Next Generation Sequencing of Immunized Mouse Splenocytes to Develop an anti-TIM3 Chimeric Antigen Receptor for Acute Myeloid Leukemia

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ABSTRACT

Generation of new chimeric antigen receptors (CARs) rely on a time consuming and/or costly process of phage display screening or hybridoma production, hybridoma screening, and immunoglobulin (Ig) sequencing. Here, we describe a novel CAR design strategy by next generation sequencing (NGS) followed by CAR synthesis and functional validation against TIM3+ targets. NGS allows for direct identification of rearranged immunoglobulin genes, mutation and repertoire analysis, and comparison of multiple immunized mice. Furthermore, by eliminating the need for hybridoma production and screening we have developed a rapid, economical system for the development of novel CARs. After immunization of four separate mice with CHO-TIM3, NGS demonstrated that nearly 70% of Ig heavy (IgH) and 61% of Ig kappa (Igκ) sequences were accounted for by 2 and 4 V-J rearrangements, respectively. This suggested that these rearrangements were enriched for TIM3-reactivity and we designed pairs of these IgH and Igκ rearrangements. By this process we generated 8 anti-TIM3 CARs (4 VHS-based CARs and 4 VH8-based CARs). After their gene-synthesis and cloning into a genetic construct we produced retrovirus and transduced Jurkat, as well as primary T cells. Robust gene-transfer was observed with most constructs (14-64%). Furthermore, we detected antigen-specific cytokine production by primary T cell or Jurkat stimulated with TIM3+ targets. We also examined in vitro killing by primary anti-TIM3 human T cells using a Real-Time cell Analysis cytotoxicity assay (Fig. 5). We are now evaluating the in vivo efficacy of anti-TIM3 CAR T cells in immune deficient animal models implanted with TIM3+ targets. This work demonstrates NGS can be used to identify new sequences that can be developed rapidly and inexpensively into CARs. Using this process we created 8 anti-TIM3 CARs and by in vitro screening, are looking towards one of them (VH8-461) for in vivo validation. We are using this same NGS CAR generation system to develop additional anti-AML CARs which can be used in tandem with anti-TIM3 CARs to treat AML.

INTRODUCTION

The field of anti-cancer T cell therapy has had major advances in recent years with the development of the chimeric antigen receptor (CAR). Our group has developed and demonstrated that CARs against CD19 have complete remission rates of greater than 90% against B cell acute lymphoblastic leukemia (B-ALL)[1]. However, the progress against B-ALL has not been matched in other types of cancers. Acute Myeloid Leukemia (AML) is the most common acute leukemia in adults, presenting greater than 20,000 new cases per annum and representing 80% of acute leukemia[2]. Additionally, prognosis is poor with as much as 70% of patients, 65 years or older, dying within 1 year of diagnosis. A recent study examining AML has shown that a TIM3 monoclonal antibody (mAb) blocks AML engraftment and eliminates leukemic stem cells (LSC)[3]. Additionally, TIM3 mAbs have anti-tumor effects such as slowing tumor progression. Gene-expression analysis demonstrates an 8-fold increase of TIM3 in LSC compared to hematopoietic stem cells. Our own evaluation of TIM3 expression on AML blasts and LSC demonstrated that TIM3 is commonly co-expressed on CD33+ blasts. These results led us to hypothesize anti-TIM3 CAR T cells can target and kill AML, which we will evaluate by the novel CARs.

OBJECTIVES

To demonstrate that NGS can be used as a quick and cost effective way to generate novel CARs by using it to synthesize an anti-TIM3 CAR T cell which can target and kill AML.

MATERIALS AND METHODS

CAR constructs and mouse T cell transduction

The SFG retroviral construct was used for all constructs and our standard SFG-m1928z CAR has been described[4]. SFG-m1928z was modified by removing the stop codon and replacing it with a glycineserine linker followed by GFP to tag the CAR directly to a fluorescent reporter. We modified this construct to replace the intracellular CD28 domain with the intracellular human 41BB. Retroviral supernatants were prepared and used to transduce activated primary T cells as described[4,5].

Intracellular cytokine staining

Jurkat cells were transduced with anti-TIM3 CARs using retroviral supernatants. GFP+ CAR cells were then sorted using a BD FACS Aria. These cells were then stimulated with either CHO-TIM3 or CHO-Empty with golgi blocker (BD) for 4hrs. Cytokine staining was then performed using Fix/Perm (BD) and FACS data was acquired using a LSRII (BD). FACS analysis was performed using FlowJo (TreeStar).

