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Immunotherapy Using Chimeric Antigen Receptor Macrophage

A thesis submitted in partial satisfaction of the requirement for the degree
Master of Science

in

Biology

by

Peng Fei Huang

Committee in charge:

Professor Yang Xu, Chair
Professor Steven Briggs, Co-Chair
Professor Cornelis Murre

2019

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The thesis of Peng Fei Huang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-chair

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Chair

University of California San Diego

2019

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ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor Professor Yang Xu for giving me the opportunity to work under him and providing the absolute best environment to grow and strive as a student. Furthermore, I would like to thank Dr. Xu for his patience, motivation and mentorship that has immensely impact me and as pushed me to grow as a student. Finally, he has provide endless amounts of effort guiding me throughout my Masters and I could not have imagined having a better advisor and mentor for my Masters

I would also like to acknowledge the rest of my thesis committee: Professor Steven Briggs and Professor Cornelis Murre for their invaluable support, which has provided the absolute best encouragement throughout my studies.

I would also like to acknowledge everyone in the Xu Lab for being patient with me even after countless mistakes and providing me the guidance to succeed. Everything I know is because of everyone in the Xu Lab's willingness to teach me and I would like to thank everyone from the bottom of my heart. In particular, I am extremely grateful for Qu Chen for guiding me throughout my journey and enlightening me about how research should be done.

ABSTRACT OF THE THESIS

Immunotherapy Using Chimeric Antigen Receptor Macrophage

by

Peng Fei Huang

Master of Science in Biology

University of California San Diego, 2019

Professor Yang Xu, Chair
Professor Steven Briggs, Co-chair

Chimeric antigen receptor (CAR) T cell immunotherapy has become one of the most prominent and leading cancer therapies due to its remarkable success in targeting hematological malignancies. Unfortunately, CAR-T cell immunotherapy has not had the same amount of success in solid tumors due to the challenging tumor's immunosuppressive microenvironment. Therefore, we hypothesize the use of macrophages as a vessel for CAR immunotherapy due to their associate with tumors as TAMs and also the tumors ability to secrete various chemokines

that can attract myeloid cells to the tumor site. THP-1 a monocytic cell line that represents a monocyte/macrophage model was transduced with an anti-CD19 scFv CAR construct. K562 a leukemia cell line that represents hematological malignancies and H460 a lung cancer cell line that represents solid tumors were transduced to overexpress the surface marker CD19. THP-1 clones expressing the anti-CD19 CAR construct were cocultured with the two tumor cell lines, which demonstrated the ability of the THP-1 CARs to specifically targeted and lysis the tumor cells that overexpressed the CD19 surface marker. Upon CAR activation, THP-1 cells were polarized towards the M1 classical activated phenotype due to the increase in expression of TNF-α, IL-1β, IL-6, IL-12β, CXCl10, HLA-DR, and CD86. Additionally, THP-1 cells did not show any change in the M2 alternative activated markers of IL10, TFGβ, CCL18, CCL22, CD206, and CD204 to suggest polarization towards the M2 phenotype. As a result, this study validates the proof of concept that macrophages could potentially be a vessel for CAR immunotherapy.

Introduction

Cancer Immunotherapy

Cancer has become one of the leading causes of death worldwide with an expected 600,000 deaths and an additional 1.7 million newly diagnosed cases of cancer in 2018.[1] Furthermore, by 2020 the annual cancer cases is expected to increase by 24.1% or more than 1 million newly diagnosed cancer cases for men and 20.6% or more than 900,000 newly diagnosed cancers cases for women since 2010.[2] As a result, these shocking statistics have revolutionized the field of cancer therapy throughout the past decades, but since cancers have been reported to possess mechanisms to evade endogenous immune responses, new innovations are required to fight this disease.[3,4] One of the most promising new innovations that have revolutionized the field of cancer therapy is immunotherapy, which works under the premise of engineering or priming the patient's own immune system to detect the cancer and effectively destroy it.[5] Throughout the years, many forms of immunotherapy have been developed and have produced amazing results, but one of the most promising types of immunotherapy is chimeric antigen receptors (CARs) therapy. CAR therapy utilizes the patients own immune system by harvesting autologous lymphocytes, T cells being the most prominent lymphocyte currently being used for this therapy, and genetically modifying them to enhance their immunological functions against the cancer.[5]

Structure of the Chimeric Antigen Receptor

With the emergence of CAR as one of the leading immunotherapies in the last decade, researchers have made attempts and successfully constructed a vast array of different constructs of CAR structures. Universally, the basic CAR structure consists of an antigen derived binding

motif, a transmembrane domain, and a signaling intracellular domain.[6] More precisely, the antigen derived binding motif is most commonly derived from an antigen binding (Fab) fragment of a monoclonal antibody that has high affinity towards targeting the antigen of interest, which has been named an single-chain variable fragment (scFv). The scFv comprises the extracellular region of the CAR structure, which is linked to the transmembrane domain that connects to the signaling intracellular domain. The intracellular domain consists of an immunoreceptor tyrosine-based activation motif (ITAM), which is usually taken from either regions of CD3ζ chain or γ chain of immunoglobulin receptor FcεRIγ.[6,7] This unique structure allows CARs to specifically bind towards the antigen of interest and cause an immune response by initiating the signal transduction pathway that leads to the lymphocyte activation.[8–11] Due to the flexibility of the CAR structure combined with the creativity of the researcher, vast amounts of different CAR structures have been produced and the different CAR structures have been classified has first, second, or third generation CARs based on their differing intracellular domain.[12,13] First generation CARs consists of extracellular scFv region and a single intracellular stimulatory domain, which displayed low levels of activation and proliferation of T cells leading to low potency against tumors.[10] To fix the restraints of the first generation CARs, the second generation CARs were designed to express a stimulatory domain combined with a co-stimulatory domain that is usually consists of CD28 or 4-1BB.[12] With the addition of a co-stimulatory domain, CAR-T cells displayed a heightened ability to expand and persist allowing for better T cell survival.[14] Finally, the third generation was designed to increase the efficacy of the CAR's ability to target the tumor by designing the CAR to consist of the stimulatory domain combined with two co-stimulatory domains, which usually consist of CD28 and 4-1BB.[13]

