Serial assessment of circulating T lymphocyte phenotype and receptor repertoire during treatment of non-muscle invasive bladder cancer with adoptive T cell immunotherapy

Xiaoli Wang1*, Guoliang Qiao1*, Ni Jiang1*, Michael A Morse2, Xinning Zhou1, Shuo Wang1, Jiangping Wu1, Yuguang Song1, Yanjie Zhao1, Lei Zhou1, Yanhua Yuan1, Amy Hobeika2, Jun Ren1,2, Herbert Kim Lyerly2

1Department of Medical Oncology, Beijing Key Laboratory for Therapeutic Cancer Vaccines, Capital Medical University Cancer Center, Beijing Shijitan Hospital, Beijing 100038, China; 2Department of Surgery, Duke University Medical Center, Durham, NC 27710, USA. *Equal contributors.

Received November 3, 2020; Accepted February 1, 2021; Epub April 15, 2021; Published April 30, 2021

Abstract: Recurrence and progression of non-muscle-invasive bladder cancer (NMIBC), frequent despite the availability of multiple treatment modalities, may be partly explained by the presence of immunosuppressive cell populations. We hypothesized that progression of disease could be prevented by the administration of an activated T cell immunotherapy (ACT) at time points when immunosuppressive populations increased in peripheral blood. In an N-of-1 study, a patient with multiple primary bladder high grade urothelial carcinomas, previously treated with standard local resection and chemotherapy but with evidence of progression, received ACT consisting of dendritic cells mixed with cytokine induced killer cells (DC/CIK), intravenously 18 times over a 6 year period at indicated time of observed increases in peripheral blood immunosuppressive CD8+CD28- cells. Peripheral blood was analyzed for T cell phenotype by flow cytometry, T cell receptor (TCR) repertoire, and circulating tumor DNA (ctDNA) by next generation sequencing (NGS) at the time of each infusion. Cystoscopy and pelvic CT scans were performed at routine intervals to assess clinical status of disease. There has been no recurrence or metastasis of urothelial carcinoma. Peripheral blood cytotoxic T cells and unique TCR clones increased and suppressive T cell populations decreased after DC/CIK infusions evidenced by the two more proof-of-concept cases. ctDNA analysis detected mutations in six genes (ARID1B, MYCN, CDH23, SETD2, NOTCH4 and FAT1) which appeared at different times, but all of them disappeared after the DC-CIK infusions. These data suggest that DC/CIK infusions may be associated with beneficial changes in T cell phenotype, TCR repertoire, decreases in circulating tumor DNA and sustained recurrence-free survival.

Keywords: Adoptive T cell immunotherapy, bladder cancer, T cell receptor repertoire

Introduction

Non-muscle-invasive transitional cell bladder cancer (NMIBC), accounting for more than 60% of bladder cancers [1], has a high recurrence rate (50% for high grade T1 disease [2]) after transurethral resection (TURBT), despite intravesical chemotherapy or immunotherapy with bacillus Calmette-Guerin (BCG) [3]. Immune checkpoint inhibitors have demonstrated efficacy in metastatic bladder cancers and preliminary data from a study of checkpoint blockade prior to cystectomy for muscle invasive bladder cancer has demonstrated a complete response rate of 39% [4]. Studies for non-muscle invasive bladder cancer unresponsive to BCG are ongoing [5]. Salvage systemic therapies include chemotherapy, immune checkpoint inhibitors (ICIs), and the FGFR inhibitor erdafitinib for patients with FGFR alterations [6]; however, the majority of bladder cancers do not respond in part due to a “non-inflamed” tumor microenvironment [7] or the presence of immunosuppressive cell populations [8]. Indeed, we observed in other malignancies such as breast cancer, that peripheral blood CD8+CD28- T cells, a suppressive cell population, are associated with disease progression in patients treated with chemotherapy [9].

