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14. ABSTRACT

My research focuses on developing Chimeric Antigen Receptor (CAR) T cells for adoptive immunotherapy. The goal of the research is to enhance the efficacy of CAR T therapy by developing potent of CAR T cells. Another goal of this research is to shorten the CAR T manufacturing period to increase the availability of this therapy in resource constraint health care settings, as well those patients with rapidly progressive disease. CAR T cells are generated by transducing activated T cells with lentiviral vectors and expanding their progeny over 9-14 days. T cells progressively differentiate over time. Transducing quiescent T cells with CAR will preserve the intrinsic stem-like properties of naïve and memory T cells. This approach will yield CAR T cells with enhanced replicative capacity, engraftment, and in vivo activity. The purpose of this work is to determine the optimal T cell subset and corresponding optimal costimulatory domain for CAR when it is expressed in quiescent T cells. The scope of this research is that CAR T cells generated by transducing quiescent T cells will preserve the intrinsic stem-like properties of naïve and memory T cells. This approach will yield CAR T cells with enhanced replicative capacity, engraftment and in vivo activity. The goal of the study is to determine how co-stimulation in the context of a CAR, expressed in quiescent T cell subsets, influences proliferation, metabolism, and effector function in xenograft models of ALL.

15. SUBJECT TERMS

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TABLE OF CONTENTS

1. **INTRODUCTION:**

My research focuses on developing Chimeric Antigen Receptor (CAR) T cells for adoptive immunotherapy. The goal of the research is to enhance the efficacy of CAR T therapy by developing potent of CAR T cells. Another goal of this research is to shorten the CAR T manufacturing period to increase the availability of this therapy in resource constraint health care settings, as well those patients with rapidly progressive disease. CAR T cells are generated by transducing activated T cells with lentiviral vectors and expanding their progeny over 9-14 days. T cells progressively differentiate over time. Transducing quiescent T cells with CAR will preserve the intrinsic stem-like properties of naïve and memory T cells. This approach will yield CAR T cells with enhanced replicative capacity, engraftment, and in vivo activity. The purpose of this work is to determine the optimal T cell subset and corresponding optimal costimulatory domain for CAR when it is expressed in quiescent T cells. The scope of this research is that CAR T cells generated by transducing quiescent T cells will preserve the intrinsic stem-like properties of naïve and memory T cells. This approach will yield CAR T cells with enhanced replicative capacity, engraftment and in vivo activity. The goal of the study is to determine how co-stimulation in the context of a CAR, expressed in quiescent T cell subsets, influences proliferation, metabolism, and effector function in xenograft models of ALL.

2. **KEYWORDS:**

CAR T cells, adoptive immunotherapy, CART19 (CD19-specific CAR), quiescent CAR T cells.

3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**

  - CAR Lentivirus production
  - Comparing the proliferation, differentiation, metabolism, and cytolytic effector function of quiescent CD19-specific CAR T cells in vitro.
  - Identifying which costimulatory domain, is most effective in xenograft models of ALL.
  - Comparing the effects of varying CAR design in different T cell subsets
  - Identifying which quiescent CAR T cell subset, with corresponding optimal costimulatory domain, is most effective in xenograft models of ALL

- **What was accomplished under these goals?**

Specific objects:
Aim 1: CAR Lentivirus Production:
I have designed and developed a panel of second generation of CARs expressing 4-1BB, CD28, ICOS, and CD27. I also included a distinct fluorescent protein following T2A as below:

1. CART19 (bbz) T2A mCherry
2. CART19 (bbz) T2A GFP
3. CART19 (28z) T2A mCherry
4. CART19 (28z) T2A GFP
5. CART19 (z) T2A mCherry
6. CART19 (z) T2A GFP

Significant results for Aim 1:
I needed to develop a comprehensive toolbox including individual CAR-19 constructs bearing unique costimulatory domains. I chose bicistronic vectors which will allow me to identify different subsets by flow

cytometry. With regard to progress, all cloning has been completed and lentiviral supernatants have been collected and titrated. The microbeads (Dynal) coated with anti-idiotype -Fc to the FMC63 scFv present within the corresponding CD19 specific CAR is also produced.

Aim 2: Comparing the proliferation, differentiation, metabolism, and cytolytic effector function of quiescent CD19-specific CAR T cells in vitro:

In this aim, I evaluated the potency of different T cell subsets (Naïve-like T cells and Tcm) and compared them to the total T cells, when quiescent CAR-T cells are used. To evaluate the potency of these cells, I sorted T cells into different T cell subsets. Each subset was transduced separately with a CD19-specific CAR with 4-1BB co-stimulatory domain. Following transduction, T cells were cultured for 5 days in IL-7 and IL-15 to permit CAR expression and support cell viability. The transduction efficiency of individual subsets was compared (**Figure 1A**). T cells were then stimulated microbeads (Dynal) coated with anti-idiotype -Fc to the FMC63 scFv present within the corresponding CD19 specific CAR. Six days following activation, the proliferative capacity and the metabolic properties of each subset was assessed.

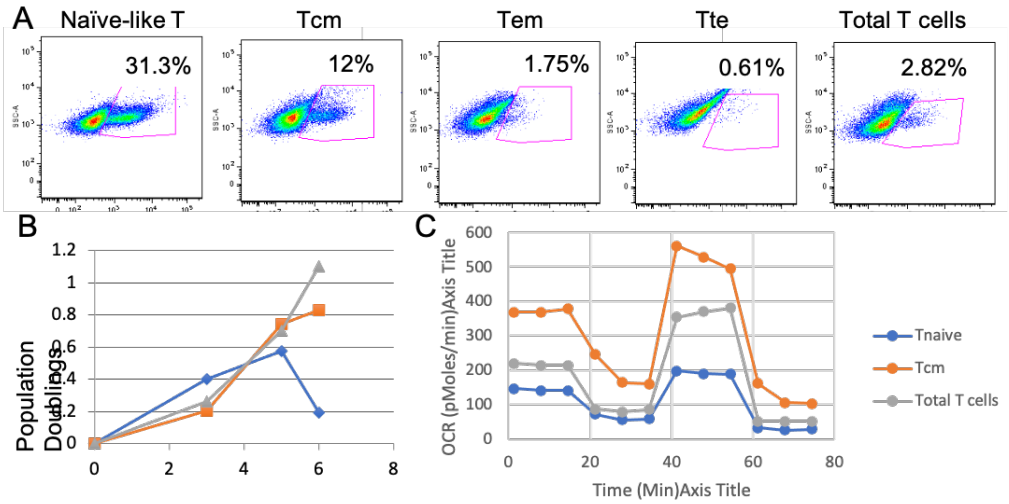

Figure 1: Phenotypic and metabolic profile of quiescent CAR T cell subsets. (A) Transduction efficiencies of non-activated CAR T cells derived from individual subsets and transduced with CAR19 T2A GFP lentivirus are shown. Representative plots from 3 individual experiments with separate donors are shown. (B) Proliferative capacity (population doublings) of individual subsets after activation with CD19 coated beads after 6 days is shown. Data is representative of 3 individual experiments with separate donors. (C) The oxygen consumption rates (OCR) of CAR T cells from individual subsets after stimulation on day 6 in culture under basal metabolic conditions and in response to mitochondrial inhibitors are shown. Data is representative of 3 individual experiments with separate donors.

Significant results for Aim 2:

As shown in **Figure 1**, naïve-like T cells had the highest expression of CAR. Using Seahorse analysis, it was revealed that Tcm cells had the highest oxidative spare respiratory capacity, SRC, compared to the other subsets. This correlates with a contingency energy source that cells can access in order to survive the energy cost of the hostile tumor environment.

Aim 3: Identifying which quiescent CAR T cell subset, with corresponding optimal costimulatory domain, is most effective in xenograft models of ALL

In this aim, I evaluated the anti-tumor potency of different T cell subsets (Naïve like T cells, Tcm, Tem) when quiescent CAR-T cells are used. To evaluate the potency of these cells in vivo, I sorted T cells into different T cell subsets. Each subset was transduced separately with a CD19-specific CAR with 4-1BB co-stimulatory domain, for 24 hours. I then transferred 0.7x106 of each subset into NSG mice bearing pre-established Nalm6 xenografts. Bulk quiescent T cells (unsorted) were used as a control as well as 0.7x106 mock transduced quiescent T cells.

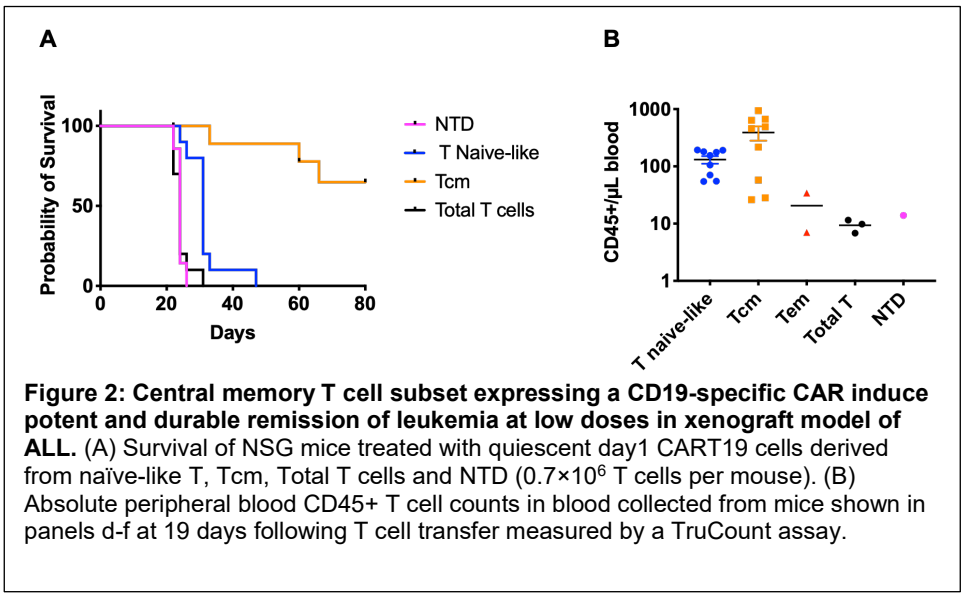

Figure 2: Central memory T cell subset expressing a CD19-specific CAR induce potent and durable remission of leukemia at low doses in xenograft model of ALL. (A) Survival of NSG mice treated with quiescent day1 CART19 cells derived from naïve-like T, Tcm, Total T cells and NTD (0.7×10^6 T cells per mouse). (B) Absolute peripheral blood CD45+ T cell counts in blood collected from mice shown in panels d-f at 19 days following T cell transfer measured by a TruCount assay.

Significant results for Aim 2:
As shown in **Figure 2a,** CAR T cell product generated from central memory T cells are the most potent in clearing tumor. Circulating CARTs are also higher when Tcm are infused relative to the other subsets (**Figure 2b**); exemplifying an important role for Tcm in persistence when nonactivated T cells are used as therapy.

Other Achievements:
The goals not met yet were reliant on the tools developed in Aim 1 and are also shaped by preliminary data generated in Aim 2. We are now set to integrate the constructs with other costimulatory domains into our experimental design and compare their efficacy, metabolic profile, and anti-tumor function.

What opportunities for training and professional development has the project provided?

I was accepted into, and attended, the "Women in 2021 Women in Cancer Immunotherapy Network (WIN)" from SITC. This was an ideal platform to network and fine-tune other professional skill development.

- **How were the results disseminated to communities of interest?**

My preliminary findings were presented in 2020 annual meeting of American Association of Blood Banks (AABB). The title of the talk was "Developing Potent CAR T Cells in Less Than 24 Hr for Adoptive Immunotherapy".

- **What do you plan to do during the next reporting period to accomplish the goals?**

Future plan of aim 2: In the next step, I will determine what is the optimal costimulatory domain for each individual subset. I will sort T cells and each T cell subset (naïve, central memory, effector memory) will be transduced CD19-specific CARs containing either 4-1BB or CD28 co-stimulatory domain. The cytolytic function of each subset with specific CAR design will be evaluated as described.

Future plan of aim 3: I have already performed a repeated in vivo experiment under condition above. This will provide results from another donor. This experiment is ongoing. In the next step, I will determine what is the optimal costimulatory domain for each individual subset. I will sort T cells and each T cell subset (naïve, central memory, effector memory) will be transduced CD19-specific CARs containing either 4-1BB or CD28 co-

stimulatory domain. The potency of each subset with specific CAR design will be evaluated in vivo by injecting limiting doses of each subsets into the Nalm6 leukemia model.

4. **IMPACT:**

   - **What was the impact on the development of the principal discipline(s) of the project?**

     Nothing to Report

   - **What was the impact on other disciplines?**

     Nothing to Report

   - **What was the impact on technology transfer?**

     Nothing to Report

   - **What was the impact on society beyond science and technology?**

     Nothing to Report

5. **CHANGES/PROBLEMS:**

   - **Changes in approach and reasons for change**

     Nothing to report

   - **Actual or anticipated problems or delays and actions or plans to resolve them**

     Last year, my lab was closed for several months due to the COVID-19 pandemic. After re-opening, we were restricted with respect to lab personnel. Additionally, our cores (animal core and flow core mainly) were partially open and also operating on a restricted schedule. This severely impeded my progress.

   - **Changes that had a significant impact on expenditures**

     Due to a University hiring freeze (which persisted into 2021) per the pandemic, I was unable to hire the technician that I had budgeted for. However, with everything moving forward and reopening, I finally identified, and hired, a suitable alternative (with a start date of August 16th, 2021).

     Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents Nothing to report

   - **Significant changes in use or care of human subjects** Nothing to report

   - **Significant changes in use or care of vertebrate animals** Nothing to report

   - **Significant changes in use of biohazards and/or select agents** Nothing to report

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**

  - **Journal publications:**

    1- Epigenetic strategies to boost CAR T cell Therapy (Accepted on July 29th, 2021, at Molecular Therapy)

    2- Ultra-Rapid Manufacturing of Chimeric Antigen Receptor (CAR) T Cells without T Cell Activation (under revision in Nature biomedical Engineering)

  - **Books or other non-periodical, one-time publications.**

    Nothing to report

  - **Other publications, conference papers, and presentations.**

    Invited talk: Developing Potent CAR T Cells in Less Than 24 Hr for Adoptive Immunotherapy. Annual meeting of AABB 2020

- **Website(s) or other Internet site(s)**

    1- Webinar invited by Nucleus Biologics: Path to Media Mastery https://nucleusbiologics.com/resources/on-demand-webinar-path-to-media-mastery/

    2- Webinar invited by Nucleus Biologics: Path to Media Mastery https://nucleusbiologics.com/resources/on-demand-webinar-path-to-media-mastery-2/

- **Technologies or techniques**
  Nothing to report

- **Inventions, patent applications, and/or licenses**
  Nothing to report

- **Other Products**
  Nothing to report

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

  ***Example**:*

Name:	Saba Ghassemi
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-2415-3576
Nearest person month worked:	4.2
Contribution to Project:	She supervised the technician in carrying out the project aims, and she write the results and progress reports.
Funding Support:	Current DOD Career Development Award

Name:	Chune Zhang
Project Role:	Research Specialist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	7.2
Contribution to Project:	She was responsible for producing lentivirus vectors and conduct the animal experiments, and carrying out the proposed research in Aims 1 and 2.
Funding Support:	Internal grant

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?** Nothing to Report

- **What other organizations were involved as partners?** Nothing to Report

8. **SPECIAL REPORTING REQUIREMENTS**

9. **APPENDICES:**

Ultra-Rapid Manufacturing of Chimeric Antigen Receptor (CAR) T Cells without T Cell Activation

Saba Ghassemi[1,2,*], Joseph S. Durgin[1], Selene Nunez-Cruz[1,2], Jai Patel[1], John Leferovich[1,2], Marilia Pinzone[2], Feng Shen[1], Katherine D. Cummins[1], Gabriela Plesa[1], Saar I. Gill[1,3], Una O'Doherty[2], Roddy S. O'Connor[1,2], Michael C. Milone[1,2,*]

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Chimeric antigen receptor (CAR) T cell therapies are able to generate deep and durable clinical responses in hematologic malignancies of the B-cell lineage. The manufacturing of these T cell-based therapies typically relies upon viral transduction of T cell-receptor (TCR) activated T cell followed by ex vivo expansion for 6 or more days prior to infusion. In addition to the required time and labor, the TCR/CD3 activation and ex vivo expansion leads to progressive differentiation of the CAR T cells with associated loss of anti-leukemic activity. Here, we demonstrate that functional CAR T cells can be generated within less than 24 hours from peripheral blood-derived T cells without the need for prior T cell activation in a process that is significantly influenced by the medium formulation and geometry of the transduction vessel. Using several CAR models, we show that T cells generated using this simple and rapid manufacturing approach exhibit superior per-cell anti-leukemic compared to CAR T cells produced in the standard protocol. These data illustrate the potential for significantly reducing the time and cost of CAR T cell production required to expand the application of this therapy to patients with rapidly progressive disease as well as resource-poor healthcare settings.

Adoptive cellular immunotherapy (ACT) using T cells that are genetically modified to express a chimeric antigen receptor (CAR) or cloned T cell receptor (TCR) yield durable clinical responses in patients with cancer.[1-6] The effectiveness of ACT led to the regulatory approval of several CD19-specific CAR T cell therapies, including tisagenlecleucel and axicabtagene ciloleucel. Both of these therapies involve the isolation of mononuclear cells containing T cells from a patient's peripheral blood, followed by T cell activation through their endogenous TCR/CD3 complex, genetic modification using a viral vector and expansion ex vivo before reinfusion. We recently showed that activated T cells undergoing rapid proliferation ex vivo differentiate toward effector cells with loss of anti-leukemic potency.[7] The ability of T cells to engraft following adoptive transfer is related to their state of differentiation with less differentiated naïve-like and central memory cells showing the greatest potency in several preclinical studies.[8-11] A number of interventions have been reported to limit the differentiation of T cells and enhance the potency of ex vivo expanded T cells such as blockade of Fas-FasL interactions,[12] inhibition of Akt signaling[13, 14] or activation of Wnt signaling.[15-17] However, eliminating the activation step and corresponding proliferative

phase of T cells ex vivo offers a far simpler and more cost-effective approach provided the barriers to gene transfer into quiescent T cells can be overcome.