Advantages and Disadvantages of Chimeric Antigen Receptor Immunotherapy

CAR constructs consists of an antigen derived binding motif linked to a transmembrane domain and an intracellular signaling domain, which have allowed CAR cells to avoid Major Histocompatibility Complex (MHC) mediated recognition to target antigen. Eliminating this limitation from the CAR cells allows for a greater range of potential cellular targets and evades the concern that tumors avoid immune surveillance by down regulating MHC expression.[15–18] On the contrary, with the benefit of evading MHC recognition, CAR cells are only designed to have an affinity towards surface markers on the tumor cells.[13] Designing the CAR construct is one of its major advantages because it gives the researcher the ability to uniquely develop a construct to specifically target any surface marker of interest, which could include any carbohydrates, lipids, or protein antigens that is expressed on the surface of the tumor.[19] Furthermore, due to CAR cells ability to not be restricted my MHC, their interactions with antigen presenting cells (APCs) is very minimal, meaning CAR cells do not receive a co-stimulatory signal from the APCs. As a result, due to the flexibility of the design of the CAR construct, a co-stimulatory signal can be designed into the structure making CAR cells not require the interaction of APCs.[20] However, that is also one of CAR therapies major challenges is being able to find a appropriate surface marker to design the CAR construct to target that only destroys the tumor cells and does not damage any health tissue. Due to the difficulties of finding tumor specific antigens to target, many of the CAR constructs developed have been more tumor “associated” antigens rather than tumor “specific” antigens. This correlates to the idea that many of these tumor associated antigen are highly expressed on the tumor cells, but not exclusively expressed on the tumors cells and is also expressed on normal healthy tissue, which could lead to on-target/off-tumor toxicity.[21]

Chimeric Antigen Receptor Immunotherapy Against Hematological Malignancies

As stated previous, one of the major disadvantages of using CAR therapy is finding a suitable target that would allow for highly specific targeting of the tumor, while disregarding any healthy tissue. Hematological malignancies of the B cells became a prime candidate for CAR therapy because of the essential characteristic that B cells highly and exclusively express CD19 on their cell surface starting at the later pro-B cell developmental stage.[22] CD19 has become nearly a perfect target to create a CAR construct against because of the specificity of the expression in majority of B cell lineage malignancies, which includes: B-cell non-Hodgkin lymphoma (NHL), Acute lymphoblastic Leukemia (ALL) and Chronic Lymphocytic (CLL).[23–25] First generation CARs that were developed to target B-cell malignancies consisted of a anti-CD19 scFv binding domain combined with only CD3ζ stimulatory domain produced very minimal antitumor effects due to the CAR's inability to persist and expand.[26] As a result, to further strengthen the CAR's ability to activate, the second generation of CARs was produced that contained a co-stimulatory domain from either 4-1BB or CD28 combined with the typical construct of the first generation CARs. Using the second generation of CARs, many clinical trails utilizing the anti-CD19 scFv CAR-T cells have been most successful against B cell Acute Lymphoblastic Leukemia (B-ALL). One of the clinical trails that consisted of 16 patients infused with the CD19 CAR-T cells demonstrated a remarkable 88% complete remission (CR).[27] With these results lead to one of the landmark clinical trails that consisted of 30 patients with ALL that were infused with the autologous CAR-T cells, which lead to a 90% remission rate, 67% event-free survival after 6 months, and a overall survival rate of 78%.[28] Despite the success of CD19 as an ideal target for CAR-T cell therapy, antigen escape, which is the loss of detectable CD19 on the surface of tumor cells, have been reported in about 10-20% of pediatric B-ALL patients

treated with CD19 targeted CAR immunotherapy.[29] Ultimately, the need for more novel targets similar to the specificity of CD19 is require to alleviate the concerns of antigen escape related to CAR immunotherapy.

Chimeric Antigen Receptor Immunotherapy Against Solid Tumors

Although CAR therapy derived to target hematological malignancies have shown promising results with remarkable clinical results, utilizing CAR therapy to target solid tumors have made limited progress due to several new challenging obstacles compared to hematological malignancies. Like before, one of the major challenges is finding a suitable target to create a CAR construct against because unlike B-cell malignancies, solid tumors do not have highly specific surface markers that is exclusively expressed only on the tumor.[30] As a result, on-target off-tumor toxicity is an extremely critical concern since CAR therapy is not MHC medicated, which could lead to stronger and faster immune response whether its against tumor or healthy tissue.[31] Furthermore, due to the genetic instability of tumor cells, highly expressed antigens that could be a candidate for CAR therapy can stop being expressed leading to more difficulties of finding a suitable target. Even if a suitable target is determined and a CAR is produced, another one of the major challenges is the inability to "traffick" the CAR cells to the tumor sites due to its microenvironment.[32] In order for the tumor to protect itself, it produces a strong immunosuppressive microenvironment which is characterizes by hypoxia, low pH, inhibitory effects of tumor derived cytokines, and upregulation of inhibitory pathways effecting CAR cell activation.[33–35] Therefore, it is important to better understand the different types of obstacles that have limited the success of CAR therapy against solid tumors in order to eventually develop a

successful system to ultimately produce the same amount of success as CAR therapy against hematological malignancies.

Chimeric Antigen Receptor Macrophage Immunotherapy

Due to the difficulties of utilizing CAR-T cell therapy against solid tumors, other avenues must be explored and one of those is harnessing the power of the human innate immune system in conjunction with CAR therapy. One of the most prominent lymphocytes within the innate system is the macrophage, which has the ability to not only attack foreign invaders but also have the ability to present antigen.[36] As a result, with the ability to antigen present, this allows macrophages to prime the T-cell response against novel antigen expressed on the tumor cells causing a secondary immune response. Solid tumors have been reported to actively recruit myeloid cells through the secretion of tumor derived chemokines and have shown be inhabitants of solid tumors, which have been reported to be called tumor associated macrophages (TAMs).[37,38] Therefore, we hypothesis that by utilizing the macrophages as the vessel for CAR immunotherapy, which could potentially be a powerful immunological agent if properly activated and redirected towards targeting tumor cells and possibly solid tumors. In this study, we used THP-1 a monocytic cell line that is a good representation of monocytes/macrophages in order to test the ability of transducing CAR constructs into the macrophages. After transduction, cytotoxicity assay was done to test the antitumor cytotoxicity ability of the CAR-Macrophages on K562, a leukemia cell line, which represented a hematological malignancy, and H460, a lung cancer cell line, which represented solid tumor cells. Finally, gene expression assay was done on the CAR-Macrophages in order to determine whether classically activated type 1 (M1) or the alternatively activated type 2 (M2) phenotype is expressed after CAR-Macrophage activation.

Materials and Methods

Plasmid

The two secondary generation CAR expression vectors was synthesized by iGene (China). The anti-CD19scFv-CD8 hinge- CD8 transmembrane domain-4-1BB- CD3ζ and CD19scFv-CD8 hinge- CD8 transmembrane domain-dectin 1-Fcr was subcloned and inserted between AgeI and EcoRI site on the lentiviral vector: lenticas9-blast (Addgene #52962). The CAR sequence replaced the coding sequence region of cas9. Human CD19 gene expression vector was modified from the PL452CNP vector. CAG-Human CD19 sequence was inserted after the CAG-NEO-IRES-PURO cassette. AAVS1 genome homologous arms were inserted upstream of the CAG-NEO-IRES PURO cassette and downstream of CAG-Human CD19 sequence.