A strategy to promote an inflamed tumor microenvironment and counter suppressive T cell
Adoptive T cell immunotherapy in bladder cancer

populations is delivery of ex vivo activated T cell products (autologous adoptive T cell immunotherapy (ACT)). A common form of ACT consists of a mixture of dendritic cells (DC) and cytokine induced killer (CIK) cells. Advantages of DC-CIK are feasibility of generation, rapid expansion ex vivo, and non-MHC restricted cytotoxicity [10, 11]. We have previously demonstrated that the combination of DC-CIK infusions and S-1 chemotherapy was safe and resulted in favorable PFS and OS in both advanced pancreatic and gastric cancers [12, 13] and observed clinical benefit for DC-CIK infusions in advanced breast and non-small cell lung cancer [12-16]. Measurement of the peripheral blood TCR repertoire using high-throughput T cell receptor Vβ sequencing (HTTCR) [17-19] has led to the observation that clinical responders have a greater number of expanded unique, rather than shared, clones in the peripheral blood.

In this report, we describe a patient with multiple recurrences of NMIBC despite standard intravesical treatment and intravenous chemotherapy who then received DC-CIK immunotherapy for 18 cycles over 63 months, guided by changes in peripheral blood immune parameters. During this time period, peripheral blood samples (n=21) collected at each cycle of T cell immunotherapy were analyzed for circulating tumor DNA (ctDNA) mutations, TCR repertoire and T lymphocyte phenotype. Serial cross-sectional imaging and cystoscopy have continued to demonstrate no evidence of recurrence. For more elucidated supportive evidences, we also supplied two more cases as the proof-of-concept to measure the CD8+/CD28- cells and ctDNA mutational frequency as well.

Material and methods

Patient

The subject was a 62-year-old man who presented to the hospital on June 2013 with a one-month history of hematuria. TURBT revealed multiple lesions measuring 0.6 × 0.5 × 0.5 cm, 0.7 × 0.5 × 0.5 cm, and 1.0 × 0.5 × 0.5 cm. Pathological analysis demonstrated high-grade NMIBC, T1N0M0. The patient received the institutional standard 2-weekly pirarubicin and 6-weekly thiotepa intravesical instillations, and 3 monthly hydroxycamptothecin instillations concurrently with gemcitabine plus cisplatin intravenous chemotherapy for 5 cycles, each at 21-day intervals. Comparing the PET/CT from August 8, 2013 with that performed on June 8, 2013, we noted metabolic activity of the bladder consistent with early progression of disease. The subject declined radical cystectomy and subsequently enrolled into this N-of-1 study after providing written informed consent approved by the Institutional Review Board of Beijing Shijitan Hospital, Capital Medical University Cancer Center, Beijing, China.

Treatment plan

The DC-CIK cell product was prepared as described in our previous studies [13, 15]. For the induction of DC-CIKs, peripheral blood mononuclear cells were mobilized by G-CSF. Apheresis was performed using the COBE Spectra cell separator (COBE BCT, Lakewood, CO, USA) until CD34+ cells reached ≥ 4.5 × 10^6/kg. A portion of the apheresis product (25-50 ml) was co-cultured with IL-4, TNF-α and GM-CSF in vitro to generate autologous DCs. PBMCs were activated in vitro with IL-2, INF-gamma and anti-CD3 antibody to generate the CIK cells. After meeting lot release criteria, the cultured cells were infused intravenously over 20 minutes.

Beginning in the first month post-TURBT, the ex vivo expanded DC-CIK cells were administered intravenously (median infusion of 1.95 × 10^9 CIK cells, consisting of ~75% CD8+ T cells) for a total of 18 infusions over a 63 month period. Blood samples were collected for TCR repertoire and T cell phenotypic analysis and ctDNA mutational analysis during this time period. Peripheral blood CD8+/CD28- T cell levels were used as predictors for determining the timing of adoptive T cell infusion. We chose 24.2% of CD8+/CD28- as the threshold to determine the timing of DC-CIK infusions [20].