Natural human immunodeficiency virus (HIV) has the ability to infect quiescent T cells in the G_0 stage of the cell cycle.[18-20] Unlike gamma-retroviruses, HIV-based lentiviral vectors can infect both dividing and non-dividing cells. However, the transduction efficiencies in quiescent T cells are typically lower than their activated counterparts. Lentiviral infection is a multi-step process involving binding of the viral particle to the T cell plasma membrane and endocytosis followed by envelope fusion, reverse transcription (RT) to form a pre-integrated DNA provirus and finally integration into the host T cell genome. Lentiviral particles that are pseudotyped with the vesicular stomatitis virus g-glycoprotein (VSV-G) to broaden the viral tropism depend upon the low-density lipoprotein receptor (LDL-R), which is ubiquitously expressed on the surface of various cells including lymphocytes, for entry.[21, 22] Limitations to efficient lentiviral transduction of quiescent T cells occur at each stage of infection. The fusion of lentiviral particles with quiescent T cells is inefficient. [23, 24] Conditioning the cell culture medium with recombinant cytokines IL-7 and IL-15 can overcome this limitation[25] and increase transduction efficiencies as well as cell survival in quiescent T cells.[26, 27] Engineering viral particles to express an IL-7 fusion protein increased quiescent T cell lentiviral transduction.[28] Post-entry, low concentrations of nucleotides and the presence of additional restriction factors such as SAMHD1 limit the rate of reverse transcription in quiescent T cells.[29-31] Collectively, these factors make lentiviral transduction of non-activated T cell inefficient.

Having previously shown that shortening the ex vivo culture of CAR T cells yields a cellular product with less differentiated T cells and significantly enhanced effector function, we hypothesized that elimination of the CD3/CD28 activation step could yield a T cell product with high functional potency. Reducing the culture duration could also substantially reduce the vein-to-vein time and significantly improve the logistics required to make CAR T cell products. Here, we have overcome some of the barriers to lentiviral transduction of non-activated T cells with a CAR, by modifying the ex vivo manufacturing protocol. This technical advance resulted in CAR T cells with potent antitumor function that are available within 24 hours of mononuclear cell collection compared with a 9-day process currently used by tisagenlecleucel. These results demonstrate the potential for vastly reducing the time, materials and labor required to generate CAR

T cells, which could be especially beneficial in patients with rapidly progressive disease and in resource-poor health care environments

Results

Transduction of non-activated T cells by lentiviral vectors

We confirmed the previously reported, low transduction efficiency of freshly isolated, quiescent T cells with VSV-G pseudotyped lentivirus vector. Primary human T cells obtained from healthy donors were mixed with an infrared red fluorescent protein (iRFP)-encoding lentiviral vector under conditions identical to activated T cells and followed for 96-hours to assess the efficiency and kinetics of transduction (**Figure 1A)**. In comparison to lentiviral transduction of T cells activated 24 hours prior with anti-CD3/28 microbeads at a multiplicity of infection (MOI) of 5 that yields > 85% transduction at 48 hours, the efficiency (**Figure 1A**) and kinetics (**Figure 1B-C**) of lentiviral transduction in non-activated T cells is substantially slower, requiring at least 72 hours to achieve detectable expression of an iRFP transgene with a transduction efficiency at 96 hours that is about 11 fold lower than activated T cells (**Figure 1A**. The slower kinetics of this process is consistent with prior observation of inefficient transduction efficiency of VSV-G pseudotyped lentivirus vector[23], and the decreased rate of reverse transcription reported for natural HIV in quiescent T cells compared with activated T cells.[39] Transduction is observed in both memory and naive subsets of CD4+ and CD8+ T cells with the highest efficiency in CD8+ cells with a memory phenotype (**Figure 1D-E)**.

We then repeated the above studies using a 3rd generation lentiviral vector encoding a CD19-specific CAR (CAR19), a cell-membrane expressed protein in contrast to the cytoplasmic nature of iRFP. CAR expression was measured by immunostaining with a monoclonal antibody recognizing the idiotype of the single chain variable fragment.[32] In a similar kinetic analysis to that performed with an iRFP-encoding lentiviral vector, we observe that non-activated T cells acquire CAR expression as early as 12-hrs with a steady increase to >80% of T cells by 96 hours (**Figure 2A**). To determine if CARs were stably expressed, the T cells were treated with either a reverse transcriptase (RT) or integrase inhibitor during the lentiviral transduction process. As shown in **Figure 2B-C**, CAR expression is unaffected by either compound in non-activated T cells in contrast with activated T cells where both RT or integrase inhibition completely abrogate CAR expression. This pseudotransduction observed in non-activated T cells is likely due to transfer of CAR protein from the lentiviral vector envelope by viral fusion to the T cell as membrane proteins expressed in

the packaging cells are well known to incorporate into HIV's envelope.[40] The absence of apparent pseudotransduction with a vector that encodes iRFP, a cytoplasmic protein, supports this envelope-mediated transfer mechanism (**Figure 2D**). Of note, the observed pseudotransduction is not exclusive to non-activated T cells. However, greater rates of pinocytosis in activated T cells may increase cell membrane turnover, and hence clearance of passively transferred proteins contributing to pseudotransduction. Importantly, CD19-specific CAR T (CART19) cells generated by lentiviral transduction of non-activated T cells in the presence of RT and integrase inhibitors show no specific cytolytic activity and cytokine production against CD19-expressing target cells (**Supplemental 1A-B)**. Based on these data, we conclude that long term persistence of CAR expression in T cells will require vector integration, which occurs at a substantially lower frequency in non-activated T cells compared with activated T cells.

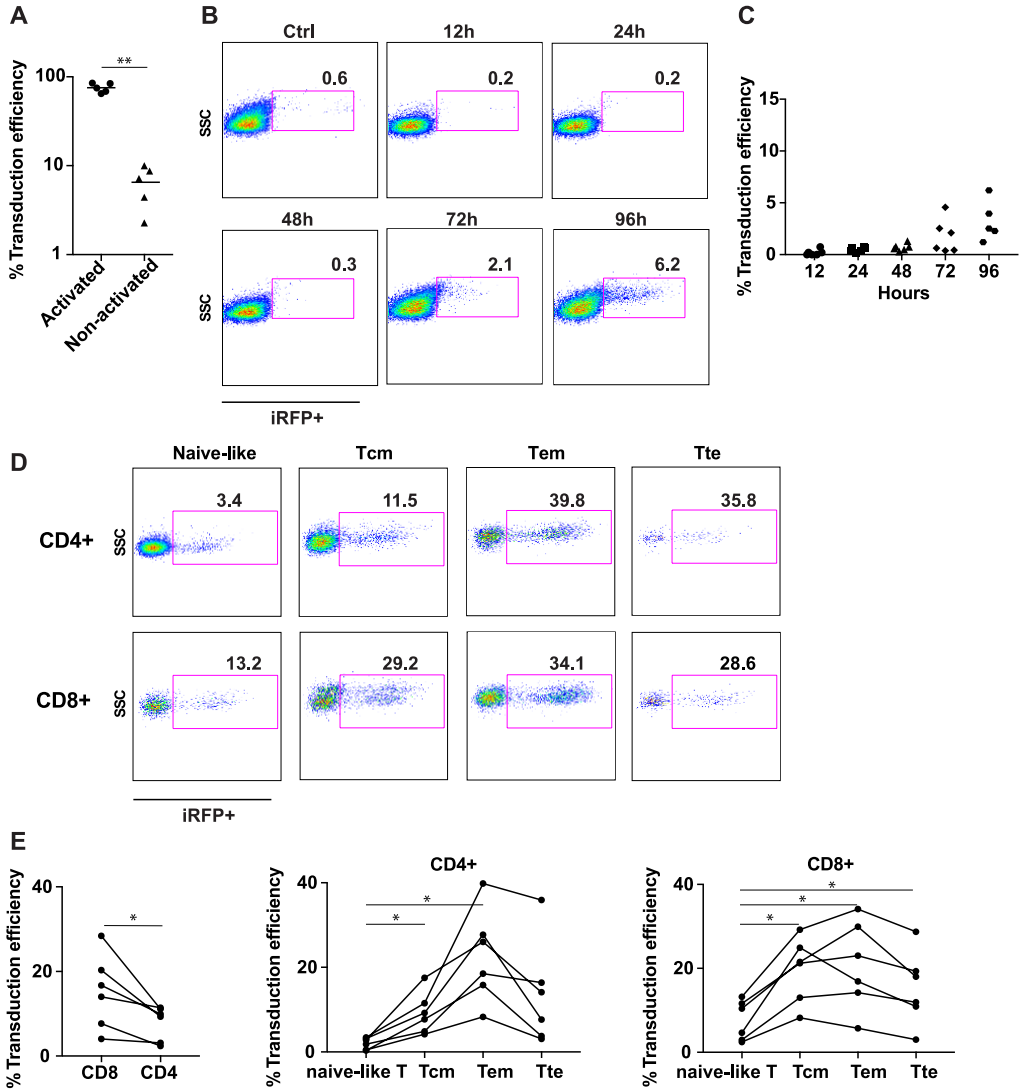

Figure 1: Lentiviral vectors transduce non-activated T cell subsets with preference for memory subsets. (**A**) Transduction efficiency of freshly isolated human T cells cultured either in IL-7 (10 ng/mL) and IL-15 (10 ng/mL) or activated with beads coated with anti-CD3/CD28 antibody, and transduced with lentiviral vector encoding iRFP for 5 days. (**B-C**) Freshly isolated human T cells cultured in IL-7 and IL-15 and transduced with lentiviral vector encoding iRFP for the indicated time interval. iRFP+ cells were quantified by flow cytometry. (**C**) Similar results were obtained in an independent experiment from six different donors. (**D**) Representative flow cytometric analysis of non-activated T cells transduced as in panel a. Naïve, central memory and effector memory T cell subsets were identified following gating on live singlets, CD3+, CD4+ or CD8+ T cells using CD45RO and CCR7 expression. (**e**) Similar results were obtained in an independent experiment from six different donors. Paired, one-way ANOVA was used, * $P < 0.05$ and ** $P < 0.01$.

108

109

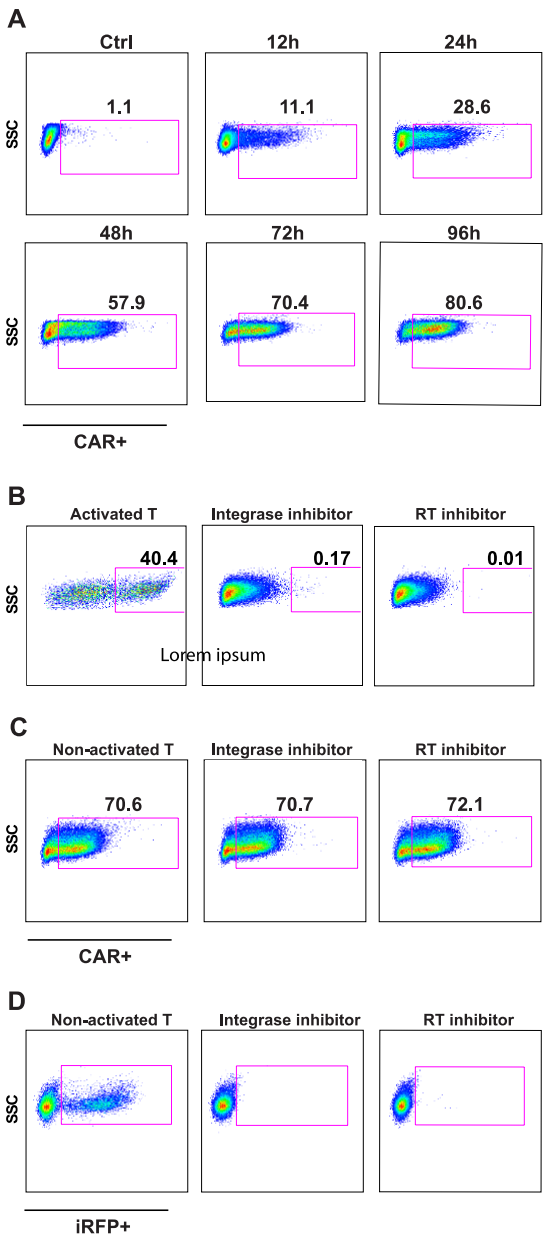

Figure 2: CAR lentivirus mediates pseudo-transduction in non-activated T cells. (**A**) Freshly isolated human T cells were cultured in IL-7 and IL-15 and transduced with lentiviral vector encoding a CD19-specific CAR. Gene transduction efficiency was measured after immunostaining with an anti-idiotype antibody for the indicated time interval. Representative flow cytometry plots of CAR expression from six separate experiments with independent donors are shown. (**B**) T cells previously stimulated with anti- CD3/CD28 microbeads and (**C**) non-activated T cells were transduced with CAR lentivirus and cocultured with an integrase inhibitor and a RT inhibitor for 4 days. CAR+ cells were quantified by flow cytometry. (**D**) Non-activated T cells were transduced with iRFP lentivirus and cocultured with an integrase inhibitor and a RT inhibitor as in panel B and C. iRFP+ cells were quantified by flow cytometry.

Although functional CAR expression was non-assessable post-transduction due to the significant pseudotransduction, we hypothesized that the transduction process would likely continue following adoptive transfer in vivo giving rise to functional CART19 cells. We therefore performed an in vivo experiment evaluating non-activated CART19 cells transduced for 24 hours (d1) as in **Figure 1** using a well-established NALM6 B-cell acute lymphoblastic leukemia mouse model.[41] As shown in **Figure 3**, a dose of $3x10^6$ non-activated CART19 cells (d1) washed and infused within 24 hours of collection was compared to CD3/CD28-activated CAR T cells that were ex vivo expanded for 9 days prior to injection, a research process comparable to that used to manufacture tisagenlecleucel, as shown schematically in Figure 3A-B. Although non-activated T cells were unable to induce a complete regression of leukemia, we show that non-activated CART19 cells controlled leukemia for an extended duration of 60 days. Given the persistence of T cells in the peripheral blood of mice (**Figure 3E-F**), our findings suggest that non-activated CAR T cells retain considerable replicative capacity to maintain function under continuous antigenic stimulation (**Figure 3D**). This contributed to a significant increase in overall survival of the leukemia-bearing mice (**Figure 3G**). These results support the functional nature of non-activated CART19 cells, and they encouraged the optimization of the transduction process to further enhance non-activated CART19 activity.

125

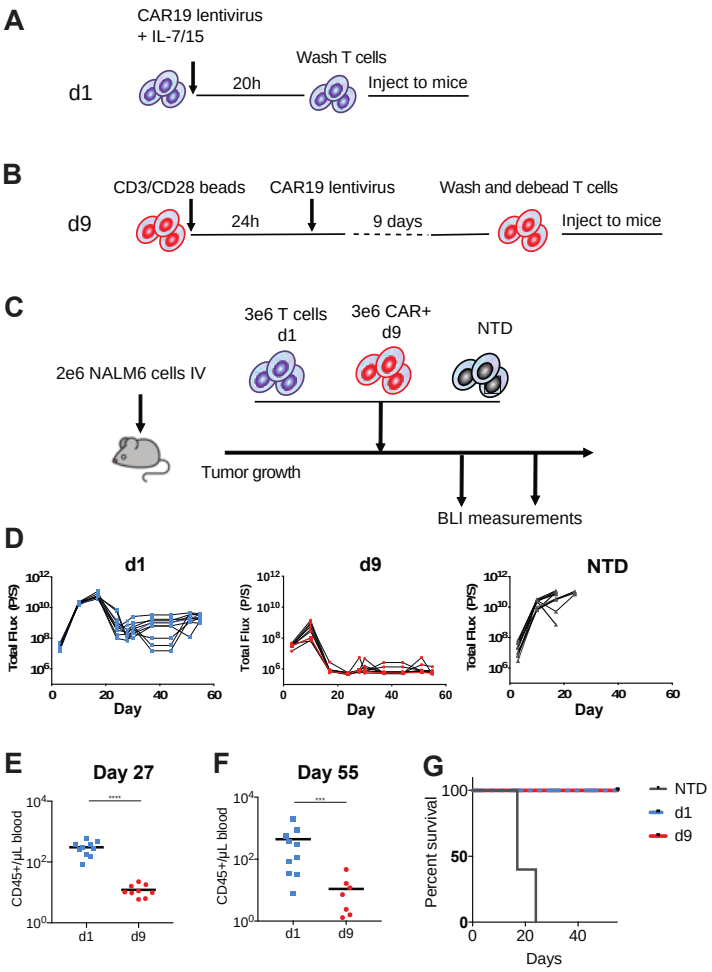

Figure 3: Non-activated T cells expressing a CD19-specific CAR control leukemia in xenograft models of ALL. (**A**) Schematic illustrating non-activated CART19 cell generation in less than 24 hours. Freshly isolated human T cells were transduced with CAR19 lentiviral vector for 20 hours in the presence of IL-7 and IL-15. Cells were then washed and infused into mice. (**B**) Schematic showing how CART19 cells are generated using standard approaches. After overnight stimulation with anti-CD3/CD28 beads, T cells were transduced with CAR19 lentiviral vector and expanded for 9 days. Cells were then washed, debeaded and infused into mice. (**C**) Schematic of the xenograft model with CART19 cell treatment in NSG mice. (**D**) Total bioluminescence flux (BLI) in mice treated with 3 x 10^6 non-activated T cells transduced as in panel A (d1), 3 x 10^6 CAR+ T cells stimulated with anti- CD3/CD28 microbeads and expanded over 9 days as in panel B (d9), and 3 x 10^6 non-transduced (NTD) control non-activated T cells (n=10 per group). (**E-F**) Absolute peripheral blood CD45+ T cell counts in peripheral blood collected from mice shown in panel D at the indicated time following T cell transfer measured by a TruCount assay. The mean of each group is indicated by the solid black line. Groups were compared using the two-tailed, unpaired Mann–Whitney test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. (**G**) Overall survival of mice by group. $P < 0.0001$ for d1 vs NTD and d9 vs NTD by log-rank test.