Cell Culture

Human monocytic THP-1 cell line were obtained from American Type Culture Collection (ATCC) and was maintained in culture in Roswell Park Memorial Institute medium (RPMI 1640) containing 10% heat inactivated fetal bovine serum (FBS), and supplemented with 10 mM Hepes (Gibco, #15630-056), 1 mM pyruvate (Gibco, #11360-039), 2.5 g/L D-glucose (Gibco, #A2494001) 50 pM ß-mercaptoethanol (Gibco; 31350–010) and 1% penicillin/streptomycin (Gibco, #10378016). Human chronic myelogenous leukemia (CML) cell line K562 were obtained from ATCC and was maintained in culture in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% heat inactivated fetal bovin serum (FBS) and 1% penicillin/streptomycin (Gibco, #10378016). Human large cell lung carcinoma call line NCI-H460 was obtained from ATCC and maintained in culture in RPMI 1640 supplemented

with 10% heat inactivated fetal bovin serum (FBS) and 1% penicillin/streptomycin (Gibco, #10378016). CD19 expression vector was transduced into K562 and H460 cells using Lipofectamine™ 2000 Transfection Reagent (Invitrogen #11668027) and selected with 2 μg/ml of puromycin, surviving clones were expanded and their expression of CD19 confirmed by flow cytometry stained.

Production of CD19 Expressing Tumor Cell Lines

2.5μg of AAVS1 Human CD19 plasmid were transduced into K562 cells in 6-well plates by Lipofectamine™ 2000 Transfection Reagent (Invitrogen #11668027) and selected with 1 μg/mL of puromycin two days after transduction for one week of treatment. Surviving clones were expanded and their expression of CD19 confirmed by flow cytometry stained. The same protocol was done on H460 cell line, but using 2 μg/mL of puromycin for selection.

Lentiviral Trnasduction and Production of CAR-Macrophage Cells

The lentiviral vectors encoding the anti-CD19 CAR gene and two of the helper vectors: psAX2 (Addgene #12260) and pMD2.G (Addgene #11259) were transduces into 293FT cells using the calcium phosphate transduction protocol. Twenty-four hours after transduction, the supernatant was collected and virus concentrated with Lenti-X™ Concentrator (Clontech #631232). THP-1 cells were transduced with the lentivirus for 24 hours before the medium was replaced with fresh medium. The THP-1 transduced cells were then subsequently analyzed with flow cytometry.

In Vitro Cell Lysis Assay with CAR-Macrophage

K562 WT and K562 CD19 cells were mixed into a 1:1 ratio and seeded onto a 96 well plate at a density of $1x10^4$ cells/well. THP-1 WT, THP-1 Zeta, and THP-1 DecFcr were first stained with CFSE (Biolegend, #423801) at a concentration of 5μM per $1x10^6$ cell/mL and then seeded along with the mixture of K562 WT and K562 CD19 at various ratios (1:1, 5:1, and 10:1). After 24-hour incubation, all cells in the well were harvested and stained with 1μL per $1x10^6$ cells in 100μL anti-CD19 antibody (Biolegend, #302229) and analyzed by a BD LSR-II machine using FACS Diva software (Becton Dickinson). The same procedure was done using H460 WT and H460 CD19.

Flow Cytometry Analysis (FACs)

Flow cytometry analysis of the surface expression of the Chimeric Antigen Receptor, $1x10^6$ cells were harvested and washed with PBS and stained with 0.01μg of biotin conjugated protein L for 30 minutes at room temperature. Afterwards the cells were washed and subsequently stained with Streptavidin conjugated Allophycocyanin (APC) (BD Bioscience, #554067) for 30 minutes in room temperature. For flow cytometry analysis of the surface expression of CD19 on K562 cells, $1x10^6$ cells were harvested and washed with PBS and stained with anti-human CD19 conjugated to PerCP/Cyanine5.5 (Biolegend, #302229) for 30 minutes at room temperature. The same procedure was done analysis the surface expression of CD19 on H460. For flow cytometry of the cell lysis assays, THP-1 clones were all stained with CFSE (Biolegend, #423801) before seeding and all cells in the wells were harvested and washed with PBS before being stained with 1μL per $1x10^6$ cells in 100μL anti-human CD19 conjugated to

PerCP/Cyanine5.5 (Biolegend, #302229) for 30 minutes at room temperature. Samples were analyzed by BD LSR-II machine using FACS Diva software (Becton Dickinson).

Reverse Transcription-Polymerase Chain Reaction and Real-Time Polymerase Chain Reaction

Total RNA was purified from THP-1 WT, THP-1 Zeta, and THP-1 DecFcr after 24 hour coculture with H460 CD19 at a 5:1 ratio with a density of $4x10^5$ H460 CD19 cells/well and $2x10^6$ THP-1 cells/well in a 24 well plate. PureLink™ RNA Mini Kit (Invitrogen, 12183018A) was used to extract RNA with 2µg of total RNA extracted was utilized to reverse transcribed cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368814). Analyze by quantitative real-time PCR was done using PowerUp™ SYBR™ Green Master Mix (Applied Bioststems, #A25742). CD45 was used as the reference gene for normalization and fold change was quantified using the threshold cycle method. The primers used are listed below

Statistical Analysis

All statistical analysis was performed using GraphPad Prism 6 Software. A non-parametric one-way ANOVA was performed to compare the cytotoxic functionality of the differing CAR constructs. P values of 0.05 or less were considered to be significant.

Table 1: Primer Sequence Used in RT-PCR Gene Expression Assay

Gene	Primer sequences (5' → 3')	
IL-1β	Forward	CCACAGACCTTCCAGGAGAATG
	Reverse	GTGCAGTTCAGTGATCGTACAGG
IL-6	Forward	AGACAGCCACTCACCTCTTCAG
	Reverse	TTCTGCCAGTGCCTCTTTGCTG
CXCL10	Forward	GGTGAGAAGAGATGTCTGAATCC
	Reverse	GTCCATCCTTGGAAGCACTGCA
CD206	Forward	AGCCAACACCAGCTCCTCAAGA
	Reverse	CAAAACGCTCGCGCATTGTCCA
CCL18	Forward	GTTGACTATTCTGAAACCAGCCC
	Reverse	GTCGCTGATGTATTTCTGGACCC
CCL22	Forward	TCCTGGGTTCAAGCGATTCTCC
	Reverse	GTCAGGAGTTCAAGACCAGCCT
IL-10	Forward	TCTCCGAGATGCCTTCAGCAGA
	Reverse	TCAGACAAGGCTTGGCAACCCA
HLA-DR	Forward	AGTCCCTGTGCTAGGATTTTTCA
	Reverse	ACATAAACTCGCCTGATTGGTC
CD86	Forward	CTGCTCATCTATACACGGTTACC
	Reverse	GGAAACGTCGTACAGTTCTGTG
IL-12β	Forward	ACCCTGACCATCCAAGTCAAA
	Reverse	TTGGCCTCGCATCTTAGAAAG