ctDNA mutational analysis

Next generation sequencing was performed on peripheral blood ctDNA by a commercial vendor (Geneplus-Beijing Institute, Beijing). Targeted sequencing was performed in 60 plasma ctDNA and 30 germ line DNA samples. The target region is about 1.1 Mb, which includes coding exons and selected introns of 1021 genes selected from four sources: 1) known oncogenes and tumor suppressor genes; 2) genes
that are targets of agents approved by the FDA or have been assessed in clinical trials; 3) genes implicated in major cancer-related signaling pathways; 4) genes identified in the findings of the TCGA network which covers 12 cancer types. Sequencing libraries were prepared from ctDNA using KAPA DNA Library Preparation Kits (Kapa Biosystems, Inc.), and gDNA sequencing libraries were prepared using the protocols recommended by the Illumina TruSeq DNA Library Preparation Kit. For samples close to the minimum input requirement, additional pre-capture PCR cycles were performed to generate sufficient PCR product for hybridization. Libraries were hybridized to custom-designed biotinylated oligonucleotide probes (Integrated DNA Technology, Coralville, USA) covering the target region sequence. DNA sequencing was carried out with the HiSeq3000 Sequencing System (Illumina, San Diego, CA).

Somatic SNVs and Indels were detected using the Mutect 2.0 algorithm (https://software.broadinstitute.org/gatk/gatkdocs/current/org_broadinstitute_gatk_tools_walkers_cancer_m2_MuTect2.php) Somatic copy number alterations and structure variations were analyzed using local algorithms.

T cell receptor (TCR) sequencing of peripheral blood T cells

DNA was extracted from peripheral blood T cells using a Qiagen DNA FFPE kit, DNA blood kit, or DNA blood mini kit (Qiagen). TCR Vβ CDR3 sequencing was performed using the deep (Peripheral blood mononuclear cell, PBMC) resolution Immunoseq platforms. Bioinformatic and bio-statistical analyses of productive clones were performed to assess the dynamics of T cells. The Shannon diversity index [21], TCR clonality [22] and Evenness [23] of T cells were used to evaluate the diversity of TCR CDR3 V-beta sequences.

Phenotypic analysis of peripheral blood immune cells

Whole blood (100 μl) was incubated in the dark with primary antibody at 4°C for 15 min. Anti-CD3-FITC/anti-CD56-RPE (Dako), anti-CD3-FITC (fluorescein isothiocyanate), anti-CD4-RPE, anti-CD8-RPE, anti-CD45R0, and anti-CD4-FITC/anti-CD25-PE (BD Bioscience) were used. After hemolysis for 10 minutes, samples were centrifuged for 10 min at 1,500 rpm at room temperature, and then washed twice in PBS and subjected to three-color flow cytometry to determine cell phenotypes using an FC500 (Beckman-Coulter), and CXP analysis software (Beckman-Coulter). Lymphocytes were identified on scattergrams as the low forward scatter/low side scatter population. Analyses was performed on > 5,000 gated events.

Clinical outcomes

At the time of each cycle of cell therapy, standard physical exam, pelvic CT and cystoscopy were performed to assess the status of the tumor.

Statistical analyses

Continuous variables were expressed as mean ± SD (standard deviation) and compared using a two-tailed unpaired Student’s t test. Categorical variables were compared using χ² or Fisher analysis. All statistical evaluations were carried out using SPSS software (Statistical Package for the Social Science, version 15.0, SPSS Inc, Chicago, IL) and GraphPad Prism 5 (Version 5.01, GraphPad Software, Inc., USA). A value of P<0.05 was considered to be statistically significant in all of the analyses.

Results

Clinical evaluation

During 72 months of follow-up, pelvic CT and cystoscopy have demonstrated an ongoing, complete remission (Figure 1A-F). The standard examination to exclude the distant metastasis were also performed. Neither local nor other sites were discovered for clinical metastasis.