Luminex cytokines

Primary mouse T cells were transduced with anti-TIM3 CARs using retroviral supernatants. CAR T cells were then stimulated for 24hrs with either CHO-TIM3 or CHO-CD123 cells after which supernatants were collected and cytokine concentration was determined using a mouse magnetic Luminex assay (R&D systems).

Real-Time cell analysis cytotoxicity assay

Primary human T cells were transduced with anti-TIM3 CARs using retroviral supernatants. Cytotoxicity assays were also run on an xCELLigence RTCA (real time cell analysis) instrument (ACEA Biosciences, San Diego, CA) according to the manufacturer's instructions. Briefly, CHO-TIM3 or CHO-Empty cells were seeded at 10,000 per well in an E-Plate 96. The next day CAR T cells were resuspended in fresh complete medium without IL2 and added onto target cells at ratios of 1:5, 1:10, 5:1, and 10:1 cells per well. Cell growth was monitored for 8 days.

RESULTS

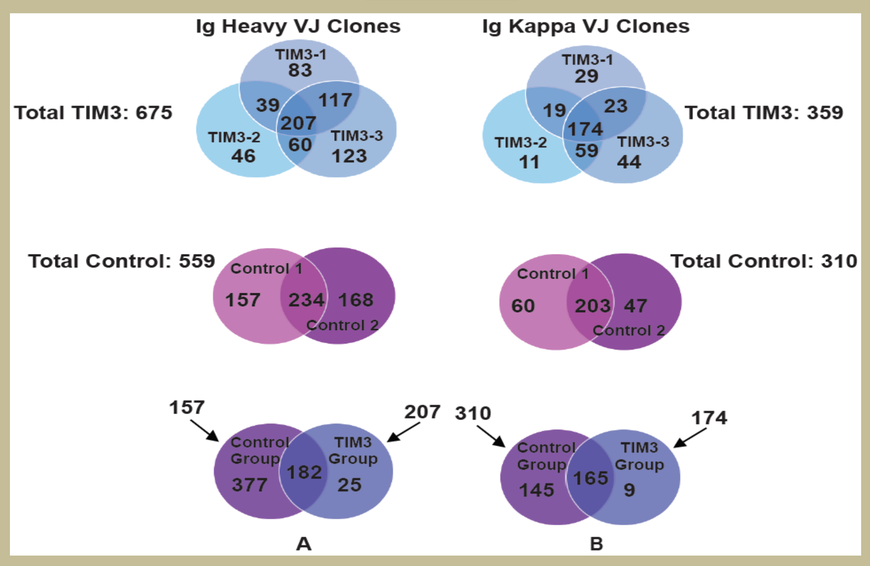

Figure 1. NGS data showing the most common sequences enriched for in immunized mice

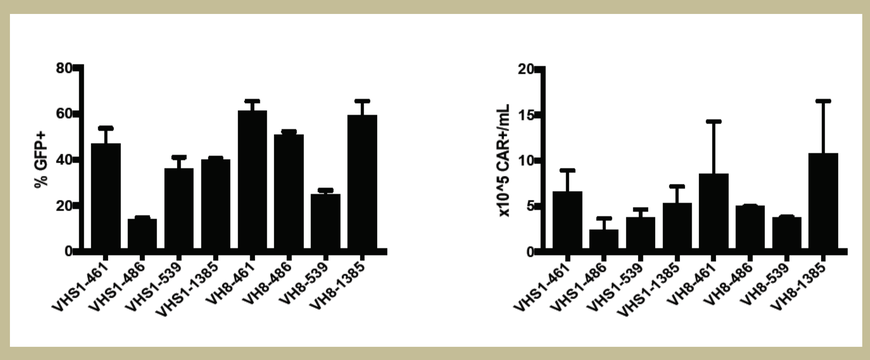

Figure 2. Constructs exhibited robust gene transfer (14-64%) and persistence in culture