Modifying the culture conditions to enhance non-activated T cell transduction

Numerous mechanisms restrict quiescent T cell infection by natural HIV. Viral attachment and entry represent a critical initial phase of the transduction process during which the RNA genome of the lentiviral vector is inserted into the host T cell's cytoplasm. While natural HIV uses both chemokine receptors and CD4 for attachment and entry,[42] VSV-G-pseudotyped lentiviral vectors use the LDL-R as their primary receptor.[22] The low transduction efficiency of non-activated T cells has been attributed to the low expression of LDL-R.[21] As the LDL-R abundance is linked to the metabolic state of the cell and LDL uptake can be enhanced by cholesterol restriction,[43] we evaluated whether a brief serum starvation prior to lentiviral vector transduction increases lentiviral vector transduction of non-activated T cells. Brief (3-6 hour) serum starvation increases iRFP expression by an average of 2-fold in non-activated T cells (**Figure 4A, and Supplemental Figure 2**). The slow kinetics of reverse transcription in non-activated T cells by both natural HIV and lentiviral vectors also contribute to the reduced transduction efficiency. Completion of reverse transcription during natural HIV infection is enhanced by high concentrations of deoxynucleosides (dNs).[19] We show that supplementing the culture medium with 50μM dNs also increases lentiviral transduction of non-activated T cells by almost 2-3 fold (**Figure 4B**). Finally, the limited diffusibility of lentivirus in large culture vessels is another major barrier limiting transduction efficiency in T cells.[44] To evaluate this, we adjusted the geometric conditions to enhance the colocalization of vector particles with T cells. By increasing the surface area to volume ratio of the culture vessel, while keeping the volume constant, we increase transduction of non-activated T cell by at least 2-fold (**Figure 4C)**. Combining these approaches, we show that transduction efficiencies can be enhanced by 2-12 fold in non-activated T cells (**Figure 4D)**.

Table 1: Integrated lentiviral vector analysis using repetitive sample Alu-gag qPCR		
Sample	RU5/cell	Proviral HIV/cell
12h	0.19	0.01
24h	0.16	0.09
48h	0.55	0.23
72h	0.69	0.6
12h (RT/Integrase inhibitor)	0.15	<LOD
24h (RT/Integrase inhibitor)	0.12	<LOD
48h (RT/Integrase inhibitor)	0.07	<LOD
72h (RT/Integrase inhibitor)	0.05	<LOD

< LOD = below limit of detection; RU5 = LTR primer only for total vector (integrated + non-integrated)

Table 1. Integrated lentiviral vector analysis using repetitive sample Alu-gag qPCR. Comparison of integrated vector (Proviral HIV copies/cell) with the total vector copies (RU5/cell, detected total integrated + non-integrated vector) with or without RT/Integrase inhibitors

Since pseudotransduction with CD19-specific CAR interferes with the ability to estimate transduction efficiency (see **Figure 2**) and vector integration is likely ongoing at the time of infusion, we estimated the efficiency of the optimized transduction process by adoptively transferring the transduced T cells into non-leukemia bearing NSG mice, which permits an estimation of the frequency of integrated vector in the absence of CAR stimulation by antigen-expressing tumor cells that would normally stimulate and enrich for CAR+ T cells. CART19 cells were generated from freshly isolated, peripheral blood T cells by serum starving the T cells for 3 hours followed by transduction in a minimal volume of lentiviral vector encoding a CD19-specific CAR for 20-hrs in the presence of 50μM dNs, IL-7 and IL-15 (10 ng/ml each), and lentiviral particles at an MOI of 5 (**Figure 4E**). Evaluating three CART19 products produced from three separate donors, we observed a mean transduction frequency of 8% (range 6-16%) based upon analysis performed at 3 weeks following adoptive transfer (**Figure 4F**), which is within the lower-end of the range of CART19 products using a 9-day manufacturing process.[37] This was confirmed by quantitative PCR analysis of integration of lentivirus vector in non-activated T cells over time (**Table 1)**. Thus, our data is consistent with integration of the lentiviral vector in the non-activated T cells occurring over several days after infusion of the non-activated CART19 cells.

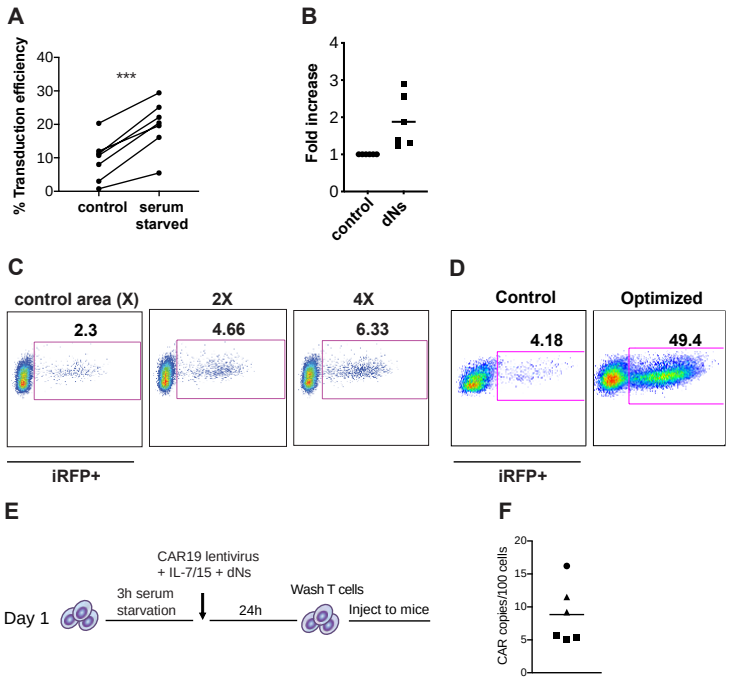

Figure 4: Transducing conditions can enhance transduction efficiency in non-activated T cells. (**A**) Freshly isolated human T cells were either serum starved by washing and resuspending in serum-free medium or maintained in complete medium for 3-6 hours and then transduced with a lentiviral vector encoding iRFP for 24 hours in the presence of IL-7 and IL-15 in complete medium. Cells were then maintained in culture for 5 days in culture prior to determining the iRFP+ cell frequency. Each dot represents transduction frequency determined by flow cytometry from an independent experiment using 6 different donor. (**B**) Relative fold change of transduction of iRFP+ cells transduced in the presence of 50μM dNs normalized to iRFP+ cells transduced in complete media without dNs. Data are shown as mean±SD of six experiments performed with different donors. (**C**) T cells were transduced with lentiviral vector iRFP cultured in either one well, or two wells, or four wells or eight wells with total culture volume held constant. Cells were then maintained in culture for 5 days in IL-7 and IL-15-containing medium prior to determining the iRFP+ cell frequency by flow cytometry. Results are representative of three independent experiments using three different donors. Unpaired Mann–Whitney test, two-tailed was used. * $P < 0.05$, *** $P < 0.001$. (**D**) Freshly isolated human T cells were transduced with a lentiviral vector encoding iRFP in an optimized condition as described in previous panels (serum starvation, dNs, and optimized geometry). T cells were then maintained in culture for 5 days followed by flow cytometric analysis for iRFP expression. Results are representative of the best transduction achieved using this process. (**E**) Schematic of generation of non-activated CART19 cells in 24 hours. (**F**) CAR+ cell frequency as estimated by quantitative PCR analysis of vector copy number in peripheral blood collected at 3 weeks following adoptive transfer of T cells. The results are expressed as a percentage of human cells by normalization to the CDKN1A gene, which has two copies in the human diploid genome. Each symbol represents a separate donor.

In vivo functional assessment of optimally transduced CD19-specific CAR T cells

We hypothesized that a nonactivated CAR T cell product preserves the intrinsic stem-like properties of naïve and memory T cells, culminating in enhanced persistence following infusion. To evaluate this hypothesis, we performed an in vivo functional "CAR stress test" using limited numbers of CAR T cells in the Nalm6 leukemia model. $2x10^6$, $7x10^5$ or $2x10^5$ total non-activated CART19 cells prepared using our optimized process were adoptively transferred into NSG mice bearing pre-established Nalm6 xenografts. $3x10^6$ activated CART19 cells prepared by anti-CD3/CD28 bead stimulation followed by 9 days of ex-vivo expansion was used as a control as well as $3x10^6$ mock-transduced, non-activated T cells (**Figure 5**). As shown in **Figure 5B-D**, complete regression of Nalm6 leukemia to BLI less than $1x10^6$ p/s was observed in all groups treated with non-activated CART19. The kinetics of the anti-leukemic response for the non-activated CART19 cells was dose dependent; the lowest dose group cleared the tumor by 18 days, whereas the highest dose group achieved tumor regression by 11 days (**Figure 5E**). The CD3/CD28 stimulated and expanded CART19 cells showed the most rapid leukemia clearance (**Figure 5D**). However, the depth and durability of the response for this donor was limited with all mice relapsing by day 17. In contrast, the non-activated CART19 cells controlled leukemia for the duration of the experiment in all mice at the highest dose and in most mice at the lower doses (**Figure 5C**). This durability was associated with an increased persistence of T cells. As seen in **Figure 5F-H**, the absolute counts of CART19 cells were significantly increased in the peripheral blood of mice treated with non-activated CART19 cells, which was proportional to the dose infused, compared to treatment with activated CART19 cells. These cells were mostly effector memory (**Supplemental Figure 3)**. To show the broader applicability of our approach, we also evaluated the anti-tumor function of CD33-specific CAR T cells generated from non-activated T cells in a xenograft model of AML. As seen in **Supplemental Figure 4**, non-activated CARTs demonstrated anti-leukemic function that was similar to CARTs generated from CD3/28-activated T cells and expanded over 9 days. Unfortunately, the durability of response could not be assessed in this model due to an allogeneic response that was evident with NTD cells after two weeks of CAR T infusion.

In summary, these findings demonstrate that as few as an estimated 12,000-32,000 non-activated CAR T cells, based upon the range of transduction efficiency generated with the optimized transduction process (**Figure 3E**), within 24 hours from collection could eradicate leukemia. The long-term engraftment

of nonactivated CAR T cells likely occurs due to their enhanced replicative capacity compared with activated CAR T cells.

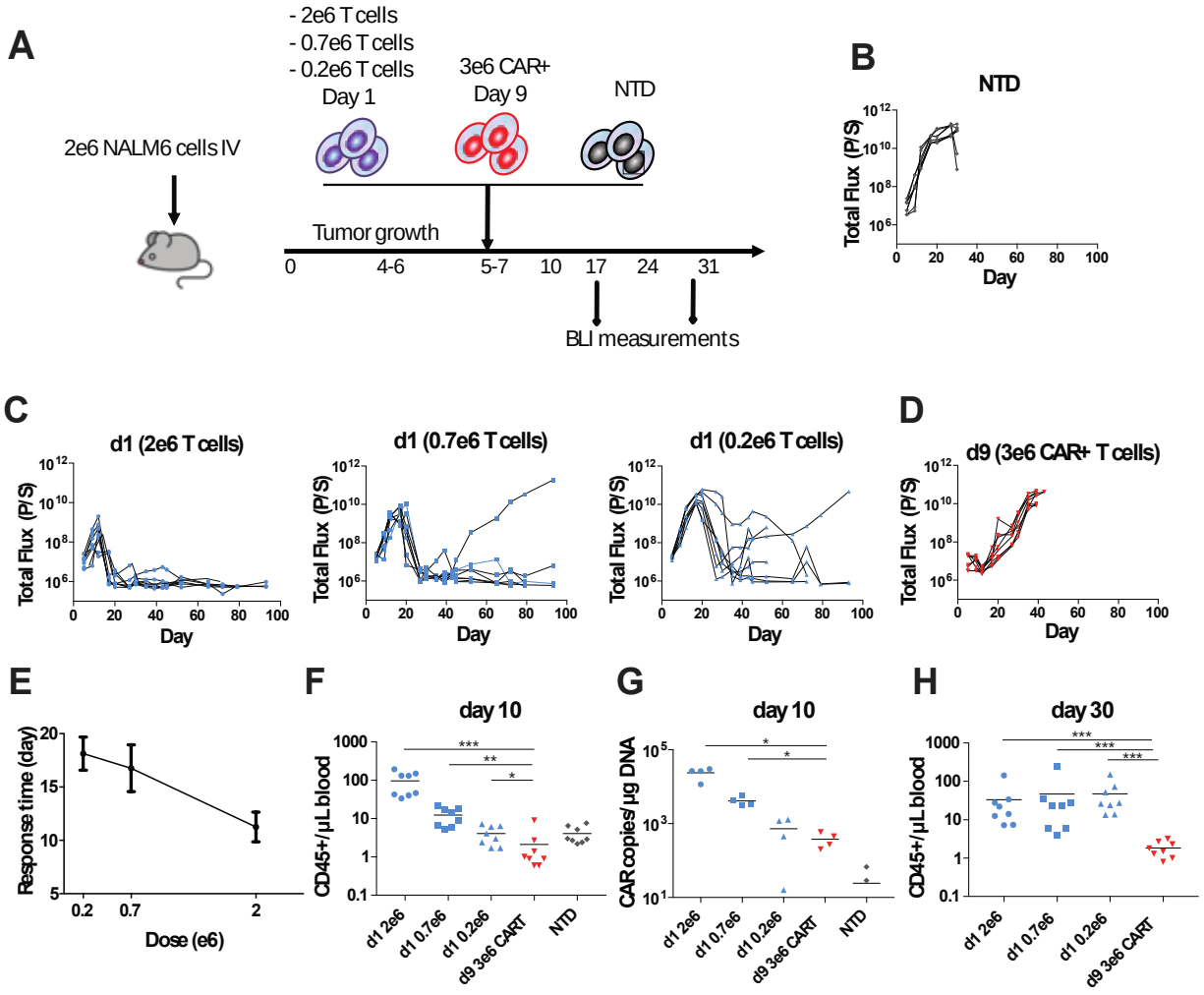

Figure 5: Non-activated T cells expressing a CD19-specific CAR induce potent and durable remission of ALL at low doses. (**A**) Schematic of the xenograft model and CART19 cell treatment in NSG mice. (**B-D**) serial quantification of disease burden by bioluminescence imaging. (**B**) Total bioluminescence flux in mice treated with non-transduced (NTD) control non-activated T cells. (**C**) Total bioluminescence flux in mice treated with a single high (2 x 10^6), medium (0.7 x 10^6) or low (0.2 x 10^6) dose of non-activated T cells transduced as in Figure 4E. (**D**) Total bioluminescence flux in mice treated with 3 x 10^6 CAR+ T cells stimulated with anti- CD3/CD28 microbeads and expanded over 9 days. There are 8 mice in each group. (**E**) Time to initial anti-leukemic response (i.e. first reduction in bioluminescence) after infusion of non-activated CART19 in relationship to T cell dose. (**F**) Absolute peripheral blood CD45+ T cell counts in blood collected from mice shown in panels B-D at 10 days following T cell transfer measured by a TruCount assay. (**G**) Vector copy number in peripheral blood collected at day 10 following T cell transfer measured by qPCR and normalized to DNA concentration. (**H**) Absolute peripheral blood CD45+ T cell counts in blood collected from mice shown in panels B-D on day 30 following T cell transfer measured by a TruCount assay. The mean of each group is indicated by the solid black line. Groups were compared using the two-tailed, unpaired Mann–Whitney test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Feasibility and functionality of non-activated CAR T cells using patient samples

After showing that non-activated CAR T cells can be generated from healthy donor lymphocytes. we extended our observations to T cells isolated from patients undergoing cancer treatment. Immunosuppressive factors in the tumor environment may impede our ability to generate functional CAR T cells using our novel approach. We therefore evaluated the anti-leukemic activity of non-activated CAR T cells generated using apheresis-collected mononuclear cells (MNCs) derived from a subject with diffuse large B cell lymphoma treated in one of our CD19-specific CAR T cell clinical trials (clinicaltrials.gov NCT02030834). Since apheresis-collected MNCs comprise numerous cell types in addition to T cells, we employed a CD4+ and CD8+ T cell positive selection strategy using cGMP-compliant anti-CD4 and anti-CD8 magnetic microbeads (**Supplemental Figure 5A**). Following selection, the enriched T cells were lentivirally transduced with CD19-specific CAR in medium conditioned with 50μM dNs, 10 ng/ml IL-7 and IL-15 for 24 hours as in the prior studies with healthy donor T cells. CD19-specific CAR T cells manufactured within the University of Pennsylvania's Cell and Vaccine Production Facility using a cGMP-compliant 9-day process were used a control. $3x10^6$ total non-activated T cells versus $3x10^6$ CAR+ day 9 T cells were adoptively transferred to NSG mice bearing pre-established Nalm6 xenografts. To control for CD19-specific cytolytic activity, $3x10^6$ mock-transduced, non-activated T cells were included as an additional control. To estimate the transduction efficiency of non-activated CART cells, $3x10^6$ transduced T cells were infused into NSG mice without leukemia (**Figure 4E**). The lentiviral infection efficiency was estimated at 32% as assessed by integration analysis performed 7 weeks following adoptive transfer. These data were corroborated by flow cytometric analysis (**Supplemental Figure 5B**). As shown in **Figure 6A-B**, day 9 CART19 cells exhibited complete regression of Nalm6 leukemia. However, all mice relapsed by day 20 consistent with the progressive loss of function and low replicative capacity of CAR T cells in this model. In contrast, non-activated CART19 cells provided sustained control leukemia in half of the mice (3 out of 6) for the duration of the experiment. The enhanced durability of anti-leukemic activity in non-activated CAR T cell-treated mice demonstrated by lower bioluminescence signal at day 49 (**Fig 6E**) was associated with an improved persistence of T cells in the peripheral blood (**Figure 6F-H).** In summary, these results demonstrate the feasibility of generating CAR T cells from patient-derived, non-activated T cells using a process that can be imported into a cGMP environment, and further demonstrated that rapidly produced,

non-activated CAR T cells exhibit more durable anti-leukemic function when compared to CAR T cells manufactured using a 9-day ex vivo expansion process following CD3/CD28 activation.