Pathological findings of original tissue

For validation of the current therapeutic determination, we have retrospectively reviewed the original pathological findings diagnosed on June 14, 2013 with the support of pathologist. The formal report of pathologist has described that there was no PD-1 expression within lymphocytes but 2% positivity of PDL-1 TPS (tumor percentage score) Figure 1G, 1H, indicating a non-inflamed tumor microenvironment unlikely to respond to immune checkpoint inhibitor treatment regardless of immune checkpoint inhibitor availability at that moment.
Variable TCR diversity is associated with the infused T cell number

In order to analyze the changing TCR diversity of T cells associated with the DC-CIK infusions, we measured the changing trends of Shannon index, clonality and evenness (Figure 2A) and compared these data with the recorded number of T cells infused over the course of the study (Figure 2B). The number of infused cells was significantly associated with the Shannon index (Figure 2C, $r^2=0.295$, $P=0.019$) but not with clonality (Figure 2D, $r^2=0.183$, $P=0.076$) or evenness (Figure 2D, $r^2=0.130$, $P=0.142$). Considering the differences observed in T cell frequency and clonality, we further investigated the overlap in T cell clones of this patient. As shown in Figure 2E, the unique TCR clones and the shared TCR clones changed after the administration of ACT. Moreover, as shown in Figure 2F, the number of unique TCR clones was significantly associated with the infused cell numbers ($r^2=0.646$, $P=0.001$).

Phenotypic analysis of peripheral blood mononuclear cells

Phenotypic analysis of peripheral blood mononuclear cells prior to the initiation of ACT and at the end of each cycle of adoptive cell therapy.
Adoptive T cell immunotherapy in bladder cancer

was performed. CD3+ and CD8+/CD28+ T cell subsets were increased after DC-CIK cell therapy (P<0.05) (Figure 3A, 3B), while the CD4+/CD25+ and CD8+/CD28- subsets were significantly decreased after DC-CIK cell therapy (P<0.05) (Figure 3C, 3D). We correlated changes of T lymphocyte phenotypes with Shannon index and unique TCR subclones as representatives of the diversity of TCR repertoire. The percentages of CD3+ (r²=0.615, P=0.001) and

Figure 2. TCR diversity after DC-CIK therapy is associated with the infused cell number. A, B: Alteration of Shannon index and infused cell number are shown; C: The Shannon index was significantly associated with the infused cell number; D: The clonality and evenness were not significantly associated with the infused cell number; E: Changes in TCR subclones after DC-CIK therapy; F: The association of unique TCR clones with infused cell number.
CD4+/CD25+ T cells ($r^2=0.484$, $P=0.001$) were significantly associated with the Shannon index (Figure 3E); however, the percentages of CD3+/CD8+ ($r^2=0.092$, $P=0.222$) and CD8+/CD28- ($r^2=0.108$, $P=0.183$) T cells were not associated with the Shannon index (Figure 3E). The percentages of CD3+ ($r^2=0.348$, $P=0.01$), CD4+/CD25+ ($r^2=0.247$, $P=0.036$) and CD8+/CD28- ($r^2=0.384$, $P=0.006$) T cells were significantly associated with the unique TCR clones (Figure 3F); however, the percentage of CD3+CD8+ T cells ($r^2=0.022$, $P=0.554$) was not correlated.
Adoptive T cell immunotherapy in bladder cancer