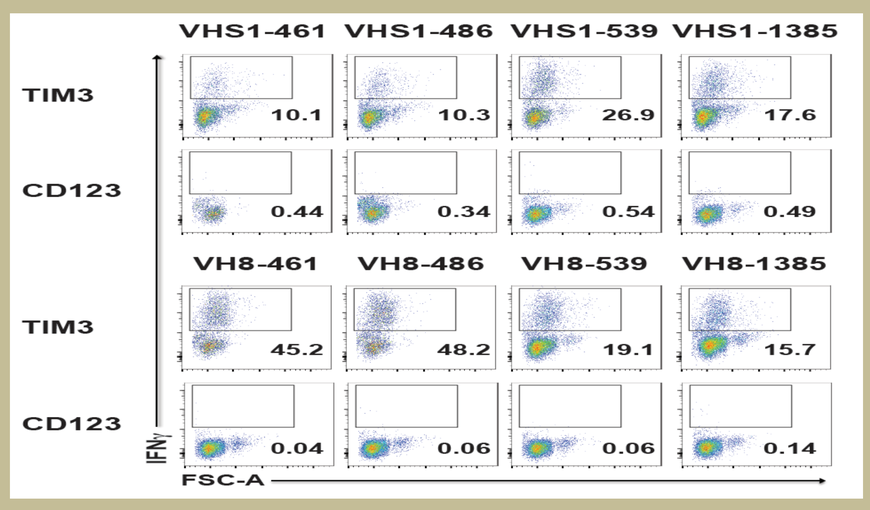

Figure 3. Jurkat cells expressing anti-TIM3 CAR VH8-461 have robust IFNγ expression after stimulation with target cells expressing TIM3

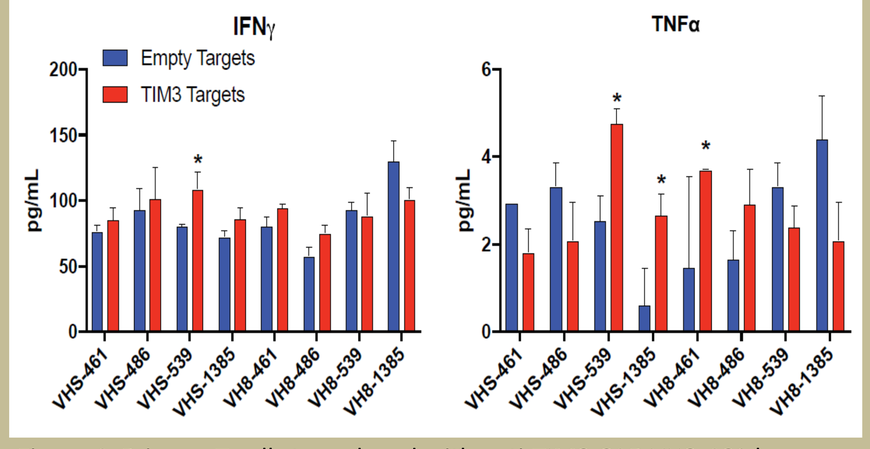

Figure 4. Primary T cells transduced with anti-TIM3 CAR VH8-461 have significantly increased secretion of cytokines after TIM3 stimulation

RESULTS

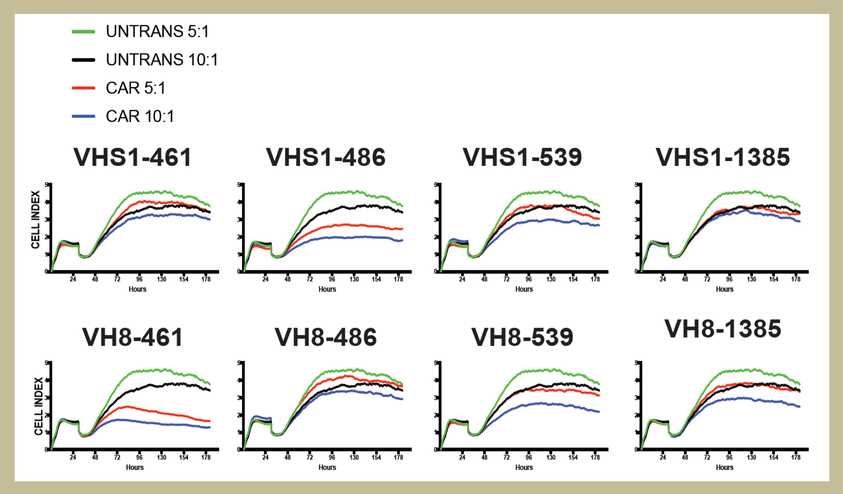

Figure 5. Primary T cells expressing anti-TIM3 CAR VH8-461 can effectively kill TIM3 target cells as measured using a Real-Time cell Analysis cytotoxicity assay

CONCLUSIONS

This work demonstrates that NGS can be used to identify new sequences that can be developed rapidly and inexpensively into CARs. Additionally, using this process we created 8 anti-TIM3 CARs and by in vitro screening selected 1 of them (VH8-461) for in vivo validation. However, a single CAR targeting TIM3 is unlikely to be successful because of T cell fratricide so we will combine it with other anti-AML CARs we are developing.

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