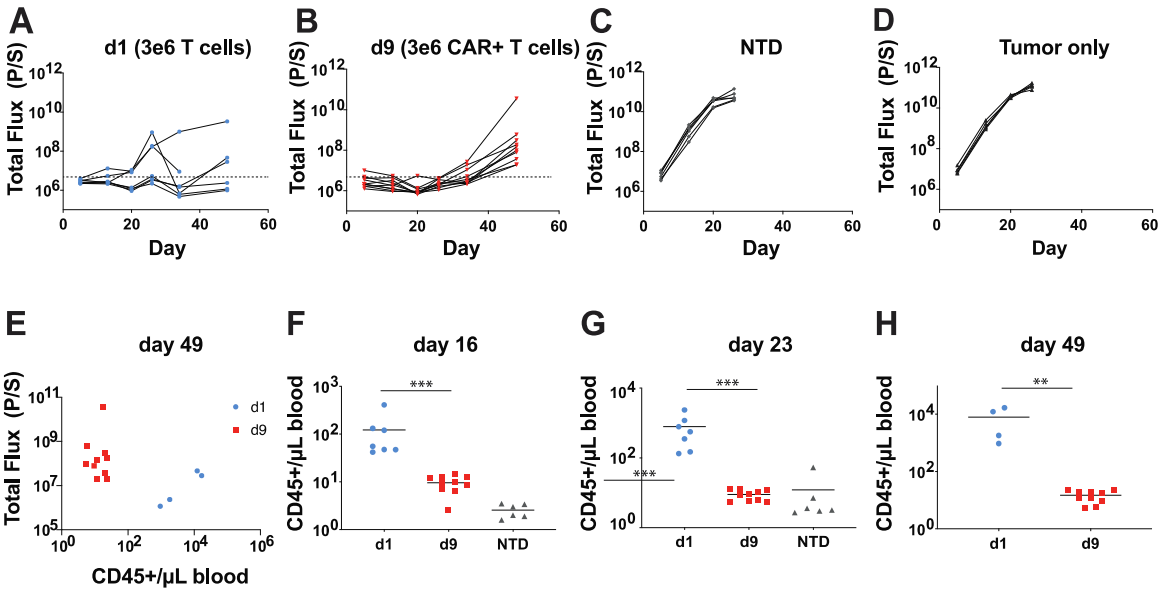

Figure 6: Non-activated CART cells generated from patient samples show potent efficacy in vivo. (**A-D**) Serial quantification of disease burden by bioluminescence imaging. (**A**) Total bioluminescence flux in mice treated with 3 x 10^6 non-activated T cells transduced (d1) as in panel B in Figure 3 (n=7), (**B**) 3 x 10^6 CAR+ T cells stimulated with anti- CD3/CD28 microbeads and expanded over 9 days (d9) (n=10), (**C**) 3 x 10^6 non-transduced (NTD) control non-activated T cells (n=10), and (**D**) no treatment. (**E**) BLI measurement of disease burden of mice drom d1 and d9 groups (panel A-B) in relation to the absolute peripheral blood CD45+ T cell counts in their blood at day 49 (**F-H**) Absolute peripheral blood CD45+ T cell counts in blood collected from mice shown in panels A-C at (**E**) 16 days, (**F**) 23 days, and (**G**) 49 days following T cell transfer measured by a TruCount assay. The mean of each group is indicated by the solid black line. Groups were compared using the two-tailed, unpaired Mann–Whitney test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Discussion

This study presents a novel approach to rapidly generate highly functional CAR T cells for adoptive immunotherapy. This approach capitalizes upon the unique ability of lentiviral vectors to transduce non-activated, quiescent T cells. Currently, ex vivo cell culture following T cell activation is an essential part of the manufacturing of CAR T cell therapies. Because TCR activation and clonal expansion promote irreversible differentiation of T cells as well as potentially other detrimental changes to the T cells through processes such as oxidative stress,[45, 46] the potency of CAR T cells may be compromised during their manufacturing process. While interventions including provision of different costimulatory receptor signals,[47] cytokines[48] or other alterations to the culture conditions (e.g. Akt inhibition)[49] help to limit this cellular differentiation, stable expression of a CAR within a non-activated T cell provides a far simpler approach to limiting differentiation. Our results demonstrate that functional transduction of non-activated T cells including memory subsets can be achieved within less than 24 hours of T cell collection. Moreover, non-activated T cells transduced with a CD19-specific CAR exhibit potent in vivo anti-leukemic efficacy at cell doses well below those effective for activated T cells.[7]

To show the potential benefit of our approach beyond CART-19 in models of ALL, we confirmed the anti-tumor potency of unstimulated CAR T-cells redirected against CD33. These findings broaden the impact of our approach to other blood-based leukemias including AML. We found that NTD cells controlled tumor burden two weeks following infusion in our xenograft model of AML. High levels of antigen presentation specific to AML, may create an allogeneic immune pressure that stimulates the “Graft vs Leukemia effect” in donor T cells. This is an expected response as bone marrow transplantation works to treat leukemia patients.

Importantly, we established that our overall process was technically feasible in line with current manufacturing procedures, facilitating a rapid transition into the clinical sector. We show using patient apheresis material that T cells can be isolated using GMP-grade antibodies and standard column-based purification methods. These cells retain maximal functional competence using our novel method of CAR gene delivery and preparatory phase that eliminates activation. Our approach is poised for a rapid implementation to Clinimax-based systems that are currently used in our manufacturing division at Penn as well as other facilities.

The approach used for transduction of non-activated T cells in our studies has only been partially optimized, and it is likely that the efficiency of the transduction process can be further enhanced. A recent study demonstrated that the use of a microfluidic chamber for transduction could significantly increase both the efficiency and kinetics of the transduction process by overcoming the diffusion barriers inherent in static cultures.[44, 50] Interference with SAMHD1, a deoxynucleoside triphosphate triphosphohydrolase that restricts HIV-1 infection in quiescent T cells is another potential strategy to enhance non-activated T cell transduction.[31, 51] Mechanistically, SAMHD1 hinders lentivirus infection by impeding the rate of reverse transcription. Loss-of-function approaches show that SAMHD1 elimination leads to increased infection efficiencies in non-activated T cells.[51] Small molecules that inhibit SAMHD1 are under development, and may have applications here.[52] In addition to the post-entry restrictions, substituting the VSV-G envelope protein with alternative glycoproteins such as the cocal virus envelope may also enhance the lentiviral entry step into quiescent T cells as this envelope protein has yielded superior transduction efficiencies in hematopoietic stem cells and activated T cells.[53]

In addition to optimizing the transduction process, the optimal composition of non-activated T cells for adoptive immunotherapy is largely unknown. Various syngeneic murine models show that memory T cell subsets have superior anti-tumor function following adoptive transfer.[8, 11, 16, 54] In the CD8+ T cell compartment, transduction efficiencies were highest within the memory T cell subsets (**Figure 1E**). It cannot be assumed that the T cell subsets found to be optimal for activated T cells will be the same when using non-activated T cells in adoptive immunotherapy. Cells with effector differentiation and function may be needed in addition to memory cells for replenishment of the tumor-specific T cell pool when starting with quiescent T cells. The optimal CAR design for non-activated T cells has also not been defined. Our study used a 2nd generation CAR incorporating the 4-1BB cytoplasmic domain; however, this design was previously selected for its function in activated T cells in which natural 4-1BB is typically expressed[55]. Alternate CAR designs may be required for optimal function in quiescent T cells. Combining T cell subsets with their preferred costimulatory domain may be the most beneficial approach for immediate effector function and long-lasting engraftment.

Intriguingly, quiescent memory T cells appear more susceptible to lentiviral transduction than their naïve counterparts. These findings suggest that prior activation supports lentiviral transduction, even when

the T cells are in a quiescent state at the time of transduction. It is possible that epigenetic alterations underlying the commitment to memory render the cells more susceptible to infection at a later date. In support of this hypothesis, H3K36me3 histone modifications promote viral integration in actively transcribed regions in non-dividing cells.[56] In addition to the unique epigenetic landscape of memory cells, transcriptional complexes are redistributed to the nuclear pore in non-dividing cells.[57] Proteins assembled at the nuclear pore may enhance HIV-1 nuclear entry in memory T cells through unknown mechanisms.

The slow kinetics of lentiviral transduction observed in our study complement the delay in reverse transcription and integration observed in natural HIV infection of non-activated CD4+ T cells.[18-20] It is likely that the majority of T cells used in our in vivo studies lacked integrated proviral DNA at the time of adoptive transfer, and the processes of reverse transcription and integration instead likely continue post-infusion. This introduces challenges to assessing CAR T cell quality. Transduction efficiency, typically measured by CAR expression at the cell surface and/or vector integration, is routinely used as one surrogate measure of product potency as well as CAR T cell dose. Translation of a rapid manufacturing approach using non-activated T cells will therefore require development of alternative methods to evaluate the transduction process such as by quantitation of reverse transcription intermediates.

There are inherent regulatory challenges in conforming the current FDA guidelines, which were largely developed for small molecule drugs and simpler biologics like protein therapeutics to highly complex, living therapeutics such as CAR-modified T cells. In the case of the rapid manufacturing process described here, one of the most significant challenges is that the “manufacturing” process is to some extent still ongoing at the time of CAR T cell harvest. Limiting the ex-vivo transduction process to 24 hr means that a number of vector particles will still be undergoing reverse transcription and integration post infusion as these processes are significantly slower in non-activated T cells. As we have shown, quantitative measures of the active reverse transcription and integration process such as the Alu repeat-based qPCR may be the most direct methods of assessing the quality of the transduction process in lieu of directly measuring CAR protein expression as traditionally done with activated CAR T cell products; however, this needs to be rigorously evaluated in future studies.

In summary, the ability to generate highly functional CAR T cells within a day has important implications for improving CAR T cell therapies. Lentiviral vectors provide a highly efficient method to produce CAR T cell products with durable engraftment and function by leveraging the unique ability of these vectors to enter and integrate into the genome of non-dividing cells. Extended ex-vivo culture of T cells is unnecessary to produce CAR T cells for therapeutic purposes. Minimizing ex-vivo manipulation, in addition to reducing costs, conserves limited resources such as human serum and manufacturing space as T cell clonal expansion occurs entirely in vivo. If the process can be reduced to a few simple steps, it also has the potential to decentralize CAR T cell manufacturing to local hospital laboratories. This will avoid many of the logistical challenges. The generation of engineered T cell products within a shorter interval between apheresis collection and re-infusion of CAR T cells could also be of particular benefit to patients with rapidly progressive disease, who may otherwise be unable to receive the therapy.[58]

Methods

Generation of Lentiviral Vectors

Replication defective lentivirus was produced by standard methods using a 3rd generation lentiviral vector transfer plasmid encoding either infrared fluorescent protein (iRFP), an anti-CD19-BBζ CAR[32], or anti-CD33-BBζ CAR[38] mixed with three packaging plasmids encoding VSV-G (pMDG.1), gag/pol (pMDLg/pRRE) and rev (pRSV-rev), and transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen).

Cells

Peripheral blood leukocytes from healthy donors were obtained from the Human Immunology Core. Informed consent was obtained from all participants prior to collection. All methods and experimental procedures were approved by the University of Pennsylvania Institutional Review Board. Healthy donor T cells were purified at the University's Human Immunology Core by negative selection using the RosetteSep T cell enrichment Cocktail (Stem Cell Technologies, Vancouver, BC). Patient-derived T cells were isolated by positive selection using CD4- and CD8-specific microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer protocols.

All cell lines (NALM-6, MOLM14 and HEK293T) were originally obtained from the American Type Culture Collection (ATCC). Cells were expanded in RPMI medium containing 10% fetal bovine serum (FBS), penicillin and Streptomycin at a low passage and tested for mycoplasma. Cell line authentication was performed by the University of Arizona Genetics Core based on criteria established by the International Cell Line Authentication Committee. Short tandem repeat profiling revealed that these cell lines were above the 80% match threshold.

Transduction of T cells

For activated T cells, cells were resuspended at $1x10^6$ T cells/mL in X-VIVO 15 (Cambrex, Walkersville, MD) supplemented with 5% human AB serum (Valley Biomedical, Winchester, VA), 2 mM L-glutamine (Cambrex), 20 mM HEPES (Cambrex), IL-7 and IL-15 (10 ng/mL each, Miltenyi Biotec). Dynabeads™ Human T-Activator CD3/CD28 beads (Thermofisher) were added to a final ratio of 3 beads to 1 cell. After 24 hours, lentiviral vector supernatant was added at a multiplicity of infection (MOI) as indicated. Cells were maintained in culture at a concentration of $0.5x10^6$ cells/mL by adjusting the concentration every other day based on counting by flow cytometry using countbright beads (BD Bioscience) and monoclonal antibodies to human CD4 and CD8[33]. Cell volume was also measured with a Multisizer III particle counter (Beckman-Coulter) every other day. For non-activated T cells, cells were resuspended at $1x10^7$ T cells/mL in X-VIVO 15 supplemented with 2 mM L-glutamine, 20 mM HEPES, IL-7 and IL-15 (10 ng/mL) for 3-6 hours followed by addition of human AB serum to 5% of the total volume and lentiviral vector supernatant to achieve an MOI as indicated. Deoxynucleotides (50 µM, Sigma), reverse transcriptase (Saquinavir, 1 µM) and integrase inhibitor (Raltegravir, 1 µM) (Cayman Chemical) were also added to the medium in some experiments as indicated.

Flow cytometry

T cell differentiation was assessed using the following antibodies: anti-CCR7–FITC clone 150503 (BD Pharmingen); anti-CD45RO–PE clone UCHL1, anti-CD8–H7APC clone SK1 (BD Biosciences); anti-CD4–BV510 clone OKT4, anti-CD3–BV605 clone OKT3, anti-CD14–Pacific Blue (PB) clone HCD14, anti-CD19–PB clone H1B19 (BioLegend). The anti-CAR19 idiotype for surface expression of CAR19 was provided by Novartis (Basel, Switzerland). Cells were washed with phosphate-buffered saline (PBS), incubated with LIVE/DEAD Fixable Violet (Molecular Probes) for 15 minutes, and resuspended in fluorescence activated

cell sorting (FACS) buffer consisting of PBS, 1% BSA, and 5 mM EDTA. Cells were then incubated with antibodies for 1 hour at 4°C. Positively stained cells were differentiated from background using fluorescence-minus-one (FMO) controls. Flow cytometry was performed on BD LSR Fortessa. Analysis was performed using Flowjo software (Tree Star Inc. version 10.1).

Quantitative (q) PCR analysis

Genomic DNA was isolated using a QIAamp DNA Micro Kit (Qiagen). Using 200 ng genomic DNA, qPCR analysis was performed to detect the integrated BBζ CAR transgene sequence using ABI Taqman technology as previously described.[32, 34] To determine copy number per µg of genomic DNA, an 8-point standard curve was generated consisting of 5 to 10^6 copies of the BBζ lentivirus plasmid spiked into 100 ng non-transduced control genomic DNA. A primer-probe set specific for the CDKN1A gene, a single copy gene in the human haploid genome, was used as a normalization control to estimate vector copies per cell. The levels of total and integrated DNA were measured by PCR as previously described.[35, 36] Briefly, for total lentiviral levels, primers against the LTR (LTR F: TTAAGCCTCAATAAAGCTTGCC; LTR R: GTTCGGGCGCCACTGCTAGA) was used. Integrated DNA was measured using primers against the human *Alu* element (Alu F: GCCTCCCAAAGTGCTGGGATTACAG) and the lentiviral *gag* gene (gag R: GCTCTCGCACCCATCTCTCTCC). Notably, a small amount of *gag* was retained in the lentiviral vector. For both reactions, a nested approach was utilized. For LTR PCR, PCR conditions for the first round were: 95 °C for 2 min; then 95 °C for 15 s, 64 °C for 45 s, 72 °C for 1 min for 12 cycles; and then 72 °C for 10 min. For Alu-gag PCR, the following PCR conditions were used: 95 °C for 2 min; then 95 °C for 15 s, 56 °C for 45 s, 72 °C for 3:30 min for 40 cycles; and then 72 °C for 10 min. Fifteen microliters of the first-round PCR reactions were run on the qPCR instrument using the primers LTR F and LTR R (probe CCAGAGTCACACAACAGACGGGCACA). PCR conditions were: 95 °C for 15 s; then 95 °C for 10 s, 60 °C for 20 s for 40 cycles. For Alu-gag PCR, genomic DNA was diluted to target 30-80% positive wells at two dilutions to minimize variance. The percent of positive wells was used to estimate the lentiviral levels using a binomial distribution.

Cytokine secretion

T cells were incubated at a ratio of 1:1 with irradiated target cells at a concentration of 10^6 T cells/mL in a cytokine free media. Supernatants were collected at 24 hours to assess cytokine production. Measurement

of Cytokine was performed with a Luminex bead array platform (Life Technologies) according to the manufacturer's instructions.[37]

Cytotoxicity assays

Cytotoxicity assays were performed using a ^{51}Cr release-assay as previously described.[7] In brief, $Na_2{}^{51}CrO_4$-labeled target cells were incubated with CART cells for 20 hours at various effector:target ratios and placed into 96 well Lumaplates (Perkin Elmer). The amount of 51Cr released from the labeled target cells was measured on a liquid scintillation counter (MicroBeta trilux, Perkin Elmer). Target cells incubated in medium alone or with 1% SDS were used to determine spontaneous (S) or maximum (M) 51Cr release. Percentage of specific lysis was calculated as follow: 100 x (cpm experimental release- cpm S release)/(cpm M release- cpm S release).

***In vivo* models**

Xenograft models of leukemia were used as previously reported.32, 38 Briefly, 6- to 10-week-old NOD-SCID γc−/−(NSG) mice, which lack an adaptive immune system, were obtained from Jackson Laboratories or bred in-house under a protocol approved by the Institutional Animal Care and Use Committees (IACUC) of the University of Pennsylvania. Animals were assigned in all experiments to treatment/control groups using a randomized approach. Animals were injected IV via tail vein with $2x10^6$ NALM6 or $1x10^6$ MOLM14 cells expressing click beetle green luciferase and eGFP, in 0.1 mL sterile PBS. CAR T cells or non-transduced (NTD) human T cells were injected via tail vein at the indicated dose in a volume of 100µL, 4 days after injection of leukemic cells. Mice were given an intraperitoneal injection of 150 mg/kg D-luciferin (Caliper Life Sciences, Hopkinton, MA). Anesthetized mice were imaged using a Xenogen IVIS Spectrum system (Caliper Life Science). Total flux was quantified using Living Image 4.4 (PerkinElmer). T cell engraftment was defined as >1% human CD45+ cells in Peripheral blood by flow cytometry. Animals were euthanized at the end of the experiment or when they met pre-specified endpoints according to the protocols except for the AML model experiment, which was terminated due to a COVID-19-related shutdown of research.

