo caused by cytokine production, which was positively correlated with the degree of the tumor burden and the infusion dose [19]. Therefore, an adverse reaction could be provoked by administering CART cells to patients with small disease burdens. Another possible means of reducing acute toxicities after CART cell infusions is to administer multiple, smaller doses of CAR-transduced T cells rather than one large dose [20]. The burden of myeloma in this patient was not large; the cell proliferation of MM itself was slow; and the process of infusion was divided into six doses, none of which were sufficient to cause extensive damage.

The research for therapeutic choices of relapsed and refractory MM is still ongoing because there is hardly any conclusive data outlining the most appropriate way of treatments for these in previous literature. The adoptive transfer of genetically engineered immune effector cells to rapidly establish T cell-mediated tumor immunity is the available choice [21]. In this approach, the patient's own T cells are targeted to tumor cells through a transgene-encoded Ag receptor consisting of a CAR, which is composed of an extracellular Ag recognition domain, most commonly a single chain fragment variable derived from a mAb that has been fused to a transmembrane domain, and a cytoplasmic signaling domain, most commonly including that of the CD3 chain [10,21–23]. When expressed in T cells, CARs efficiently redirect T-cell specificity and cytotoxicity toward cells expressing the targeted Ag in HLA-independent manner [24,25]. Previous research has shown that genetically modified NK-92 cells expressing the CAR anti-CD138 were identified to induce an intensified anti-myeloma immune response and to reinforce the lysis and apoptosis of myeloma cells in cell lines and mice [16]. One study reported treating four patients of progressive MM with iodine-131-labeled anti-CD138 monoclonal antibody (mAb). One patient of the four received partial response, the other three patients did not achieve response, their M-protein stayed in the peak. Moreover, one patient suffered from 3/4-grade hematological toxicity for 39 days after the treatment [9]. Efficacy was noted in this study, although long-term outcomes should be followed up separately. There were clinical responses in our research showing that the advanced MM patients were returned to stable conditions after CART-138 infusions. In one patient (UPN 1), 10.5% of CD138⁺ myeloma cells were observed in peripheral blood before treatment with our protocol. After treatment, when the patient was in remission, the level tended to be 1-3%. Detailed cell surface marker analysis was performed to assess the phenotype of the expanded cells. The data indicate that CART-138 cells can specifically recognize and kill unmanipulated, autologous MM cells in vivo.

To act on and within the tumor, genetically modified T cells must home to the site of the malignancy [10]. The trafficking of modified T cells was detectable by qPCR in the bone marrow 24–to 48 h after the final infusion among

![Fig. 3. Clinical responses after CART-138 cell infusions.](image)
the patients with lymphoma treated by CD20-specific adoptive immunotherapy [11,26]. The CART-19 cells were observed at high level within five weeks after therapy in the BM of the patients with ALL and CLL [27]. In our study, a high expansion of CART-138 could be detected after eight weeks, and the cells were able to enter into the BM at a high copy number. In UPN1, the expansion of CART-138 cells in the BM increased persistently, although only until seven weeks after the infusions. Therefore, the reduction of myeloma in BM was not detected at the beginning of the infusions, and even the proportion of plasma cells was increased. Although there was no obvious decrease in the plasma cell ratio of serial BM aspirates, cytoplasmic swelling and membrane budding of these cells, but not of other cell lineages, could be wildly observed in all patients receiving CART-138 therapy, possibly implying the existence of a CART-138 triggered immune attack on CD138 + cells. For patients with the SD response, these morphological changes, possibly induced by immune attack, appeared to be more consistent and wild. There are many possible explanations for the limited therapeutic antitumor activity observed in this trial. First, the high IgG level in blood of patients could have increased the blood viscosity, thereby affecting the CART cells. Second, an ineffective blood of patients could have increased the blood viscosity, thereby affecting the CART cells. Third, the localization of the T cells to the tumor sites and inadequate transgene-encoded antigen surface expression could also affect the response of tumor cells to treatment. Third, the cytokine microenvironment in tumors may affect the function of CART [28,29].

In summary, adoptive immunotherapy with CART-138 is a safe, feasible, and well-tolerated treatment for the MM patients, and may constitute a promising strategy for the relapsed or refractory MM. The present results are encouraging but also identify areas for improvement to be addressed in future CART-138 therapy trials for MM.

Conflict of interest

The authors wish to confirm that there are no conflicts of interest associated with this publication and there has been no financial support for this work that could have influenced its outcome.

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References