Table 1: Primer Sequence Used in RT-PCR Gene Expression Assay, Continued

TNFα	Forward	CCAGGCAGTCAGATCATCTTCTC
	Reverse	AGCTGGTTATCTCTCAGCTCCAC
CD204	Forward	GCAGTGGGATCACTTTCACAA
	Reverse	AGCTGTCATTGAGCGAGCATC
TGF-β1	Forward	CAACAATTCCTGGCGATACCTC
	Reverse	AAAGCCCTCAATTTCCCCTC
CD45	Forward	ATTACCTGGAATCCCCCTCAAA
	Reverse	TTGTGAAATGACACATTGCAGC

Chapter 1: Generation of K562 and H460 Cell Line Overexpressed with CD19 Surface Marker

Flow Cytometry Analysis Shows High Expression of CD19 Surface Marker On K562 and H460 Cell Lines After Lentiviral Transduction

One of the major challenges of successfully utilizing CAR therapy to its absolute potential is the obstacle of finding a appreciable target that has high specificity towards the tumor cells and very little to no expression on any healthy tissue.[20] To strengthen the CAR model shown in Figure 2A, which utilizes the well validated anti-human CD19 scFv as the extracellular binding domain, a CD19 expression vector was encoded into a lentiviral vector and transduced into the K562 chronic myelogenous leukemia (CML) cell line and H460 large cell lung cancer cell line (Figure 1A). Before transduction, the surface expression of CD19 on the K562 and H460 cell lines were stained with anti-human CD19 antibody followed by analyze through flow cytometry, which showed that K562 WT and H460 WT does not express CD19 (Figure 1B,C). After transduction of the CD19 expression lentiviral vector, the clones underwent antibiotic selection to selectively eliminate the non-expressing cells and purify the clones to have a high expression level of the surface membrane marker CD19. The purified clones that expressed CD19 were then denoted as K562-CD19 and H460-CD19. After the antibiotic selection, the CD19 expression clones were stained with anti-human CD19 antibody and analyzed through flow cytometry. Compare to the WT clones, the CD19 expressing clones have high levels of expression (>95%) of CD19, which is a high enough efficiency to process to use for the testing of the CAR models.

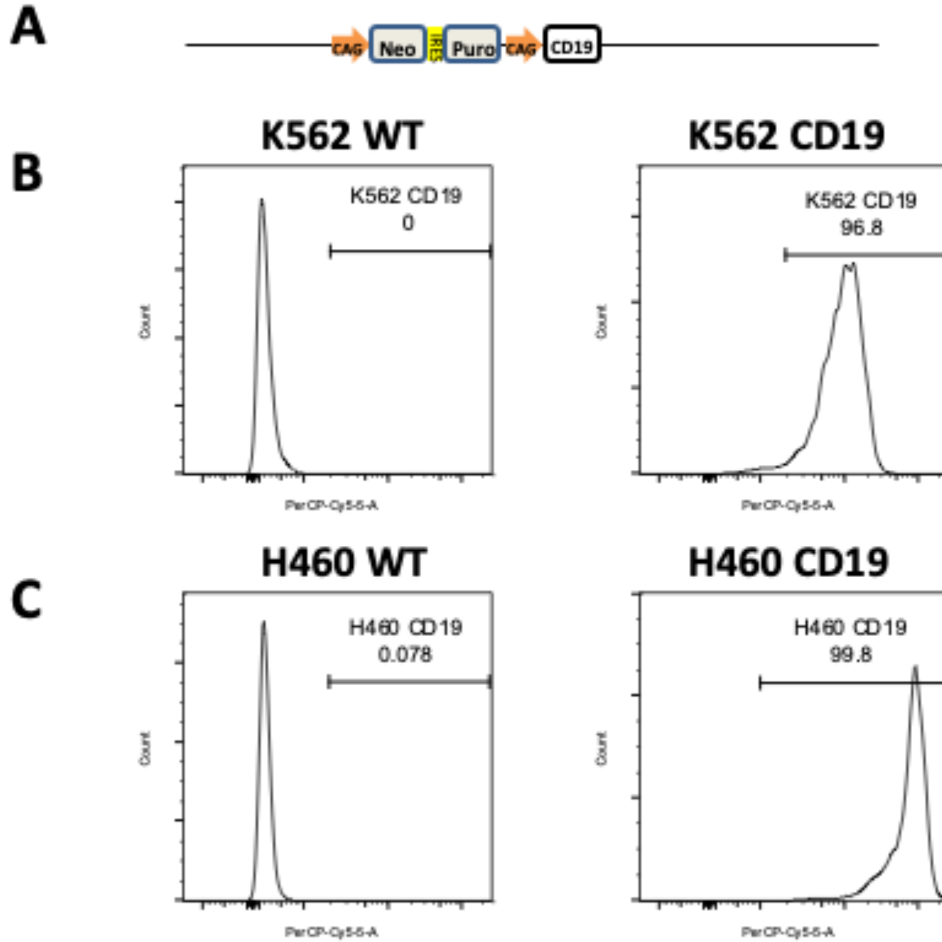

Figure 1: Generation of CD19 Expressing K562 and H460 Cell Lines
A. CD19 expression vector that was used to transduce into the tumor cells line to produce a transgenic clone overexpressing CD19.
B,C. Surface Expression of CD19 was analyzed using flow cytometry of the transfected clone compare to the WT.