Data availability

The main data supporting the findings of this study are available within the Article and its Supplementary Information. The raw data generated in this study are available from the corresponding author upon reasonable request.

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Author contributions

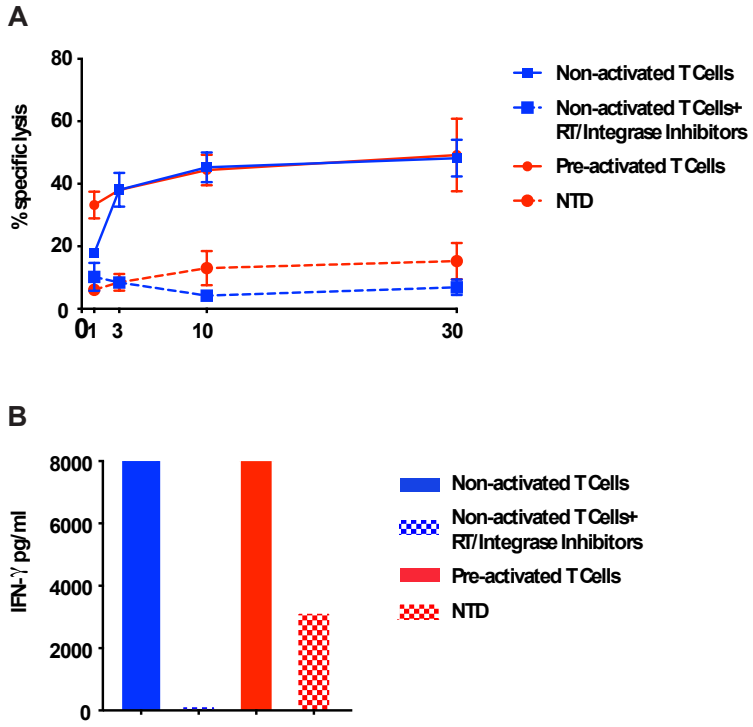
S.G. and M.C.M. designed the study. Concept: S.G., M.C.M., J.S.D., R.S.O., U.O. and S.I.G. provided conceptual guidance. S.G., J.S.D, R.S.O., S.N.C., J.P., K.C., F.S., M.P., G.P. and J.L. performed the

experiments. S.G. and M.C.M analyzed the data. S.G. and M.C.M. wrote the manuscript. R.S.O and J.S.D. read and made comments on the manuscript.

Competing interests

M.C.M. is an inventor on patent applications related to CAR technology and has received licensing royalties from Novartis corporation; S.G. and M.C.M. are inventors on patent applications related to methods of manufacturing CAR T cells. The remaining authors declare no competing financial interests.

Supplementary Information (SI).



602
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605 **Supplemental Figure 1: Non-activated T cells expressing CD19-specific CAR exert potent effector**
606 **function following antigen exposure in vitro.** (**A**) ^{51}Cr release after 20 hours using non-activated
607 CART19 cells with or without presence of RT and integrase inhibitor, as well as CD3/28 activated CART19
608 cells and NTD control, cocultured at the indicated E:T ratio with Nalm6 cells. Mean values of triplicate
609 culture are shown. (**B**) IFN gamma measured by a Luminex analysis in same groups as panel a cocultured
610 with NALM6 leukemia cells for 24 hours at a 1:1 E:T ratio prior to collection of culture supernatant for
611 cytokine analysis. Result are representative of three independent experiments using three different donors.
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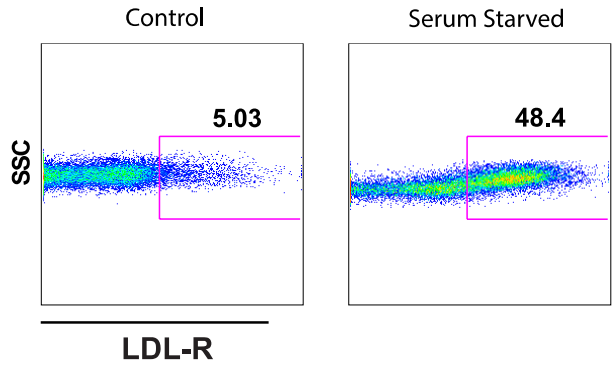
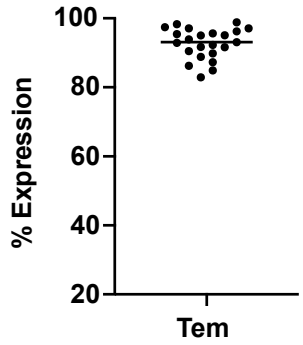
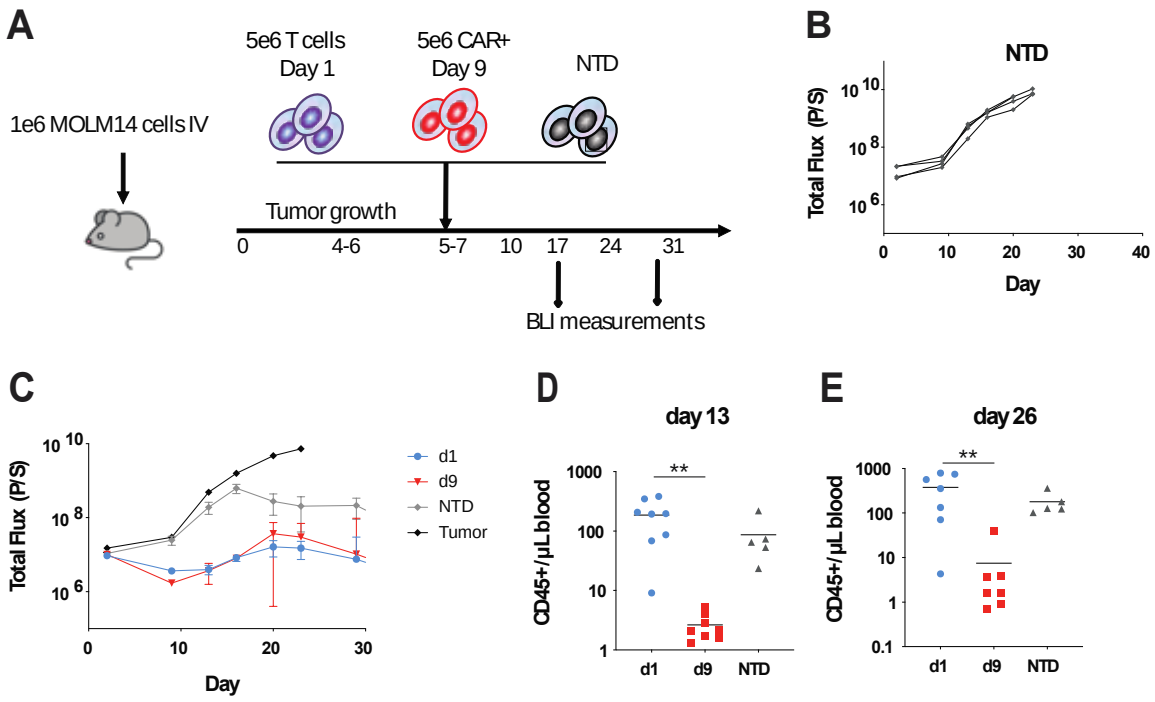


Supplemental Figure 2: LDL-R upregulates after serum starvation. Freshly isolated human T cells were serum starved by washing and resuspending in serum-free medium for 6 hours. Expression of LDL-R on the surface of T cells measured by flow cytometric analysis. Results are representative of three different donors.



Supplemental Figure 3: Characterization of non-activated CAR T cells after infusion Phenotypic analyses of non-activated CAR T cells within the peripheral blood of mice (from Figure 5H) 30 days following adoptive transfer. Tem is gated as CD45RO+ CCR7-



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632 **Supplemental Figure 4: Non-activated T cells expressing a CD33-specific CAR exhibit antileukemic**
633 **effect in vivo in Aml xenograft model.** (**A**) Schematic of the xenograft model and CART33 cell treatment
634 in NSG mice. (**B-C**) Serial quantification of disease burden by bioluminescence imaging. (**B**) Total
635 bioluminescence flux in mice with no treatment. (**C**) Total bioluminescence flux in mice treated with $5x10^6$
636 non-activated T cells transduced as in panel A (d1), $5x10^6$ CAR+ T cells stimulated with anti- CD3/CD28
637 microbeads and expanded over 9 days (d9), and 5×10^6 non-transduced (NTD) control non-activated T
638 cells (n=10 per group). (D-E) Absolute peripheral blood CD45+ T cell counts in blood collected from mice
639 shown in panels C at (**D**) 13 days, and (**E**) 26 days following T cell transfer measured by a TruCount assay.
640 The mean of each group is indicated by the solid black line. Groups were compared using the two-tailed,
641 unpaired Mann–Whitney test. * $P < 0.05$, ** $P < 0.01$.

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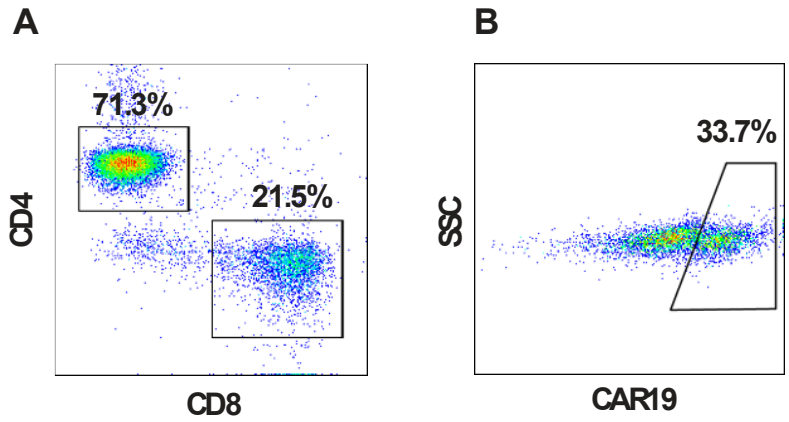

Supplemental Figure 5: Characterization of the apheresis and non-activated CART after infusion(**A**) The purity of T cells after positive selection by CD4 and CD8 magnetic microbeads measured by flow cytometry. (**B**) The transduction efficiency of CD45+ T cells from peripheral blood of non-leukemia bearing NSG mice measured by flow cytometry.

Epigenetic strategies to boost CAR T cell therapy

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Abstract

Chimeric antigen receptor (CAR) T cell therapy has led a paradigm-shift in cancer immunotherapy, but still several obstacles limit CAR T cell efficacy in cancers. Advances in high throughput technologies revealed new insights into the role that epigenetic reprogramming plays in T cells. Mechanistic studies as well as comprehensive epigenome

maps revealed an important role for epigenetic remodeling in T cell differentiation. These modifications shape the overall immune response through alterations in T cell phenotype and function. Here, we outline how epigenetic modifications in CAR T cells can overcome barriers limiting CAR T cell effectiveness, particularly in immunosuppressive tumor microenvironments. We also offer our perspective on how select epigenetically-modifications can boost CAR T cells to ultimately improve the efficacy of CAR T cell therapy.

Introduction

For years, the cornerstone of cancer therapy has been surgery, chemotherapy, and radiation therapy [1]. More recently targeted therapies, in particular cell based therapies such as adoptive T cell and CAR T cell therapies, have led to tremendous successes against cancer [1,2]. Although CAR T cell therapy has been approved by the US food and drug administration (FDA) in several hematological malignancies, their success in solid tumors has been limited. There are several roadblocks impeding CAR T cell therapy in solid tumors including i) antigen heterogeneity/loss that renders the CAR obsolete, ii) the presence of immunosuppressive cells and molecules within the tumor microenvironment (TME), iii) poor persistence of ex-vivo expanded CAR T cells, iv) impaired penetration and trafficking of CAR T cells to tumor site [3].

Despite efforts to overcome these barriers, there still is no approved CAR T cell therapy for solid tumors. Combining CAR T cells with immune checkpoint blockades, oncolytic viruses, bispecific T cell engagers and cytokines have increased the efficacy of adoptively transferred cells in preclinical model. There are still some unaddressed aspects of CAR T cell biology and functionality, such as understanding how epigenetic reprogramming and gene regulation enhance CAR T cell antitumor function [4-6]. The central dogma of molecular biology has come under scrutiny by the emerging field of epigenetics. In recent years epigenetic studies on cancer as well as immune cells have generated plenty of useful data. The current challenge is to translate this knowledge to a favorable clinical outcome. In this review we will discuss how epigenetic modifications of CAR T cells and tumor cells can boost the efficacy of CAR T cell therapy. Table 1 lists some challenges to CAR T cell therapy and summarized some epigenetic modification-based overcoming solutions.

1 Epigenetic mechanisms

The term 'Epigenetic' defined as "on top of" genetic, describes changes in gene expression independent of the genetic code itself. Epigenetic modifications can be divided into four main areas: i) genomic or DNA modifications that are a result of cytosine methylation or hydroxy-methylation, ii) histone modifications that are a result of acetylation, methylation, phosphorylation, and etc. iii) non-coding RNA associated modifications that are responsible for miRNA (and other ncRNAs) associated gene expression, and iv) higher-order associated modifications [7,8]. The placement, turnover, and activity of DNA or histone modifications can be conducted by "writers", "erasers", and "readers" respectively. While writers and erasers are responsible for DNA and histone epigenetic modifications, reader proteins translate these modifications to cell behaviors including: gene expression, DNA repair, and replication through employing their specific domains that recognize certain epigenetic modifications [9,10].

1.1 DNA-associated modifications

DNA can be modified by several mechanisms and direct nucleotide methylation is the most common of these modifications. CpG islands involved cytosine base methylation at carbon 5 . DNA can be marked by de novo mechanisms (catalyzed via DNMT3A/B/L) and once stabilized can be inherited through sequential cell divisions and maintained by the activity of DNA methyltransferase 1 (DNMT1) [11]. Another family of proteins, named ten-eleven translocation (TET), is responsible for demethylation events [12]. Methylation at promoters often causes a decrease in gene expression and suppression of gene transcription. In contrast, highly expressed genes show high levels of methylation inside the genes (i.e., introns), however, low degree of methylation at promoter or regulatory sites of the genes is observed [11,13].

1.2 Histone-associated modifications

At the next level, DNA is wrapped around core proteins called histones. These cationic proteins can be posttranslationally modified by acetylation, methylation, phosphorylation, ubiquitination, SUMOylation (Small ubiquitin-related modifier), and lactylation [14-16]. Histone modifications can be permissive, poised, and repressing, depending on which histone tail residue is modified. For example, Histone acetylation (Histone 3 lysine 27) is an activating or permissive histone modification by increasing chromatin accessibility at promoter or enhancer sites of genes. Another well studied histone modification is methylation. In contrast to histone acetylation, histone methylation is more complicated. For instance, lysine 4 of histone 3 trimethylation (H3K4me3) is associated with permissive promoters or enhancers

and euchromatin structure of genome, but mono-methylation of lysine 4 of histone 3 (H3K4me1) is associated with poised enhancer sites [17]. In contrast, trimethylation of lysine 27 of histone 3 (H3K27me3) gives rise to heterochromatin and repression of gene transcription [17]. Other modifications including histone ubiquitination, SUMOylation and lactylation can also occur. These modifications are essential for maintaining gene expression and overall chromatin structure in cells. It has been shown that mono-ubiquitination is associated with transcriptional activation while de-ubiquitination suppresses transcription. Consistently, histone SUMOylation is associated with transcription repression [18].

1.3 Non-coding RNAs-associated modifications

Non-coding RNAs provide another level to epigenetic regulation. Interestingly, the majority of human transcripts don't encode for proteins but play important roles in cell differentiation and function. Regulatory ncRNAs can be divided in two subgroups based on their size: short chain non-coding RNAs (include miRNA, siRNA, piRNA) and long non-coding RNA (lncRNA) [19]. Recent studies have shown that ncRNAs and more specifically miRNAs and siRNAs can effectively silence genes by altering histone deacetylation, methylation, and DNA methylation [20-22]. In the following sections we will discuss the how modulation of miRNA either through their repression or overexpressing (restoring) can alter the function of (CAR) T cells. There are many tools to inhibit miRNAs including antisense anti-miR oligonucleotides (AMOs), locked nucleic acid (LNA) anti-miRNAs, antagomirs, miRNA sponges, miRNA masks and miRNA-targeting small molecule inhibitors [23]. Nearly all these tools use complementary single strand oligonucleotides to sequester the targeted miRNA. Other tools that can be used to enhance miRNA expression are including miRNA mimics and miRNA expression vectors [23].

1.4 Higher-order-associated modifications

Eukaryotic DNA wraps around core histone proteins to form a nucleosome. The dynamics of higher order chromatin organization and condensation plays a major role in the regulation of replication, DNA repair, recombination and more importantly acts as an epigenetic modifier in gene transcription and expression [24]. Importantly, chromatin accessibility changes in line with T cell differentiation [25]. Chromosomes are separated into discrete topologically associating domains (TADs). TADs are highly self-interacting regions in the DNA molecule. This means DNA sequences within a TAD interact with each other more frequently than other DNA sequences [26-28]. Each TAD restricts the spread of heterochromatin or euchromatin by boundaries. These boundaries are enriched with a transcriptional repressor known as

CCCTC-binding factor (CTCF), a protein acts as an insulating factor. TADs are mainly conserved throughout development across species; however, the dynamics of their intra-interactions occurs in a tissue- and cell-specific manner [28,29]. Figure 1 illustrates the various epigenetic levels in the eukaryotic cells.

2 Epigenetic mechanisms in T cells

In recent years, epigenetic studies demonstrated how epigenetic mechanisms regulate T cell activation, maturation, and exhaustion [8,30]. Epigenetic mechanisms important for T cell differentiation are discussed below.