Loss of mutations in ctDNA following DC-CIK infusions

We explored the changing ctDNA mutational landscape during study participation by sequencing ctDNA and gDNA from blood samples obtained at each cycle of infusion. Approximately 10 Gb and 2 Gb sequencing data were generated for each ctDNA sample and gDNA sample, respectively. The average depth of coverage was 1323-fold (706-2094) for ctDNA samples. A total of 21 blood samples, collected at an average of 2.5-month intervals, were subjected to complete ctDNA mutational status. A panel of 1021 genes was selected as the target region. Somatic SNVs and InDels were detected using the MuTect 2.0 algorithm (https://software.broadinstitute.org/gatk/gatkdcs/current/org_broadinstitute_gatk_tools_walkers_cancer_m2_MuTect2.php) Somatic copy number alterations and structural variations were analyzed using local algorithms. Six mutated genes (ARID1B, MYCN, CDH23, SETD2, NOTCH4 and FAT1) were identified in 5/21 samples (Figure 4). Mutated ARID1B and MYCN disappeared after 3 cycles of T cell infusions and remained absent for 23 months. When the level of CD8+/CD28 increased to 30.6% (above the threshold value of 24.2%), new missense mutations in SED2 and NOTCH4 were detected. The patient received two cycles of DC-CIK infusions and these mutations disappeared. Subsequently new mutations in the FAT1 gene accompanied at the value of CD8+/CD28 as 19.0 (initial 30.6 at the time of infusion) which decreased to 15.2% at then end of T cell infusion treatment (Figure 4) coincident with loss of the mutation in FAT1. A gene mutation of CDH23, rare in bladder cancer, emerged in the ctDNA when there were elevated CD4+/CD25+ (Treg) but this mutation disappeared from ctDNA after DC-CIK infusion. For the two more proof-of-concept cases, there were similar data for mutational gene disappearance and mutational frequency analysis (See Tables S1, S2). These data suggest that ctDNA mutation frequency increases with higher immunosuppressive CD8+/CD28 levels and decreases after DC-CIK infusions coincident with decreases in CD8+/CD28 and CD4+/CD25+ T cells. These data suggest that during time periods of enhanced activity of immunosuppressive T cell populations, microscopic residual disease may progress but can be controlled by ACT with DC-CIK infusions.

Discussion

Immunotherapy has provided new options for treating urothelial carcinomas but responses occur in less than one out of four patients treated with immune checkpoint blockade [24, 25], frequently due to a non-inflamed tumor microenvironment. The percentage of PD-1 and/or PD-L1 has become critical biological predictive markers to consider the prescribe of such blockers. We have retrospectively reviewed the pathological findings to determine the histopathological expression. Indeed, in the current...
Adoptive T cell immunotherapy in bladder cancer

case, no expression of PD-1 was seen intra-lymphocytes of tumor whereas PD-L1 was identified 2% positivity in tumor cells. As we might be aware of immune checkpoint inhibitor unavailability in the year of 2013. Therefore we have continued to explore the immunotherapy as we had previously reported that infusions of mixtures of dendritic cells and cytokine induced killer cells (DC-CIK) demonstrated efficacy in several solid tumor malignancies [12-16, 26].

We designed an N-of-1 study to characterize the changes in immune cell profiles of a patient with NMIBC who refused radical cystectomy and instead opted to participate in a study of immunotherapy with DC-CIK after completing standard interventions. Peripheral blood samples were serially collected during their 6 years of treatment. This allowed us to correlate immune cell alterations with standard clinical imaging results. Specifically, we had the opportunity to perform a detailed analysis of T cell phenotype and receptor repertoire from multiple samples from the same patient and studied their relationship to the number of cells infused. Simultaneously, we incorporated circulating tumor DNA mutation analysis to explore whether tumor progression would be detectable at the molecular level during times when suppressor T cell populations were highest.

A unique aspect of this case was the use of peripheral blood CD8+/CD28 - and CD4+/CD25+ cells as an indicator for timing of DC-CIK infusions. CD4+/CD25+ T cells include populations of regulatory T cells. Accumulating evidence indicates that CD8+/CD28 - T cells are associated with numerous inflammatory disorders and are found in tumor microenvironments and the circulation of cancer patients. Previously, we observed that increases in these suppressor cell types were associated with inferior outcomes in breast cancer patients and were predictive of cancer cell release into the circulation [20]. In the current study, when suppressor cell subtypes increased, circulating tumor DNA mutations (in genes with relevance for tumor aggressiveness [27-30]) appeared and after delivery of DC-CIK, these suppressor cells decreased and the mutations disappeared. We have collected two more proof-of-concept cases that are supportive of this finding. The first is a patient with T4N3M1 bladder cancer whose disease was under control for 2 years while receiving intermittent DC/CIK infusions. Interestingly, when adoptive cellular immuno-