Chapter 2: Generation of Chimeric Antigen Receptor Macrophage to Specifically Target Tumor Cells

Flow Cytometry Analysis Shows High Expression of the CAR Constructs on THP-1 Cell Line After Lentiviral Transduction

Due to the flexibility of constructing a vast array of different CAR structures, researchers have experimented with using different types of intracellular signaling domains in order to optimize the CAR therapy to their needs. Therefore, we have utilized the well-validated anti-human CD19 scFv as the extracellular binding domain for both CAR constructs used in this study.[28] Both constructs are classified as second generation CAR constructs, but one is the well validated T cell specific CAR construct focusing of T cell signaling, while the other is specific towards macrophage signaling. Both CARs contain an extracellular anti-human CD19 scFv, CD8α hinge region, and a CD8α transmembrane region, while the differing regions are the intracellular signaling domains. The T cell specific CAR construct consist of a CD3ζ stimulatory domain combined with 4-1BB co-stimulatory domain, while the macrophage specific CAR construct consists of the signaling domain of Dectin-1 and the Fcγ signaling region of an Fc receptor (Figure 2A). The CAR constructs were encoded into a lentiviral vector and transduced into the THP-1 cell line that is a representation of monocytes/macrophages. After transduction, the THP-1 clone that contain the T cell specific CAR construct was denoted as THP-1 CD19 Zeta and the THP-1 clone that contain the macrophage specific CAR construct was denoted as THP-1 CD19 DecFcr. The transduction efficiency of the CAR construct was based on staining of Protein L-Biotin followed by an APC-streptavidin incubation and analyzed using flow cytometry (Figure 2B). Shown in Figure 2B, the control none transduced THP-1 WT displayed no visible

binding of the Protein L stain that is specific to the kappa light chains on the anti-human CD19 scFv created from the CAR construct. On the other hand, after transduction, THP-1 CD19 Zeta and THP-1 CD19 DecFcr both display high levels of Protein L staining compared to the THP-1 WT control, which means that the CAR construct is highly expressed in the THP-1 clones. Both transduction efficiencies were >95% for both clones, which is a high efficiency to proceed to test their functionality assays.

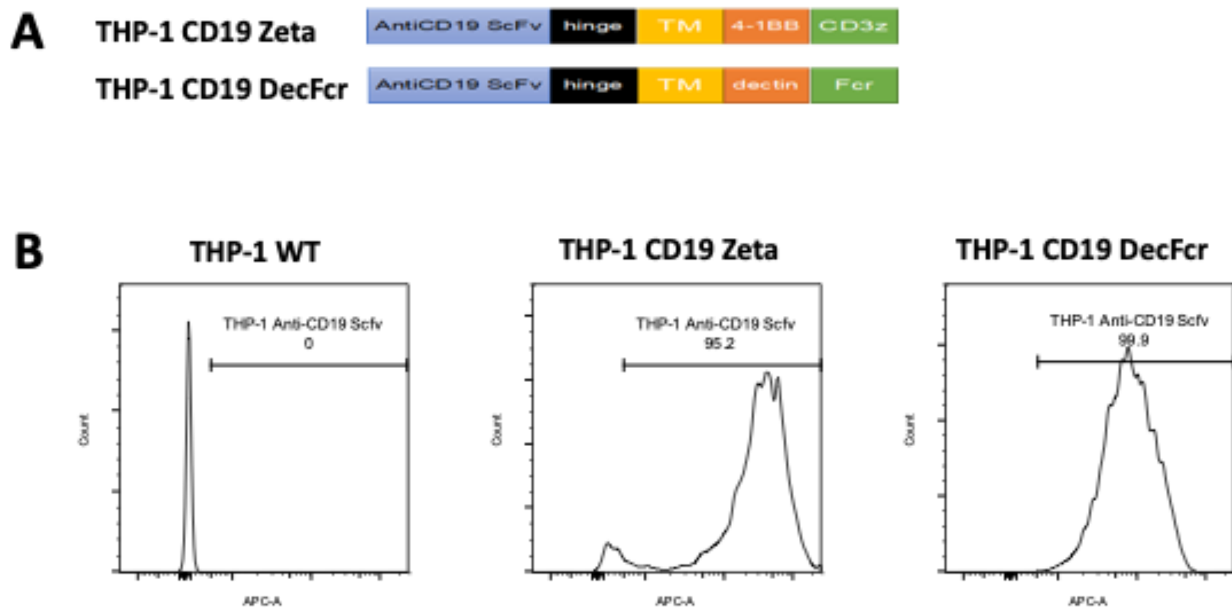

Figure 2: Generation of Chimeric Antigen Receptor Macrophage
A. Expression vector of the second generation CAR constructs. THP-1 CD19 Zeta consisted of anti-CD19 ScFv connected to a transmembrane domain and the stimulatory signaling domain of CD3ζ and co-stimulatory domain of 4-1BB. THP-1 CD19 DecFcr consisted of anti-CD19 ScFv connect to a transmembrane domain and the stimulatory signaling domains of Dectin-1 and Fc receptor.
B. Surface expression of the CAR construct was using analyzed using flow cytometry stained with protein L-biotin, subsequently followed by Streptavidin-APC.

Chapter 3: Chimeric Antigen Receptor Macrophage Against Hematological Malignancies

Flow Cytometry Analysis Shows Anti-CD19 CAR-Macrophage Has the Ability to Suppress Hematological Malignancies In Vitro

From the data presented from various CAR related clinical trails, CAR immunotherapy has only been reported to have the most success on eliminating hematological malignancies.[28] Therefore, to evaluate the ability of THP-1 transduced with anti-CD19 CAR against hematological malignancies, the leukemic cell line K562 was transduced to over express CD19 as a target for the CAR construct. K562 is a non-adherent leukemia cell line that would be a good representation of a hematological malignancy in vitro. To assess the function of the THP-1 CAR constructs, a 1:1 ratio of K562 WT and K562 CD19 mixture was created and cocultured with THP-1 CARs at various ratios. After a 24-hour incubation, the cells were run through flow cytometry to assess the ability of the THP-1 CARs antitumor ability against a hematological malignancy represented by the cell line K562. The mixture of K562 WT and K562 CD19 displayed two distinct peaks on the histogram graphs each corresponding to the their respective clones (Figure 3A). With the addition of THP-1 WT the ratio of K562 WT and K562 CD19 had very minimal to no change even had higher ratios of effector cells to tumor cells. However, upon addition of the THP-1 transduced with the anti-CD19 CAR construct, the K562 overexpressed with CD19 were specifically lysed, but none of the K562 WT controls were affected upon the coculture. Increasing the ratio of effector cells to tumor cells gradually increased the antitumor ability of the THP-1 CARs (Figure 3B).