2.1 DNA-associated mechanisms in T cells

In naïve T cells, effector genes are generally methylated; while, naïve/memory-associated genes exist in an unmethylated form. In contrast, effector T cells are characterized by methylated naïve/memory-associated genes and unmethylated effector genes. As T cells transition into central and/or stem cell memory subsets, their effector genes can be remethylated [8,31,32]. Histone methyltransferases and DNA methyltransferases (DNMT1, DNMT3A) are highly active in exhausted T cells. Inhibiting DNMT3A can prevent terminal exhaustion of T cells and increase the development of less-differentiated T cells in chronic inflammations such as chronic viral diseases or cancers [33]. Also, inhibition of DNMT3A at early stages of T cell differentiation into effector cells leads to the development of central memory T cells [34,35]. These studies indicate that upon T cell activation and differentiation, based on the cell fate, several genes are required to be methylated and demethylated to form effector or memory cells [8]. Indeed inhibiting de novo methylation of effector related-genes in naïve T cells upon activation can reduce effector cell population and increase memory-like cell population [35]. Moreover, inhibition of de novo methylation of genes in effector or memory T cells can prevent formation of terminally exhausted T cells upon chronic antigen exposure [36]. In another interesting study, it was demonstrated that decreased methylation at the CX3CR1 locus in effector memory T cells promotes their migratory activities [37]. Taken together, these findings show that DNA methylation is a critical epigenetic mechanism underlying T cell fate and behavior. Figure 2 illustrates epigenetic alterations in T cells during their differentiation program.

2.2 Histone-associated mechanisms in T cells

As we mentioned above, histone methylations can be divided into three groups, permissive histone modifications such as H3K4me3, repressive histone modifications like H3K27me3, and poised histone modifications such as H3K4me1 and H3K27me3 at enhancer sites or H3K27me3 and H3K4me3 at promoter sites [17]. Several studies have shown the importance of histone methylation in T cells. In naïve T cells H3K4me3 and H3K27me3 modification are frequently observed. H3K4me3 modifications are also commonly found in memory T cells [38]. For example, H3K4me3 modification at a number of loci including TCF7, LEF1, KLF2

supports the formation of central memory T cells (T_{CM}) and stem cell memory T cells (T_{SCM}). Increase in H3K27me3 at memory-related genes induces the differentiation of effector memory T cells (T_{EM}) [39]. Interestingly, increases in H3K4me3 and decrease in H3K27me3 at the Gcnt1 locus enhance the trafficking of memory T cells to tumor sites in an IL-15 dependent manner [40]. Moreover, while acetylation and phosphorylation of histones H3 and H4 are decreased upon T cell activation (in proximal regions of the IL-2 promoter) [41], downregulation of diacetylated histone H3 in T cells in chronic viral infection led to development of exhausted T cells. These findings suggest that inhibition of histone H3 deacetylase may enhance the function of T cells [42]. Histone acetyltransferase binding to ORC1 (HBO1)-mediated acetylation of histones H3 and H4 promotes the development of exhausted T cells. This might be explained by the role of TOX transcription factor [43]. TOX interacts with acetyltransferase in the HBO1 complex (KAT7) and enhances the gene expression of inhibitory receptors in T cells. More specifically, H3K27 acetylation at intronic and intergenic regions is shown to be associated with T cell exhaustion [44]. In aggregate, it seems that inhibition of H3K27 acetylation and/or using histone acetyltransferase inhibitors (HATi) can reverse T cell exhaustion. Increasing evidence supports a role for HATi and histone deacetylase inhibitors (HDACi) in modulating T cell fate and function. For instance, adding HDACi and IL-21 to cultures of differentiated CD8+ T cells led to enhanced central memory T cell development [45]. Another study showed that HDAC1 and 2 inhibitors promote differentiation of CD4+ T cells to cytotoxic CD4+ T cells which are enriched for gene signatures characteristic of cytotoxic CD8+ T cells [46]. In line with this finding, treating mice with HDAC1 inhibitor results in an upregulation of pathways and genes responsible for CD8+ T cell cytotoxic activity [47]. Altogether, it seems that application of HATi or HDACi can be useful for the generation of more functional CAR T cells. Still, current HDACi and HATi are mainly pan HDAC inhibitors or pan HAT inhibitors. These findings highlight a need for the development of new epidrugs targeting specific HDACs and HATs. Also, more studies are needed to evaluate the effects of HATi and HDACi on T cell differentiation and function. Besides the methylation and acetylation of histones, SUMOylation is important for Treg differentiation and its loss maintains repressive histone remodeling at FOXP3 promoter [48]. Recent studies have also shown that lactylation of histones is associated with metabolic changes in M1-polarizing macrophages differentiating in response to LPS treatment [49]. In agreement with this notion, a study has reported that inhibition of lactate dehydrogenase (LDHi) combined with IL-21 can induce stem cell memory phenotype in the treated T cells [50]. Although speculative, the authors suggest that LDHi may affect H3K79 methylation and

histone lactylation [49]. The biological role of histone ubiquitination, SUMOylation and lactylation remains to be fully elucidated, and further research is required to address the exact function of these modifications in T cells.

2.3 Non-coding RNA-mediated mechanisms in T cells

Non-coding RNAs and transcription factors also regulate gene expression and chromatin remodeling. Intuitively, silencing "non-T cell genes" via posttranscriptional gene suppression is a viable means to direct T cell differentiation during development. Similarly, miRNAs modulate T cell activation, proliferation, and transition to central memory versus effector differentiated subsets by silencing or inducing target genes. Silencing inhibitory receptor transcripts by miRNAs (e.g. miR-138) can overcome T cell intrinsic dysregulations (e.g. T cell exhaustion) and promote tumor regression in the context of cancer immunotherapy [51]. It's increasingly accepted that activated T cells undergo transcriptional changes as well as miRNAome modifications to support cell proliferation and effector functions [52]. The miRNAome repertoire continuously adapts during the ordered T cell differentiation process. [53,54]. Furthermore, several miRNAs involved in silencing essential transcripts for T cell effector function can be repressed by miRNA inhibitors in the context of cancer immunotherapy. For instance, the miR-17-92 cluster is highly active during T cell proliferation and differentiation. MiR-17-92 promotes mammalian target of rapamycin (mTOR) signaling by targeting mTOR inhibitory molecules such as PTEN and PHLPP2. Notably, miR-17-92 overexpression promotes T cell differentiation and survival. In addition, miR-17-92 deficient T cells are impaired in proliferation and differentiation [55].MiR-17-92 manipulation has resulted in changes in Id3 expression, a DNA-binding inhibitor which is a key regulator in memory differentiation, thus, miR-17-92 levels can balance the effector/memory ratio in T cells [56-59]. Consistently, miR-15/16 clusters have been shown to restrain memory T cell formation and differentiation by targeting several memory and survival associated mRNAs [60]. Several other miRNAs influence T cell activation, expansion, survival, and antitumor response including miR-155, by targeting SHIP-1 and promoting PI3K-AKT and STAT signaling [61,62], miR-146a, which targets NF-κB and interrupts T cell effector function [63-65], miR-23a, which upregulated in CTLs in response to TME-secreted TGF-β, can target the transcription factor Blimp-1 which is required for T cell differentiation and cytotoxicity. Therefore, targeting miR-23a and maintaining desired levels of Blimp-1 can prevent tumor-dependent immunosuppression [66]. MiR-139, miR-150, and miR-342 have also shown to target eomesodermin (EOMES), CD25 and perforin [67]. Additionally, miR-150 can

target c-Myb and its downregulation leads to memory T cell development [67,68] while its overexpression has been shown to reduce T cell proliferation [69]. MiR-214 enhances T cell proliferation upon activation by targeting PTEN [70], and miR-181a is a tolerogenic miRNA and target serine/threonine phosphatases in T cells [71,72]. Other forms of ncRNAs including siRNAs have also been studied in T cells. For instance, siRNA-mediated depletion of EOMES, which is enriched in terminally exhausted T cells [73], results in enhanced killing capacity and cytokine production in CD8+ T cells [74]. Altogether, there are plenty of ncRNAs which can shape T cell phenotype and function. Several studies have demonstrated that non-coding RNAs including miRNAs can regulate other epigenetic mechanisms (DNA and histone modification). For instance, it has been shown that miR-29 can inhibit the activity of de novo DNA methylation [75,76] while miR-17-5p and miR-20a can induce heterochromatin formation [77]. Other types of non-coding RNAs such as piRNAs and lncRNAs also have been shown to regulate gene expression by DNA and histone modifications [reviewed in [51]].

2.4 Higher-order mechanisms in T cells

Often viewed as "higher order levels of epigenetic remodeling", cell type specific DNA remodeling in TADs can bring distal enhancer regions near a promoter to further regulate gene expression. This type of epi-reprogramming regulates cytokine expression in CD4+ T cells and CD8 expression in CD8+ T cells [78-80]. Chromatin remodeling factors including CTCF, and cohesion complexes play pivotal roles in the regulation of chromatin interactions, accessibility, nucleosomal sliding and ultimately gene expression. Loss of these proteins can disrupt chromatin interactions resulting in aberrant gene expression [81,82]. For instance, CTCF and cohesion ablation in thymocytes impairs T cell differentiation [83,84]. Interaction of epigenetic readers, writers, and erasers with chromatin remodeling proteins can facilitate and regulate gene expression. In a study, it has been demonstrated that following TCR stimulation of CD8+ T cells, chromatin remodeling by STAB1 results in recruitment of a histone deacetylase complex at enhancer sites of Pdcd1. This complex downregulates PD-1 expression. Interestingly, tumor-derived TGF-β downregulates STAB1 expression through binding of SMAD proteins to STAB1 promoter. It also releases the pdcd1 promoter from STAB1 repression by competing with STAB1 for the binding to enhancer of pdcd1. This competition results in increased pd-1 expression [85]. Additionally, recent studies have found that the HMG-box transcription factor TOX is a central regulator of T cell exhaustion [43,44,86]. In the absence of TOX, the development and formation of exhausted T cells is blunted [86]. These findings suggest that TOX can promote chromatin remodeling and alter genomic

architecture to govern the epigenetic development of exhausted T cells [43,86]. Table 2 summarizes various epigenetic mechanisms in T cells.

Taken together, these studies shed light on the several layers of epigenetic reprogramming in T cell immunobiology. As these layers converge on T cell differentiation, it's likely that epigenetic modifications can be increasingly employed in context of cancer immunotherapy. In theory, epigenetic remodeling of (CAR) T cells can overcome several challenges to CAR T cell therapy through increasing the persistence and survival of T cells, diminishing T cell exhaustion, improving their infiltration, and promoting memory phenotype formation.

3 Epigenetic reprogramming of CAR T cell differentiation to produce less differentiated CAR T cells

Human and murine studies have shown that less differentiated CD8+ T cells exhibit superior antitumor function [87-89]. As the general CAR T expansion protocol gives rise to terminally differentiated cells, optimizing the CAR T cell manufacturing process to produce less differentiated cells is greatly needed. Almost all CAR T cell production protocols need ex vivo expansion that result in the generation of more differentiated T cells with low proliferative capacity *in vivo* [90]. Manipulation and modification of T cell-specific signaling pathways (following stimulation) can uncouple expansion from differentiation. In support of this principle, disruption of TET2 (a chromatin modifier that encodes methyl cytosine dioxygenase enzyme that facilitate DNA demethylation to activate gene expression) in a T cell clone resulted in potent antitumor activity and proliferation of anti-CD19 CAR T cells in a patient [91]. Interestingly, a metabolic by-product, S-2-hydroxyglutarate (S-2HG), limits effector differentiation and supports increased memory formation and prolonged persistence of CAR T cells [92,93]. This by-product competitively inhibits α-ketoglutarate-dependent proteins such as TET family proteins [8]. Thus, inhibition of *ex vivo* CAR T cell differentiation by TET2 elimination or inhibition can lead to enhanced CAR T cell durability. Furthermore, inhibition of the histone acetylation reader BRD4, a member of the bromodomain and extra terminal domain (BET) protein family, by JQ-1 shows similar results with S-2HG treatment [94]. In general, during T cell differentiation into effector phenotype, several "memory" genes including FOXO1, KLF2, LEF1, TCF7, IL2RA, CD27, TNF, CCR7, and SELL are repressed. This repression is mediated by repressive DNA methylation and repressive histone modifications (H3K27me3). Conversely, effector related transcription factors and genes (e.g.

EOMES, TBX21, PRDM1, GZMA, GZMB, PRF1, IFNG, and KLRG1) are upregulated by downregulating repressive modifications at genic and intragenic regions [31,37,95-98]. Reversing these alterations via miRNAs (specific) or pharmacologic treatments), may give rise to less differentiated CAR T cells with enhanced potential.

4 Epigenetic reprogramming to promote central memory and stem cell memory phenotypes in the CAR T cells

Memory cells, and more specifically central and stem cell memory CAR T cells, are the preferred subset in the context of cancer immunotherapy. Central memory as well as stem-like T cells have higher persistence and superior antitumor activity compared to effector memory cells. Epigenetic reprogramming of CAR T cells to promote central memory, and retain stem cell, differentiated progeny will improve the efficacy of CAR T cell therapy.

Transcription factors play an important role in T cell differentiation. In the context of chronic infections, Blimp-1 drives the differentiation of short-lived T cells with an effector phenotype. Interestingly, Blimp-1 co-localizes with two epigenetic modifiers (it would be good to mention these?) at the loci for IL-2 and CD27. Blimp-1 promotes effector cell differentiation through epigenetic repression of memory associated genes similar to other repressive epigenetic modulators [35,99-101]. Genetically disabling of Blimp-1 in CD8+ T cells promoted memory cell formation and more importantly enhanced proliferative capacity of CD8+ cells in response to IL-2 [102]. Therefore, genetic, or epigenetic disruption of Blimp-1 in CAR T cells by CRISPR/Cas-9, miRNAs (e.g. miR-23a), or siRNAs may also improve cell proliferation, memory cell formation, and antitumor activity. As discussed above, inhibition of DNMT3A at early stages of T cell differentiation leads to memory T cell development [34,35]. Potentially, DNMT3A inhibitors might become useful in the manufacturing of CAR T cells. As mentioned before, miR-15/16 can target several memory-associated mRNAs including Bcl2, CD28, and IL-7R transcripts, and attenuate memory T cell formation and differentiation as well as effector functions in CD8+ T cells [60]. In addition, miR-150 targets c-Myb. C-Myb regulates anti-apoptotic pathways and promotes CD8+ T cell memory differentiation [68]. Since miRNAs are involved in T cell differentiation, they can influence memory/effector formation. Thus, overexpression of some miRNAs as well as downregulation of memory restraining miRNAs such as miR-15/16 clusters and/or miR-150 may become an important strategy for restoring function in CAR T cells and the development of memory CAR T cells. Inducing central memory CAR T cells by miRNA has been examined by Zhang et al, [103]. They have shown that miR-143 can regulate memory T cell

differentiation through metabolic changes (glucose restriction) in CD8+ T cells. They also showed that overexpression of miR-143 leads to reduced expression of exhaustion marker KLRG1 and increased expression of memory-related marker CD127 in T cells. To show the advantages of overexpression of miR-143, they transfected miR-143 mimics in anti-HER2 CAR T cells. They observed that tumor cell lysis was increased in miR-143 overexpressed group. Complementary loss-of-function approaches showed that antagomirs to miR-143 decreased T cell-mediated cytotoxicity. Mechanistically, miR-143 regulates the expression of glucose transporter 1 (Glut-1) by binding to its 3' UTR transcript. The same results were also observed where Glut-1 was targeted by siRNA in the T cells. Additionally, their data showed that miR-143 can promote central memory T cell formation and induce higher antitumor response by inhibiting glucose metabolism [103]. Although epigenetic manipulation of genetically engineered tumor specific T cells by miRNAs holds great promise, caution should be taken particularly in case of adoptive transfer to patients. In addition, further studies are required to assess differentiation and effector function of CD4+ cells. Future studies will reveal if epidrugs that repress terminal effector-related genes as well as miRNAs or DNA/histone modifiers that induce memory-related genes can be used in CAR T cell therapy aiming to promote and maintain CAR T cells with memory phenotype.

5 Epigenetic reprogramming to enhance CAR T cell infiltration

Tumor cells employ several mechanisms to escape from CAR T cell surveillance. Insufficient trafficking of CAR T cells to the tumor site is one barrier to adoptive immunotherapies. Indeed, enhancing CAR T cell infiltration to the tumor site can overcome this immune evasion tactic. Several factors contribute to insufficient trafficking of CAR T cells to tumor site such as mismatched chemokine receptor with secreted chemokine in tumor bed, downregulation of adhesion molecules, and physical barriers at tumor site [104]. Also, overexpression of chemokine receptors in CAR T cells can enhance infiltration capacity of CAR T cells into tumor bed. Zhou et al., demonstrated that the concurrent downregulation of PD-1, TIM3 and LAG3 in anti-Her2 CAR T cells, increases their infiltration to tumor sites. Increased chromatin accessibility at the CD56 locus as well as upregulation of chemokines CXCL9, CXCL10, and CXCL12 enhanced the ability of PD-1/TIM3/LAG3 modified CAR T cells to infiltrate tumors[105]. CASTAT5 (a constitutively active form of STAT5) expression can also enhance infiltration and trafficking of CD4+ T cells to tumor site by epigenetic remodeling [106], indicating the importance of STAT proteins in higher-order epigenetic modifications. In several cancers, increased expression of CXCR3 observed in TILs was

correlated with more infiltration. Therefore, inducing CXCR3 in CAR T cells by epigenetic mechanisms can be useful in cancers expressing CXCL9, CXCL10, and CXCL11 chemokines. Consistently, it has been shown that miR-155 deficient T cells have impaired function as well as trafficking [107] as miR-155 targets suppressor of cytokine signaling 1 (SOCS-1) in T cells [62]. It seems miR-155 upregulation in CAR T cells might be an interesting strategy to promote trafficking and effective antitumor response of CAR T cells. Forced-expression of CCR2 has been shown to enhance trafficking and survival of CAR T cells in cancer [108,109]. Interestingly, let-7 miRNAs target and inhibit CCR2 as well as CCR5 expression in T cells [110]. Targeting of let-7 might therefore be a possible option for improving of CAR T cell infiltration. CX3CR1 enhances immune cells infiltration and tumor regression in CX3CL1+ tumors [111]. T cells genetically engineered to overexpress CX3CR1 have significantly enhanced infiltration [112]. Although until now no experimentally approved miRNAs that can target CX3CR1 in T cells have been reported. miR-27a-5p targets and inhibit expression of this chemokine receptor in NK cells [113]. Thus, targeting miRNAs that inhibit CX3CR1 expression can be an attractive approach for enhancing infiltration of CAR T cells. Another chemokine receptor supporting infiltration is CXCR6. This chemokine receptor is expressed on naïve T cells at low levels. Murine T cells deficient for CXCR6 are unable to properly infiltrate into mammary tumors [114]. Another interesting strategy would be to selectively modify tumor cells (epigenetically) to release chemokines that enhance CAR T cell infiltration. Table 3 highlights important T cell miRNAs that can be potentially modulated for effective CAR.