therapy was unavailable for 6 months due to the COVID-19 pandemic, the patient had transient progression of disease. We have illustrated his serial CD8+/CD28 - levels in Figure S1. In this case the analysis of ctDNA was recorded that there were no mutations reported during the DC-CIK infusions only an EPAS1 mutation sequenced when he was in condition of discontinued immunotherapy (Table S1). The second case is a patient with advanced bladder cancer who had received the immune checkpoint inhibitor pembrolizumab (Keytruda) from Jan 2019 to May 2019 followed by adoptive T cell immunotherapy due to mild disease progression. The ctDNA summary was presented in Table S2. All of the mutations detected prior to initiating DC-CIK were cleared following therapy. Although other mutations appeared over time, they had low mutant allele fractions and disappeared with further DC-CIK infusions. Unfortunately, both of those two cases were lacking T-cell receptor immune repertoire data. Apparently these two cases demonstrated that ctDNA mutations and CD8+/CD28 - levels decrease with DC-CIK therapy which suggest that the DC-CIK were responsible for sustaining the complete remission by modulating suppressor populations and/or by direct anti-tumor effects.

Our finding that there was a significant correlation between suppressive T cell populations and TCR repertoire profiles (such as Shannon index and unique T cell clonality) (as shown in Figure 3E, 3F) supports the former mechanism, in that the effector T cell repertoire may be too limited to control tumor when there are greater levels of suppressor cell subtypes present. Further, our finding that the TCR diversity and unique TCR clones in peripheral blood after DC-CIK administration were significantly associated with the number of infused cells [26] suggests that DC-CIK can provide at least a temporary supply of additional anti-tumor T cells. This is consistent with our previous report that T cell immunotherapy is able to restore the TCR repertoire with increased unique T cell clones which could enhance the specific T cell immune response to tumor antigens.

Conclusions

This N-of-1 study supports the hypothesis that a threshold level of suppressor cells (above which new mutations in ctDNA were observed)
Adoptive T cell immunotherapy in bladder cancer

can be identified that could serve as a criterion for the timing of T cell immunotherapy; however, because this report consists of data from a single patient, a larger study would be required to conclusively determine whether infusions of T cells at routine intervals or in reaction to waning immunity (or detection of new or increasing frequency of mutations in ctDNA) would be the more effective strategy.

Acknowledgements

We would like to acknowledge the expert technical assistance of Xuefeng Xia and Xin Yi Ph.D from Geneplus-Beijing Institute, Beijing China. Supported by Key Project of Beijing Municipal Committee of Science and Technology-Capital Clinical Featured Application Funding (Z15110-0004015183); National Natural Science Foundation of China (81602528); Enhancement Funding of Laboratory of Beijing Key Laboratory for Therapeutic Cancer Vaccines (2020-JS01).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jun Ren, Capital Medical University Cancer Center, Beijing Shijitan Hospital, 10 Tieyi Rd, Beijing 100038, China. Tel: +86-10-63926317; Fax: +86-10-63926298; ORCID: 0000-0001-5399-000X; E-mail: jun.ren@duke.edu; renjun@bjsjth.cn; Dr. Herbert Kim Lyerly, Department of Surgery, Duke University Medical Center, 203 Research Drive, Suite 433, Box 2606, Duke University Medical Center, Durham, NC 27710, USA. Tel: 919-681-8350; Fax: 919-684-6408; E-mail: kim.lyerly@duke.edu

References

Adoptive T cell immunotherapy in bladder cancer


Adoptive T cell immunotherapy in bladder cancer

Table S1. ctDNA mutational frequency of the first supportive patient of proof-of-concept