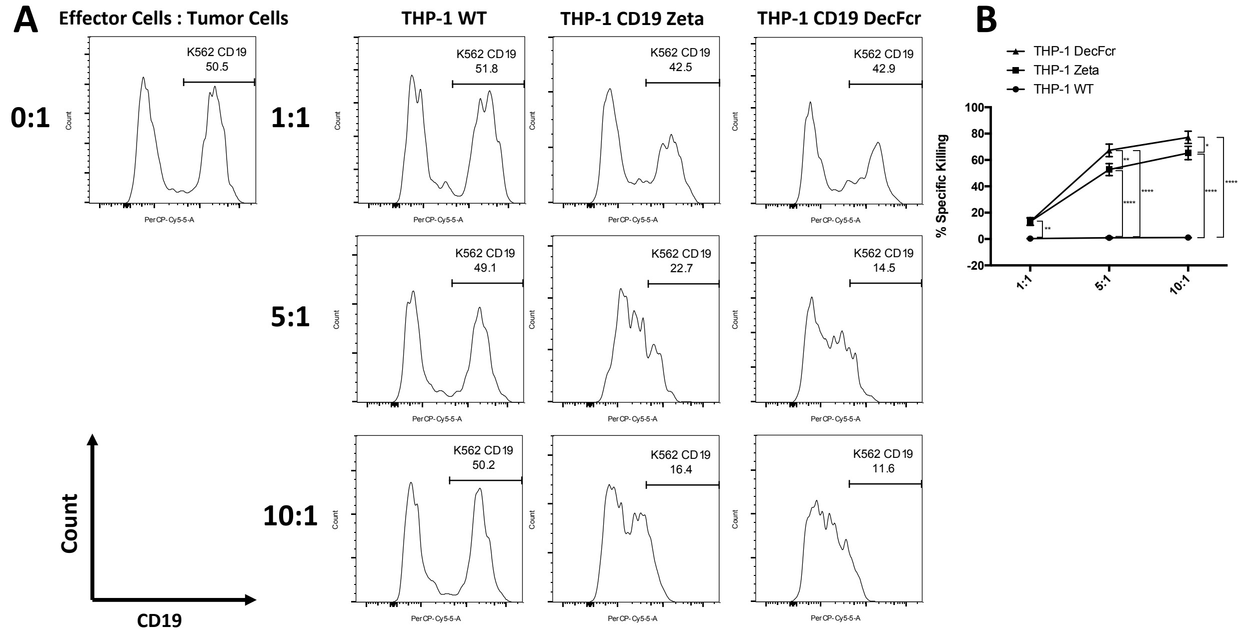

Figure 3: Chimeric Antigen Receptor Macrophage In Vitro Lysis of K562

A. A mixture of a 1:1 ratio of K562 and K562 CD19 was make to form two distinct peaks on the flow cytometry graph. Afterwards THP-1 WT, THP-1 Zeta, and THP-1 DecFcr were cocultured with the mixture of K562 and K562 CD19 in various ratios to test the antitumor functionality of the CAR-Macrophage.

B. The CAR-Marcophage mediated antitumor efficacy was quantified based on the flow cytometry data shown with the mean and standard deviation as the error bar. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Chapter 4: Chimeric Antigen Receptor Macrophage Against Solid Tumors

Flow Cytometry Analysis Shows Anti-CD19 CAR-Macrophage Has the Ability to Suppress Solid Tumors In Vitro

Although, there is significant progress made in CAR immunotherapy against hematological malignancies, solid tumors has been presented as a challenging obstacle for CAR immunotherapy to solve.[33–35] Therefore, to evaluate the ability of THP-1 transduced with a anti-CD19 CAR construct against solid tumors, the lung cancer cell line H460 was transduced to overexpress CD19 was a target for the THP-1 CARs. H460 is an adherent lung cancer cell line that would be a good representative for solid tumors in vitro. Similarly to the previous functional assay with K562, to assess the function of THP-1 CAR against H460, a 1:1 ratio of H460 WT and H460 CD19 mixture was created and cocultured with various ratios of effector cells to tumor cells. After a 24 hour incubation, the cells were run through flow cytometry to assess the functional abilities of the THP-1 CAR against solid tumors represented by the cell line H460. The mixture of H460 WT and H460 CD19 creates two distinctive peaks represented their respective clones on the histogram graphs (Figure 4A). The effects of using only the THP-1 WT clone has very minimal to no effect at all on the tumor cell line with it having a stable peaks even with the addition of higher amounts of effector cells. On the other hand, THP-1 clones that were transduced with the anti-CD19 CAR construct specifically targeted the H460 that were overexpress with CD19, but none of the control H460 WT. Increasing the amount of THP-1 CAR effector cells greatly increase the antitumor ability of the THP-1 CARs (Figure 4B).

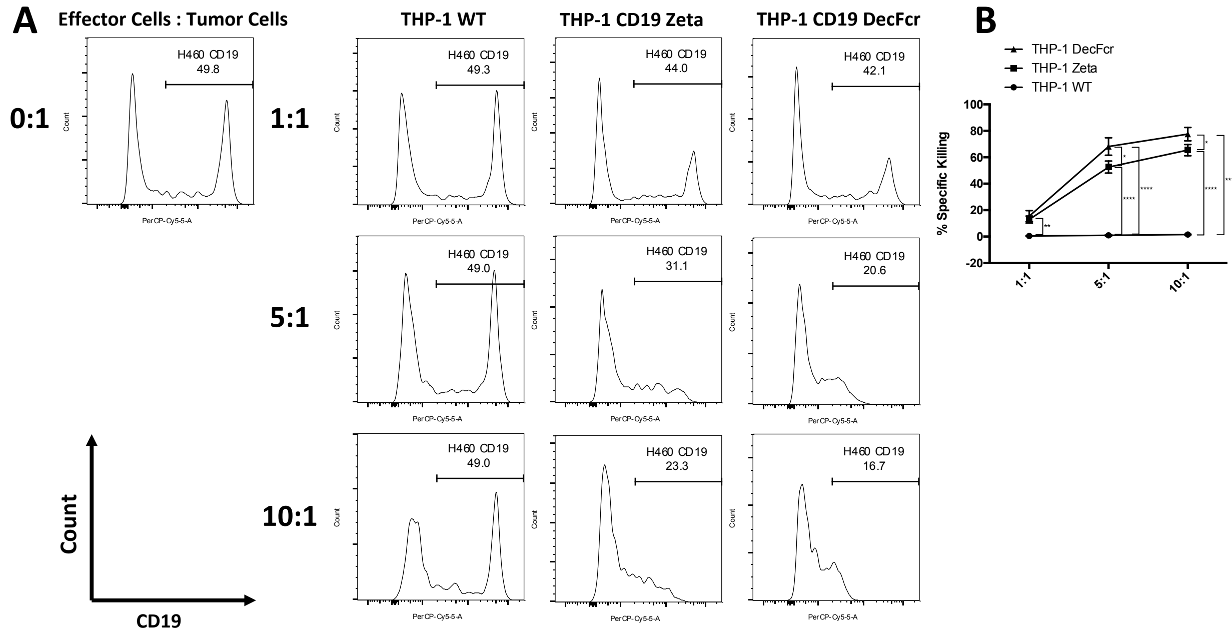

Figure 4: Chimeric Antigen Receptor Macrophage In Vitro Lysis of H460

A. A mixture of a 1:1 ratio of H460 and H460 CD19 was make to form two distinct peaks on the flow cytometry graph. Afterwards THP-1 WT, THP-1 Zeta, and THP-1 DecFcr were cocultured with the mixture of H460 and H460 CD19 in various ratios to test the antitumor functionality of the CAR-Macrophage.