6 Epigenetic reprogramming to improve CAR T cell persistence

Among several barriers in CAR T cell therapy, poor persistence of infused CAR T cells is a critical challenge. In general, suboptimal persistence is correlated with poor clinical remission in the cancer [115]. Moreover, CAR T cells with impaired persistence show limited antitumor efficacy. One factor that limits persistence is replicative senescence which can lead to reduced proliferative capacity and counter long-term therapeutic efficacy [116]. Telomere shortening, damage, and erosion contribute to senescence [117]. Like other cells, after each replication cycle the length of telomere is shortened in CAR T cells. In this regard, Bai et al., demonstrated that increasing telomere abundance in anti-CD19 CAR T cells is associated with prolonged persistence and efficient antitumor response [118]. Several studies have discussed STAT signaling as a key pathway in the promotion of cell persistence [4]. CAR T cells expressing CASTAT5 show higher persistence and expansion rates as well as superior

antitumor function. The authors demonstrated that these responses are a consequence of chromatin and epigenetic remodeling in the T cells as a majority of non-coding genomic regions become highly accessible. Furthermore, the expression of various genes associated with T cell dysfunction such as Runx2, Id2, Nr4a2, and TOX were reduced, while several other genes such as Gata1, Jun, Junb, Fos, Fosl2, and EZH2 were upregulated and activated [106]. Therefore, epigenetic modification of the STAT5 pathway maybe a promising strategy to promote the generation of highly persistent and polyfunctional CAR T cells. Bcl-2 proteins can be divided into two groups based on inducing (pro-apoptotic) or inhibiting (anti-apoptotic) apoptosis in the cells. Indeed, anti-apoptotic members such as Bcl2 can enhance CAR T cell persistence [119]. Another approach to enhance CAR T cell persistence is by downregulating pro-apoptotic proteins such as Bad and/or Bax. Altogether, it seems that targeting of Bcl-2 family proteins by siRNA or other epigenetic mechanisms (e.g. miRNAs) can modulate CAR T cells persistence. However, possible adverse effects related to hyper-activation of CAR T cells must be assessed in the future studies. Since persistence is related to several other mechanisms in (CAR) T cells such as memory cell formation, other transcription factors influencing effector/memory formation can also regulate CAR T cells persistence. Kagoya et al., demonstrated that inhibition of histone acetyltransferase p300 (writer) and BET protein BRD4 (reader) not only leads to downregulation of BATF transcription factor and induce prolonged persistence but also promote expression of memory associated markers and genes in T cells [94]. Moreover, upon TCR or CAR stimulation of naïve or memory precursor cells, BMI1 expression is induced in (CAR) T cells. In contrast, BMI1 expression is lost in terminally differentiated T cells. BMI1 is a member of the Polycomb repressive complex 1 (PRC1) family that reads H3K27me3 histone tails. This protein represses expression of genes that promote senescence and apoptosis (p16INK4A and p14ARF) in many cells like T cells [120,121]. Reduced levels of BMI1 leads to defects in T cell expansion [122]. Therefore, maintaining BMI1expression at balanced levels or its overexpression in CAR T cells can be an ideal approach to enhance persistence and function in CAR T cells. Besides epigenetic modifications of DNA and histones, miRNAs can affect (CAR) T cells function and persistence. Forced expression of miR-155 in CD8+ T cells enhances their antitumor activity, persistence, and proliferation [123]. Sasaki and colleagues demonstrated that miR-17-92 is downregulated in T cells from glioblastoma patients conferring lower persistence rate of tumor specific T cells and thereby diminished tumor control[124]. Thus, re-expressing this miR cluster in T cells from cancer patients might promote T cell persistence. In line with this notion, Ohno and colleagues demonstrated that miR-17-92

overexpression in anti-EGFRvIII CAR T cell improves their persistence, proliferation and antitumor function in a glioblastoma xenograft model. Epigenetically reprogramming of patient-derived T cells may be an innovative strategy to generate of more functionally effective CAR T cells [125]. Mechanistically, miR-17 and miR-19b are parts of miR-17-92 cluster and shown to be critical for Th1 response by targeting TGFβRII and PTEN, respectively. These miRNAs not only promote T cell proliferation and IFN-γ production, but also protect T cells from activation induced cell death (AICD) [55,126]. Future studies will likely reveal the impact of miRNA-mediated posttranscriptional gene silencing as well as epigenetic modifications to Bcl-2 on CAR T cell persistence and effector function in clinical settings.

7 Epigenetic reprogramming to overcome CAR T cells exhaustion

Due to the immunosuppressive tumor microenvironment and prolonged antigen exposure, infused CAR T cells can become progressively dysfunctional. The ability of "exhausted" T cells to eliminate cancer cells wanes; hence, prevention from exhaustion will greatly enhance the rate of tumor regression and remission [127]. Until now, one of the main strategies to overcome exhaustion was immune checkpoint blockade. Recent work published by Beltra and colleagues reported that exhausted T cells have four distinct developmental stages, the two first subsets are TCF1+, an intermediate subset which is $TCF1^-$ and shows higher levels of effector function compared to other subsets, and fourth is composed of terminally exhausted T cells [128]. Following PD-1 therapy, the intermediate subset expands more than other subsets. While the cells can regain some elements of functional competence, they never regain their central memory status. Therefore, more effective tools to revert exhaustion in T cells is needed. As T cells undergo differentiation, their DNA methylation status is modified by DNMTs [8]. Also, data has been shown T cells from cancer patients are largely dysfunctional [129]. And this arises from dysregulation in methylation status of its epigenome [130]. DNA methylation can lead to exhaustion and limit T cell-based immunotherapy [36]. T cells from cancer patients are largely dysfunctional due to dysregulation in the methylation status of their epigenome [129,130]. In line with these notions, targeting a major component of the de novo DNA methylation program: DNMT3A, either pharmacologically (decitabine, a DNMTi) or genetically, preserves the proliferation of these functionally incompetent T cells. Treated T cells express naïve and memory-related genes at higher levels and downregulate exhaustion-related genes despite prolonged antigen exposure [131-133]. During exhaustion, increase in the expression of exhaustion-related transcription factors such as bZIP-IRF family

with or without an increase in expression of JunB or BATF is a consequence of dysregulation of AP-1 transcription factor binding motif. In exhausted cells, AP-1/IRF complexes cause limit the formation of the AP-1/Fos-Jun heterodimer. Consistent with this, a recent study showed that overexpression of c-Jun led to the formation of exhaustion-resistant CAR T cells with an increase in antitumor function and decreased expression of PD-1 and CD39. This finding reveals that c-Jun overexpression can activate AP-1 and prevent the formation of exhaustion related complexes in chromatin [134]. Several studies have demonstrated that NR4A and TOX family of proteins are upregulated in T cell exhaustion [25,43,135-139]. Therefore, targeting of NR4A family proteins and TOX family TFs can lead to reduced expression of inhibitory receptors and promotion of effector function in CAR T cells [86]. Mechanistically, TOX is induced by NFAT2 in a calcineurin-dependent manner. At later phases of the differentiation program, TOX operates in a feed forward loop and becomes calcineurin independent. Therefore, sustained levels of TOX are associated with exhaustion [43]. Also, persistent levels of TOX and NR4A interfere with NFAT. "Partnerless" NFAT or NFAT that doesn't cooperate with AP-1 at their cognate response elements in genetic loci, induces the expression of inhibitory genes in T cells to promote exhaustion [86]. Moreover, since T cell studies have shown the importance of TOX transcription factor and acetyltransferase HBO1 complex in chromatin remodeling and promoting T cell exhaustion, targeting of HBO1 complex in CAR T cells by specific miRNAs or CRISPR/Cas-9 may become a beneficial strategy to revert exhaustion and enhance antitumor efficacy of CAR T cells in the clinical practice. Indeed, expression of memory-related or effector-related transcription factors lead to an epigenetic rewiring of T cells. Importantly, miR-28, and miR-138 can target immune checkpoints like PD-1 and CTLA-4 and revert an exhausted phenotype [140,141]. MiR-28 has been identified as a key miRNA in rescuing IL-2 and TNF-α secretion in TME [140].

8 Interplay between epigenetic modifications and CAR T cells metabolism

Metabolic fitness is an important determinant of efficacy in CAR T cells [142]. It's well established that T cells alter their metabolic activity to support their growth and differentiation. Naïve and early-memory T cells rely on oxidative phosphorylation (OXPHOS) and mitochondrial metabolism [143,144]. Following TCR stimulation, activated T cells rapidly shift their metabolism from OXPHOS to glycolysis. Restricting glucose metabolism to the cytoplasm (aerobic glycolysis) channels metabolites into anabolic reactions supporting macromolecular biosynthesis [144,145]. Primary human T cells also rely on glutamine metabolism, both oxidative and reductive, to support acetyl-CoA synthesis [146]. Acetyl-CoA

provides the functional acetyl group for histone acetylation, providing means to control gene expression at the IFN-γ locus in activated T cells [147,148]. Understanding which substrates fuel the TCA cycle and hence short chain CoA replenishment is important as mitochondrial function is compromised in T cells traversing solid tumor environments [149]. Intrinsic deficits in mitochondrial function, that occur in terminally differentiated T cells, exhausted T cells, and T cells traversing hypoxic tumors will undoubtedly impact epigenetic reprogramming and gene expression. Deficits in mitochondrial function and energy-generating capacity also occur following chemotherapy treatment [150].

Competition for nutrients in solid tumors has important implications for T cell function. Extracellular glucose levels diminish in tumors, and interstitial lactate levels increase in a reciprocal manner [151]. Histone lactylation is an under-studied epigenetic remodeling event with important implications for T cell function. To date, 28 lactylation sites on histone proteins have been identified. A recent study provided evidence that lysine lactylation regulates ARG1 expression in differentiating macrophages [49]. Histone lactylation regulates cancer associated fibroblast differentiation in the context of pancreatic ductal adenocarcinoma [152]. With the development and increased use of small molecules targeting lactate production (LDHi) and lactate secretion (inhibitors to Monocarboxylate transporters), additional studies are needed to understand their impact on epigenetic remodeling on adoptively-transferred T cells in solid tumors.

Restricting epigenetic remodeling events (via metabolic regulation) to distinct T cell subsets may give rise to metabolically fitter (CAR) T cell progeny with superior antitumor activity. Metabolites other than acetyl-CoA influence epigenetic regulation in T cells. S-adenosyl-methionine (SAM) regulates DNA and histone methylation in T cells by providing methyl groups, whereas the tricarboxylic acid (TCA) cycle intermediate, and glutamine derivative, α-ketoglutarate regulates DNA and histone demethylation in an oxygen-dependent manner [153,154]. Suppressing α-ketoglutarate-mediated demethylation of DNA using S-2HG increases the formation of CD8+ central memory CAR T cells [93]. Glycolysis replenishes nicotinamide adenine dinucleotide (NAD+) a fundamental metabolite regulating histone deacetylation [155]. NAD+ is an important cofactor for sirtuins, a family of enzymes that regulate gene expression in a redox-sensitive manner [93]. microRNAs also regulate CAR T cell metabolism by epigenetic effects. miR-143 overexpression in CAR T cells decreases glucose uptake and induces expression of carnitine palmitoyl transferase 1a (CPT1A). CPT1A is a rate limiting enzyme in fatty acid oxidation (FAO). In line with energy partitioning principles, increasing

FAO leads to a corresponding decreased reliance on glucose and glycolysis. Of note, FAO is a metabolic pathway previously implicated in the production of memory CAR T cells [103,156]. However, the role of FAO in memory differentiation is controversial as it's largely based on studies using nonspecific doses of etomoxir. Some tumors such as renal cell carcinoma display a unique preference for glutamine over glucose [157]. Thus, future studies are necessary to uncover what fuels mitochondrial function during memory T cell differentiation *in situ*, and how the resultant metabolic pathways converge on epigenetic reprogramming. Taken together, reprogramming of CAR T cell metabolism through using epidrugs is an interesting strategy for the generation of metabolically fitter CAR T cell with superior antitumor activity. However, more studies are needed to fully elucidate possible crosstalk between epigenetics and metabolism in (CAR) T cells.

9 Epigenetic strategies in CAR T cell combination therapy

To overcome hostile conditions found within tumors, combining CAR T cell therapies with various cancer treatment modalities has been gained increasing attention. With the renewed interest in combinatorial approaches, the role of both tumor cell immunobiology and the status of endogenous immune system must be considered. In the following sections we discuss how such combination therapies, in particular epigenetic modifiers, can significantly improve the efficacy of CAR T cell therapy.

9.1 Epigenetic strategies to upregulate desirable antigens in tumors

Antigen loss and/or antigen heterogeneity are important barriers impeding efficacy in CAR T cell therapy. Upregulation or induction of tumor-associated antigens (TAAs) or cancer testis antigens (CTAs) can overcome antigen heterogeneity and prevent antigen loss in the tumors. Epigenetic drugs, especially those can facilitate DNA demethylation and/or histone acetylation, have the potential to upregulate all antigen processing machinery components; increasing TAA abundance, as well as costimulatory molecule expression such as CD40 and CD80 and MHCI/II [158-160], stress and death-induced ligands including DR5 and TRAIL [161,162]. DNMT inhibitors (DNMTi) and histone deacetylase inhibitors (HDACi) enhance cancer cell recognition by increasing the expression of CTAs, which are expressed in embryonic and germ cells whereas in somatic cells their promoters are methylated. DNMTi promotes DNA demethylation and permitting the re-expression of CTAs in cancer cells in several solid tumors [66,163-165]. HDACi also induces the expression of CTAs at lower levels compared to DNMTi [166]. As we discussed previously, inhibition of DNMT3a in T cells can reduce the

abundance of exhausted T cells and promote the generation of central memory cells [33,34]. Following treatment with decitabine, CAR T cells showed superior antitumor activity [132]. A recent study demonstrated that pretreatment of lymphoma cells with decitabine leads to CD19 overexpression and enhanced CAR T cell function. They also reported that two patients who received decitabine before CAR T cell therapy achieved complete responses [167]. Viral tumor antigens can also be expression following DNMTi [168]. Over time, and consequent to viral infection, Human Endogenous Retroviruses (HERVs) are integrated into the genome of germ cells. While most HERVs are non-coding, several HERVs encode functional proteins such as HERV-K HML-2 isoforms[169]. HERVs are expressed followed by DNMTi and HDACi [170]. Anti-HERV-K CAR T cells have shown antitumor promise in xenograft models of melanoma [171]. Ewing sarcoma (EwS) is a solid tumor characterized by low antigen expression and high antigen heterogeneity. Expression of Enhancer of Zeste Homolog 2 (EZH2; an enzyme that contributes to the histone methylation) in Ewing sarcoma cells is associated with reduced antigen presentation and decreased immunogenicity [172]. Pharmacological targeting of EZH2 at doses reducing H3K27 trimethylation could selectively and reversibly induce GD2 surface expression in tumor cells. They concluded that combination therapy with CAR T cells plus EZH2 inhibitors may significantly enhance antitumor activity of CAR T cells [173].

Similarly, Kunert et al., reported DNMTi and HDACi treatment led to CTA MAGE-C2 overexpression in breast cancer cells. CTA MAGE-C2 antigen is a corresponding target for genetically engineered T cell expressing MAGE-C2-TCR [174]. Taken together, epigenetic modification-based combination strategies such as using DNMTi and CAR T cells, can make tumor cells more immunogenic probably through overexpression of MHCI and MHCII on tumor cell surface as well as increasing expression of costimulatory molecules including CD40 and CD80. Therefore, these combination therapies not only can synergistically activate the endogenous immune system but also can prevent and/or revert antigen loss. Moreover, CAR T cell qualities in terms of functionality and longevity can be improved through inducing memory phenotypes and repressing exhaustion by application of epidrugs.

9.2 Epigenetic strategies to overcome hostile TME features

In addition to the role of HERVs as target antigens, epigenetic modulation of HERVs can promote a type I and III interferon response which can boost the overall antitumor immune response. DNMTi and HDACi promote the transcription of HERVs in a bidirectional manner leading to formation of dsRNAs in the cells. DsRNAs can activate RIG-I and MDA5 receptors. Together with IRF3, IRF7, and NF-κB, dsRNA/RIG-I and dsRNA MDA5

complexes can activate transcription of interferons and interferon receptor interaction. These processes lead to the induction of interferon stimulated genes (ISGs) through JAK/STAT signaling pathways. Furthermore, ISGs can induce various immunomodulatory genes and increase expression of chemokines and cytokines to attract and active immune cells. A recent study demonstrated that low doses of DNMTi can increase the expression of ISGs, chemokine and cytokines, antigen processing and presentation, and CTAs [66]. Different investigations have reported that cancer cells treated with DNMTi and HDACi can increase the infiltration of CD8+ T cells to tumor sites through activation of interferon type I response [175], and overexpression of CXCL9 and CXCL10 in breast cancer tissue [176]. Moreover, lysine-specific histone demethylase 1A (LSD1) ablation in cancer cells was shown to induce interferon type I responses as well as HERVs expression and more interestingly enhance CD8+ T cell infiltration in tumors [177]. Moreover, inactivation of Nuclear Receptor Binding SET Domain Protein 1 (NSD1), a histone methyltransferase catalyzer, can reduce CD8+ T cell infiltration in several cancers. In line with this finding, inactivating mutations in NSD1 mimic the characteristics of cold tumors [178]. Thus, modifying LSD1 as well as NSD1 are interesting targets to pursue in the context of CAR T cell cancer therapy.