<table>
<thead>
<tr>
<th>Gene</th>
<th>c.HGVS</th>
<th>p.HGVS</th>
<th>Cluster</th>
<th>Mutation Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1/17/19</td>
</tr>
<tr>
<td>NAP1L1</td>
<td>c.472dupG</td>
<td>p.D158Gfs*2</td>
<td>1</td>
<td>7.4%</td>
</tr>
<tr>
<td>TP53</td>
<td>c.359A&gt;T</td>
<td>p.K120M</td>
<td>2</td>
<td>3.8%</td>
</tr>
<tr>
<td>CTNN2A</td>
<td>c.892A&gt;T</td>
<td>p.R298W</td>
<td>3</td>
<td>2.5%</td>
</tr>
<tr>
<td>RAD51D</td>
<td>c.346-2A&gt;T</td>
<td></td>
<td>3</td>
<td>2.5%</td>
</tr>
<tr>
<td>RHOA</td>
<td>c.405G&gt;C</td>
<td>p.K135N</td>
<td>3</td>
<td>2.3%</td>
</tr>
<tr>
<td>KLB</td>
<td>c.856A&gt;T</td>
<td>p.T286S</td>
<td>3</td>
<td>2.3%</td>
</tr>
<tr>
<td>ERCC1</td>
<td>c.739A&gt;T</td>
<td>p.K247*</td>
<td>3</td>
<td>2.0%</td>
</tr>
<tr>
<td>MTO1</td>
<td>c.1246A&gt;T</td>
<td>p.T416S</td>
<td>3</td>
<td>1.9%</td>
</tr>
<tr>
<td>PTCH2</td>
<td>c.2172T&gt;A</td>
<td>p.H724Q</td>
<td>3</td>
<td>1.9%</td>
</tr>
<tr>
<td>PIK3CB</td>
<td>c.161A&gt;G</td>
<td>p.Y54C</td>
<td>3</td>
<td>1.9%</td>
</tr>
<tr>
<td>RINT1</td>
<td>c.1001A&gt;T</td>
<td>p.E334V</td>
<td>3</td>
<td>1.9%</td>
</tr>
<tr>
<td>TET2</td>
<td>c.3874A&gt;T</td>
<td>p.S1292C</td>
<td>3</td>
<td>1.8%</td>
</tr>
<tr>
<td>HIST1H1C</td>
<td>c.596A&gt;T</td>
<td>p.K199M</td>
<td>3</td>
<td>1.6%</td>
</tr>
<tr>
<td>FBXW7</td>
<td>c.1513C&gt;G</td>
<td>p.R505G</td>
<td>3</td>
<td>1.3%</td>
</tr>
<tr>
<td>JAK3</td>
<td>c.565A&gt;T</td>
<td>p.S189C</td>
<td>3</td>
<td>1.3%</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>c.382C&gt;T</td>
<td>p.R128W</td>
<td>3</td>
<td>1.2%</td>
</tr>
<tr>
<td>VEGFA</td>
<td>c.590A&gt;T</td>
<td>p.Q197L</td>
<td>3</td>
<td>0.8%</td>
</tr>
<tr>
<td>HRAS</td>
<td>c.297G&gt;C</td>
<td>p.Q99H</td>
<td>4</td>
<td>ND</td>
</tr>
<tr>
<td>TET2</td>
<td>c.5650A&gt;G</td>
<td>p.T1884A</td>
<td>5</td>
<td>ND</td>
</tr>
<tr>
<td>RPS6KB2</td>
<td>c.979T&gt;C</td>
<td>p.F327L</td>
<td>6</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table S2. ctDNA analysis and mutational frequency of the second supportive patient of proof-of-concept

<table>
<thead>
<tr>
<th>Gene</th>
<th>c.HGVS</th>
<th>p.HGVS</th>
<th>Cluster</th>
<th>Mutation Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3/15/19</td>
</tr>
<tr>
<td>EPAS1</td>
<td>c.2555G&gt;A</td>
<td>p.G852E</td>
<td>EX16</td>
<td>ND</td>
</tr>
</tbody>
</table>

Figure S1. CD8+CD28 T cell frequency at various time points.