B. The CAR-Marcophage mediated antitumor efficacy was quantified based on the flow cytometry data shown with the mean and standard deviation as the error bar. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Chapter 5: Macrophage Polarization After CAR Activation

RT-PCR Gene Expression Assay Shows That Upon CAR Activation, THP-1 CARs Polarize Towards the M1 Classical Activated Phenotype

One of the defining characteristics of macrophages is that they possess high levels of plasticity, which have allowed them to polarize to specific phenotypes in response to environmental factors that they encounter.[39] The two distinct phenotypes that macrophages can polarize to be: the classically activated or pro-inflammatory type 1 (M1) or the alternatively activated or anti-inflammatory type 2 (M2). Macrophages that are polarized to the classical pro-inflammatory M1 phenotype during times of injury or infection is connected to the expression of various cytokines which include TNFα, IL-1β, IL-6 and IL-12. On the other hand, the alternative M2 anti-inflammatory phenotype is connected with TAMs due to their association with inhibition of T cells caused by their expression of IL-10 and TGFβ.[37,38] Therefore, in order to test the polarization of the THP-1 CARs upon encountering tumor cells, the various THP-1 clones were coculture with H460 CD19 in order to activate the THP-1 cells. After a 24-hour incubation, the THP-1 cells were harvested and RNA was extracted to perform gene expression assay through RT-PCR. M1 macrophage polarization was then assessed through the measurement of various classical M1 markers including: IL-6, IL-1β, IL-12β, CXCL10, and TNFα, which are pro-inflammatory cytokines, and HLA-DR and CD86, two M1 related surface markers (Figure 5A). An increase in pro-inflammatory cytokines and surface marker expression upon coculture with tumor cells, however the relative fold change deferring between the two different CAR constructs could be due to the differing intercellular signaling motifs. M2 macrophage polarization was also analyzed through the measurement of various alternative M2 markers,

including: IL10 and TGFβ, which are M2 related cytokines, CCL18 and CCL22, which are M2 related chemokines, and CD204 and CD206, two M2 related surface markers (Figure 5B). Upon coculture with the tumor cells, THP-1 is not observed to express high levels of M2 related markers.

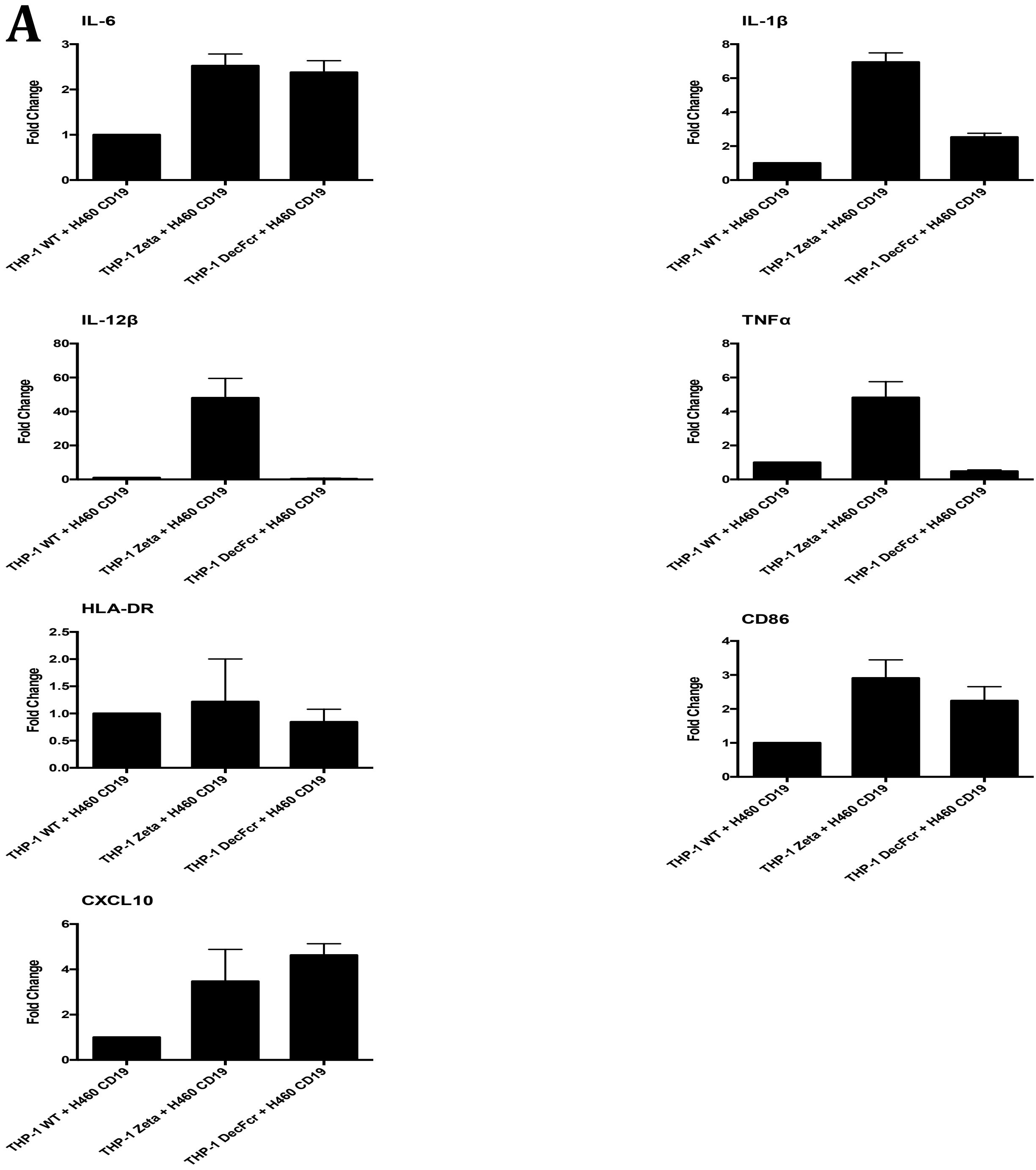

Figure 5A: Chimeric Antigen Receptor Macrophages Polarizes to M1 Phenotype Upon CAR Activation

THP-1 WT, THP-1 Zeta, and THP-1 DecFcr were cocultured with H460 CD19 for 24 hours to induce CAR activation and total RNA was extracted from the CAR-Macrophages. RT-PCR geneexpression assay was done of the extracted RNA to determine the effects of CAR activation onmacrophage's phenotype. A. M1 classical activated or pro-inflammatory marker expressions. B. M2 alternative activated or anti-inflammatory marker expression