The epigenetic status of the PD-L2 locus in tumor cells is another relevant candidate to investigate. Methylation of several CpG sites in the PD-L2 locus correlates with CD8+ T cells infiltration in tumor sites [179]. CCL5 and CXCL9 were previously shown to play a pivotal role in T cell infiltration, whereas CCL5 is often epigenetically silenced in cancer cells. A recent study revealed that decitabine stimulates CCL5 re-expression in tumor cells and enhances T cell infiltration to tumor tissues [180]. Various studies have demonstrated that DNMT inhibitors could enhance the antitumor function of T cells by modulating the immunosuppressive activity of myeloid-derived suppressor cells (MDSCs) and macrophages within the TME [175,181-183]. Treating B16 melanoma-bearing mice with HDACi revealed that the epigenetic modification of tumor cells not only enhances the efficacy of adoptively transferred cells but also promotes the infiltration of anti-gp-100 T cells following the increased expression of gp-100 and MHC molecules [184]. In addition to inhibiting histone writers, disrupting histone readers can be also a good therapeutic option in several cancers. For example, in an ovarian cancer model and squamous cell carcinoma, treatment with JQ-1, a BET inhibitor, could remarkably decrease PD-L1 expression on tumor cells and tumor-associated immune cells [185,186]. It is well-documented that the TME contains several suppressive cells including Tregs, M2 macrophages, MDSCs, tumor-associated neutrophils,

as well as cancer-associated fibroblasts [187]. These cells exploit several mechanisms to hinder effective immune response in TME. These mechanisms include: the secretion of immunosuppressive molecules (IL-10, TGF-β, arginase, IDO, PGE2, adenosine), and increased surface expression of inhibitory molecules. These soluble and insoluble molecules can inhibit antitumor function of CAR T cells through various ways. For example, tryptophan is an essential amino acid for the activation, proliferation, and survival of CAR T cells [188]. Indole amine 2,3-dioxygenase 1 (IDO) is a rate-limiting enzyme that catabolizes tryptophan to kynurenine [189]. The production of kynurenine and catabolization of tryptophan limit CAR T cell function and their antitumor activity [190,191]. IDO is expressed in several cancers and its expression is correlated with poor T cell infiltration and clinical outcome [192]. Various studies have documented that IFN-γ can induce IDO expression as well as PD-L1 [193]. In recent studies, miR-153 and miR-448 were shown to suppress IDO1 expression effectively in colorectal xenograft models [194,195]. Furthermore, miR-153 overexpression in cancer cells could enhance CAR T cell killing capacity *in vitro* and suppress tumor growth in murine colorectal cancer xenograft model through suppression of IDO1 expression [195]. Prostaglandin E2 (PGE2) is an immunosuppressive component of TME. This soluble eicosanoid can increase cancer cell proliferation and inhibit an effective antitumor T cell response. MiR-21 and miR-155 have been shown to stimulate PGE2 dependent signaling in tumor cells through augmentation of the active form of PGE2 [196,197]. In contrast, miR-708 and miR-137 can inhibit PGE2 production and decrease the levels of this molecule in tumors [198,199]. There are several other miRNAs such as miR-126 and miR-503-5p that can target tumorigenic molecules like VEGF and inhibit angiogenesis [200-203]. MiR-30a-5p and miR-422a target CD73 [204,205], an ecto-5'-nucleotidase which can generate adenosine from AMP. Extracellular adenosine is a key immunosuppressive metabolite that restricts activation of CAR T cells and impairs their antitumor responses. In line with these findings, it has been reported that genetic and pharmacological targeting adenosine A2 receptor could significantly improve antitumor function of CAR T cells [206]. It has been also shown that miR-22 targets Galectin-9, a ligand of inhibitory receptor TIM-3 on T cells [207]. There many other miRNAs that can target TGF-β and their receptors [208-210], ROS and HIF1-α signaling pathways [211,212], as well as other immune checkpoint ligands such as PD-L1 [213,214]. In an interesting study, it has been demonstrated that miR-141 can inhibit the recruitment of immunoregulatory cells to tumor sites [215]. Altogether, these exciting findings highlight the potential of combining miRNAs and CAR T cells to elicit favorable immune responses and, thereby, better clinical outcomes.

Both cancerous and healthy cells are able to secret extracellular vesicles containing lipids, proteins, RNAs, and DNAs which are called exosomes. These extracellular vesicles facilitate crosstalk between cancer cells and immune cells. This crosstalk has been implicated in tumorigenesis process and response to therapy [216]. Cancer cells can secret and transport immunosuppressive molecules (e.g. miRNAs) to immune cells and, as a consequence, disarm these antitumor cells. In line with this concept, a recent study revealed that miRNAs in melanoma-derived exosomes can alter cytokine secretion and TCR signaling in CD8+ T cells. Some of these exosomal miRNAs (e.g. miR-181a/b and miR-498) were able to directly bind to 3' UTR of TNF and decrease TNF-α secretion. Exosomal miR-3187-3p inhibits the expression of CD45, which is known as signaling gatekeeper in TCR signaling [217]. In addition to direct control of T cell response in TME, exosomes can indirectly alter T cell antitumor response. It has been shown that exosomal miR-23a-3p can target PTEN in macrophages and reduce the expression of PTEN and phosphorylation of AKT. It can also increase PD-L1 expression on macrophages in hepatocellular carcinoma cancer [218]. Moreover, TAM-derived exosomes containing miR-29a-3p and miR-21-5p have been shown to inhibit antitumor response and enhance tumor growth by targeting the 3' UTR of STAT3 in T cells [219]. STAT3 is crucial transcription factor in Th17 differentiation. Thus, STAT3 targeting by miRNAs secreted from TAMs can increase Treg/Th17 ratio and promote immunosuppression [219]. In contrast, it has been discovered that activated T cells can secrete cytotoxic extracellular vesicle containing miR-298-5p. This miRNA can inhibit metastasis, and tumor invasion through activation of caspase-3 and induction of apoptosis in mesenchymal tumor stromal cells [220]. In aggregate, blocking the secretion or generation of cancer derived exosomes or more specifically inhibiting the expression of oncomiRs including miR-23a-3p, miR-29a-3p, miR-21-5p, miR-181a/b, miR-498 and miR-3187-3p and/or promoting immunostimulatory miRNAs including miR-298-5p may be an important therapeutic strategy in the context of CAR T cell therapy. Figure 3 illustrate different layers of epigenetics on the CAR T cells within the TME. Altogether, understanding the mechanisms of immune evasion and epigenetic alterations in tumor cells can provide an important rationale for using epidrugs in combination with adoptive cell therapies. However, further investigations are yet needed to elucidate the molecular epigenetic mechanisms responsible for immune evasion mechanisms and genetic alterations in tumor and immune cells. Table 4 represents a list of miRNAs that can be potentially targeted in tumors in the context of CAR T cell therapy.

Concluding remarks

Although CAR T cells have shown remarkable strides in the treatment of patients with advanced cancers, there are still challenges limiting their overall efficacy in solid tumors. In this review we discuss how utilizing epigenetic mechanisms and modifications can overcome these challenges (Figure 4). For example, overexpression or restoration of memory-related genes as well as the downregulation or suppression of exhaustion-related genes may enhance CAR T cells persistence and long-lasting immune surveillance.

Epigenome of patient-derived T cells is also a critical factor for generation of fully functional CAR T cells with superior antitumor activity. As accumulating data suggest, T cells derived from cancer patients may be epigenetically dysregulated compared to T cells derived from healthy individuals [221-223]. Therefore, the caution should be taken in translation of *in vitro* and *in vivo* studies to clinical trials. It seems that epigenome mapping of T cells isolated from patients might be a solution to overcome this challenge. With the help of epigenome data extracted from patients T cells, it would be possible to reprogram epigenetically dysregulated T cells into fully functional and epigenetically fit CAR T cells. Moreover, the use of allogeneic CAR T cells from healthy donors might be another alternative strategy to overcome this limitation.

Epigenetically modulating CAR T cells or cells in their surrounding microenvironment might also help prevent antigen loss and heterogeneity but also make CAR T cell therapy more effective in the context of a harsh tumor microenvironment. However, it should be noted that our knowledge in this field is still poor and many details are unknown.

It is now well established that epigenetic modifications are major players in the progression of cancer and pivotal regulators of (CAR) T cell functionality which altogether are determinative factors in the clinical outcome of adoptive (CAR) T cell therapy. An ever-growing understanding of epigenetic processes will bring us closer to the prospect of safer and more effective T cell-based therapies. Epigenetic modifications with DNMTis and HDACis may lead to more efficient clinical outcomes. For example, by using DNMTis in CAR T cell production protocol, it is possible to achieve a higher-level of central memory CAR T cells. Another attractive epigenetic-based approach involves the use of CAR T cells in combination with DNMTis. This strategy may prevent antigen loss or heterogeneity and, simultaneously promote an endogenous antitumor immune response. In line with this strategy, using ncRNAs such as miRNAs are also promising and showing multiple advantages compared to other gene engineering strategies. As miRNAs can target multiple

molecules at the same time, and also target DNMTs (e.g. miR-29), the manipulation of a single ncRNA might entirely alter CAR T cell function. Indeed, due to their small size they can be readily used in multicistronic CAR platforms. However, it should be noted that as epigenetic regulators such epidrugs can affect multiple pathways within the cells, safety cautions should be considered. In summary, it can be concluded that epigenetic modifications have the potential to revolutionize the CAR T cell therapy and present the next, and more effective, generation of T cell-based cancer immunotherapies.

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Figure legends

Figure 1| Epigenetic levels in the eukaryotic cells. Four levels of epigenetic mechanisms exist in the eukaryotic cells. Higher-order epigenetic modifications can alter gene expression by chromatin remodeling. Histone modifications can shape euchromatin or heterochromatin formation and regulate gene expression. DNA methylation can both induce and repress gene expression based on methylation position in genome. And lastly ncRNAs can both induce and repress gene expression by targeting RNAs. These mechanisms, all together, shape cell behavior and fate in human beings.

Figure 2| Epigenetic alterations in T cells during their differentiation. Each phenotype of T cells has distinct epigenetic phenotype. In the naïve T cells, gene expression for both effector and stem genes is in the minimal levels; however, memory T cells show upregulation in gene expression by alteration in DNA methylation and histone modifications. Effector T cells show repression of stem related genes, while, exhausted or dysfunctional T cells are characterized by low gene expression level in both effector and stem genes. *Abbreviations: T_N: Naïve T cell; T_{SCM}: Stem cell memory T cell; T_{CM}: Central memory T cell; T_{EX}: Exhausted T cell.*

Figure 3| CAR T cells in TME. DNMTs and HDACs in tumor cells can suppress TAA and MHC expression as well as suppress transcription of Th1 associated cytokines and chemokines necessary for T cell infiltration. PD-1/PD-L1 interaction can induce CAR T cell exhaustion by epigenetic remodeling. Tumor-derived exosomes inhibit TCR signaling and cytokine production in (CAR) T cells leading to, impaired tumor killing ability of these cells. In the other hand, tumor suppressor miRs can promote immune cell functions and inhibit tumor cell invasion and angiogenesis. Tumor suppressor miRs can also inhibit Treg recruitment to tumor site. These miRs are mainly downregulated in several tumors.

Figure 4| Epigenetic modifications that can be done to overcome roadblocks in CAR T cell therapy. DNA and histone modifications as well as miRNAs can be modified by epidrugs and other tools such as antagomiRs to increase expression and presentation of tumor antigens, enhance trafficking of CAR T cells and other immune cells, modify immunosuppressive tumor microenvironment, and modulate T cell intrinsic features including promoting memory phenotype development, reverting exhaustion, and enhancing persistence of CAR T cells.

Table 1: Challenges to CAR T cell therapy in solid tumors and epigenetic solutions

Challenges			*Overcoming strategies based on epigenetic modifications*	*Ref*
Antigen heterogeneity		Heterogeneous expression of antigens in the tumors	Using DNMTi and HDACi to induce expression of CTAs and HERVs	3,163-165
Impaired trafficking		Mismatched chemokines and their receptors	Using histone, DNA, and miRNAs modifications to overexpress chemokines and their receptors	3,107,175,176
Hostile tumor microenvironment	***Physical barriers***	Extracellular matrix, stromal cells, vasculature	Using histone, DNA, and miRNAs modifications to repress immunomodulatory molecules	3,175,181-183
	Immunosuppressive cells	Treg, MDSC, iDC, M2 macrophage		
	Soluble immunosuppressive molecules	Inhibitory enzymes (arginase, IDO1), ligands (PDL-1/2, FasL), others (IL-10, TGF-β)		
	Immunosuppressive condition	Low PH, hypoxia		
T cell intrinsic regulation		PD1, TGIT, A2ar, CTLA-4, TIM3, LAG3	Using histone, DNA, and miRNAs modifications to downregulate inhibitory molecules	3,43,60,86,102,103,224

DNMTi: DNA methyltransferase inhibitor, HDACi: histone deacetylase inhibitor, HERVs: human endogenous retroviruses.

Table 2: Summery of various epigenetic mechanisms in T cells

Epigenetic level	*Mechanism*	*Effect on T cells*	*Ref*
DNA-associated mechanisms	Methylation, hydroxymethylation	De novo DNA methylation can induce exhaustion	33
		Methylation can induce differentiation of CD8+ cells to effector cells upon stimulation	31
		Methylation and remethylating of pdcd1 locus is associated with naïve and memory phenotype (respectively) while exhausted T cells showed fully unmethylated pdcd1 locus genotype	32
		Inhibition of de novo DNA methylation (DNMT3a) at early stages of effector memory differentiation accelerate central memory T cell development	34
		Reduced methylation of CX3CR1 chemokine leads to effector memory T cell differentiation	37
Histone-associated mechanisms	Methylation, acetylation, phosphorylation, etc.	Trimethylation of both H3K4 and H3K27 is associated with naïve phenotype and trimethylation of H3K4 is associated with memory/effector phenotype	38
		Trimethylation of H3K27 is upregulated in effector T cells whereas trimethylation of H3K4 shown to be upregulated in memory T cells (Tscm, Tcm)	39
		Inhibition of H3 histone deacetylation results in development of memory cells from exhausted phenotype	42
		Acetylation of H3 and H4 histones by HBO1 complex lead to exhaustion	43
		Inhibition of histone lactylation might induce stem cell memory development	49,50
		H3 and H4 histone acetylation and phosphorylation decrease in proximal promoter region of IL-2 upon T cell activation	41
		Enrichment for H3K4me3 and decrease of H3K27me3 at the Gcnt1 gene results in enhanced trafficking of memory cells	40

		H3K27 acetylation at intronic and intergenic regions is concomitant with exhausted phenotype	44
Non-coding RNA mediated mechanisms	miRNA, lncRNA, siRNA	miR-17-92 levels is associated with T cell differentiation and phenotype status	55,58
		miR-15/16 clusters can target memory related genes and their overexpression can induce apoptosis and terminally effector T cell development	60
		miR-150 reduce proliferation of CD4+ cells	69
		Repression of PD-1 and CTLA-4 by miR-138 induce tumor regression	51
		A group of miRs downregulated/upregulated in effector T cells compared to memory and naïve T cells	53,54
		miR-146a downregulation results in less apoptosis and increased proliferation and enhanced effector function	63,64
Higher-order mechanisms	Genomic architecture	TOX family transcription factors interplay with exhaustion	43,44,86

Table 3: Selected T cell miRNAs that can be potentially modulated in CAR T cells

Source	***miRNA***	***Target***	***Mechanism of action***	***CAR T study***	***Ref***
T cells	miR-214	PTEN	Enhance proliferation upon stimulation	No	70
	miR-143	Glut-1	Promote memory development	Yes	103
	miR-146a	NF-κB	Regulating and reducing effector function	No	63,64

miR-23a	Blimp-1	Reducing T cell differentiation and effector function	No	[66]
miR-150	IL-2Rα, ARRB2	Reducing effector function and proliferation	No	[67,69]
miR-155	SHIP-1, SOCS-1	Enhance proliferation, persistence, trafficking, and function of CD8+ T cells	No	[61,62,107,123]
miR-17-92	PHLPP2, PTEN	Enhance cell proliferation, persistence, and differentiation	Yes	[125]
miR-15/16	Several	Inhibit memory T cell formation and differentiation	No	[60]
miR-181a	DUSP5, DUSP6, PTPN11, PTPN22	Induce tolerance	No	[71]
miR-139 miR-342	EOMES, perforin	Reduce effector function	No	[67]
miR-28 miR-138	CTLA-4, PD-1	Decrease immune checkpoint expression	No	[51,140]
Let-7	CCR2, CCR5	Impair trafficking	No	[110]

Table 4: A list of miRNAs that can be potentially targeted in the context of CAR T cell therapy

Source	*miRNAs*	*Target*	*Mechanism of action*	*Cancer*	*CAR T study*	*Ref*
Tumor-derived Exosomes	miR-3187-3p	CD45 (PTPRC)	TCR signaling alteration	Melanoma	No	[217]
	miR-181a/b miR-498	TNFα	Cytotoxicity reduction	Melanoma	No	[217]

	miR-23a-3p	PTEN	PD-L1 expression in macrophages	HCC	No	[218]
	miR-29a-3p miR-21-5p	STAT3	Secreted from TAMs Upregulation of Treg/Th17 ratio	Several	No	[219]
	miR-298-5p	N.D - Caspase 3	Secreted from T cells, induce apoptosis via caspase 3 activation on MSCs	Fibrosarcoma	No	[220]
Tumor cells	miR-141	CXCL1	Inhibit Treg recruitment	NSCLC	No	[215]
	miR-448	IDO1	Enhance proliferation and antitumor function of CD8+ T cells	CC	No	[194]
	miR-153				Yes	[195]
	miR-155	PTGES/PTGES2	Increase PGE2 production and cell proliferation	Breast	No	[197]
	miR-137	COX-2	Inhibit COX-2 and PGE2 production	Retinoblastoma	No	[198]
	miR-21	HPGD	Promote expression of active PGE2	OTSCC	No	[196]
	miR-708	COX-2 & mPGES-1	Inhibit PGE2 production	NSCLC	No	[199]
	miR-126	VEGF-A	Reduce cell proliferation, inhibit angiogenesis	Several cancers	No	[200,202,203]
	miR-503-5p	VEGF-A	Inhibit angiogenesis	CC	No	[201]
	miR-30a-5p	CD73	Inhibit adenosine production	NSCLC	No	[204]
	miR-422a	CD73	Inhibit adenosine production	HNSCC	No	[205]

miR-22	Galectin-9	Suppress cell growth, invasion, and metastasis	HCC	No	[207]
miR-873	PD-L1	Attenuate stemness and resistance of tumor cells	Breast	No	[213]

HCC: Hepatocellular carcinoma, NSCLC: Nonsmall-cell lung carcinoma, CC: colon cancer, OTSCC: Oral tongue squamous cell carcinoma, HNSCC: Head and neck squamous cell carcinoma, TAM: Tumor-associated macrophage, MSC: Mesenchymal stromal cells.