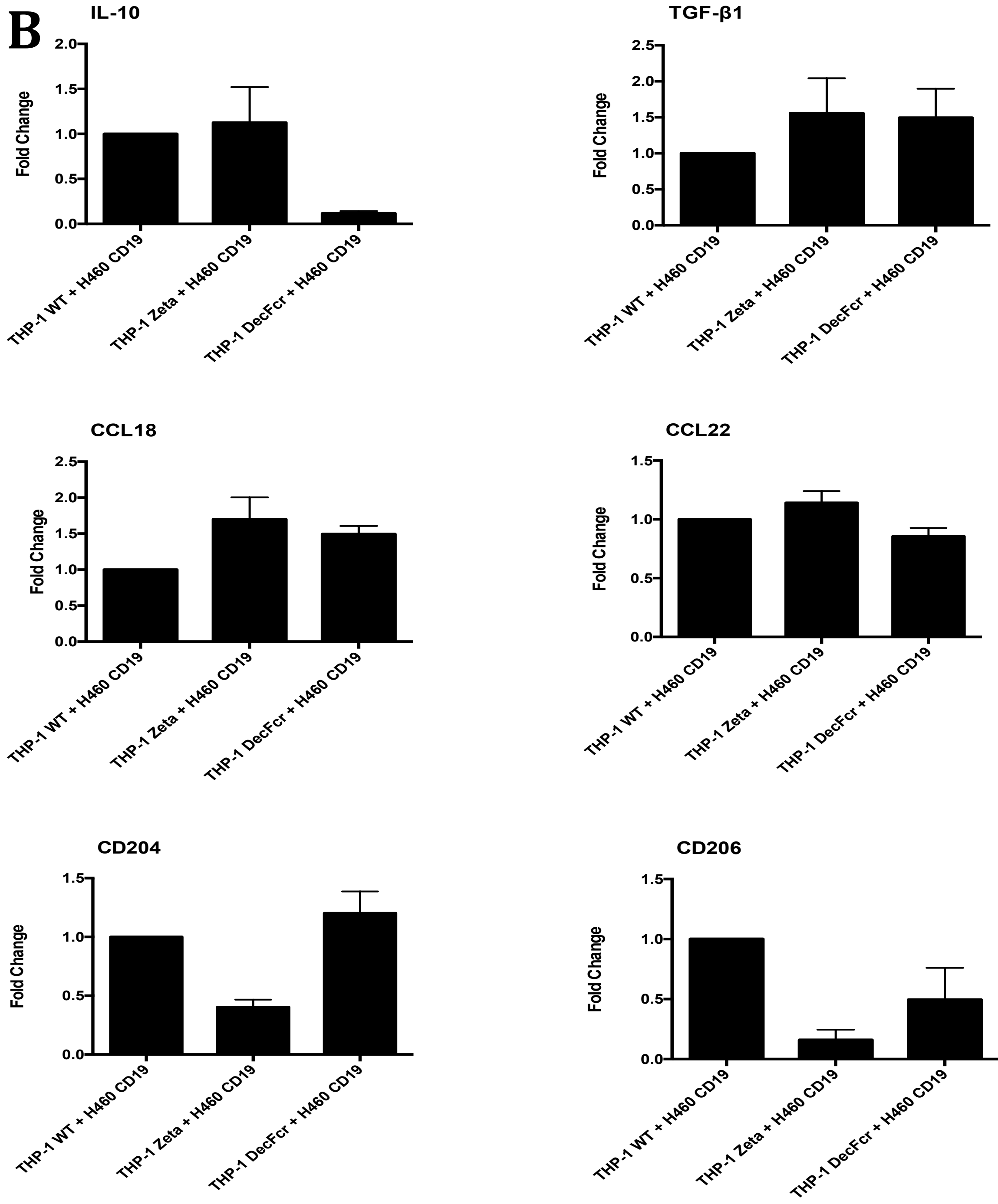

Figure 5B: Chimeric Antigen Receptor Macrophages Polarizes to M1 Phenotype Upon CAR Activation, Continued

Discussion

Due to the success of CAR-T cell therapy in various different clinical trails against hematological malignancies, CAR immunotherapy has become one of the leading pioneers towards fighting cancer.[28] Unfortunately, CAR immunotherapy has not found all the answers just yet, and has encountered various challenging obstacles when confronted by solid tumors.[33–35] Many of the challenges researchers have encountered are due to the hostile immunosuppressive microenvironment that protects the solid tumors. The immunosuppressive microenvironment has a hostile environment targets T cells that try to target it due to the hypoxia, low pH, inhibitory effects of tumor derived cytokines, and upregulation of inhibitory pathways that have diminished the effects of CAR immunotherapy.[33–35] As a result, many researchers have tried to optimize the construct of various CARs creating generations of differing constructs in order to combat the many problems that solid tumor microenvironments have presented.[30] Furthermore, one of the major problems of CAR-T in solid tumor models is their ability to traffick to the actual tumor site. Therefore, in this study rather than focusing on optimizing T cells for CAR therapy, we suggest utilizing macrophage as the vessel for CAR therapy. Tumors have been reported to actually secrete various forms of cytokines that actually recruit myeloid lineage lymphocytes to the actually tumor sites and tumor-associated macrophages (TAMs) have played an important role in the development of various solid tumors.[37,38] With the ability to actually penetrate through the hostile solid tumor microenvironment could enhance the antitumor activity of CAR immunotherapy, and additionally with macrophages ability to present antigen, could potentially create a secondary immune response. In order to assess the ability of macrophages ability to utilize CAR immunotherapy, THP-1, a monocytic cell line, was used as a monocyte/macrophage representative for the transduction of the anti-CD19 CAR constructs. Once transduced with high

expression of the respective CAR constructs, the THP-1 CARs were cocultured with the leukemic cell line K562 as a representative for hematological malignancy and the lung cancer cell line H460 as a representative for solid tumors. Coculture of the THP-1 CARs with the tumor cells displayed remarkable antitumor functionality of the THP-1 CARs due to their ability to specifically target the tumor clones overexpressed with CD19 and not any of the tumor WT clones (Figure 3,4). Therefore, macrophage definitely has the ability to be utilized as a vessel for CAR immunotherapy and has the potential overcome the challenges of the solid tumor microenvironment.

The plasticity of macrophages allows them to polarize to specific phenotypes based upon the environmental factors that are presented to them. The two specific phenotypes that macrophages can polarize into are the classical activated or as known as the pro-inflammatory type 1 macrophages (M1), or the alternative activated or as known as the anti-inflammatory type 2 macrophages (M2).[39] M1 macrophages are characterized by their polarization after injury or infection that causes the secretion of pro-inflammatory cytokines, phagocytosis of microbes, and initiates an immune response. On the other hand, M2 macrophages are characterized by their ability to heal wounds, repair tissues, and tumor-associated macrophages (TAMs) have been classified as M2 macrophages due to their ability to suppress immune response with the secretion of suppressive cytokines.[38] Therefore, the THP-1 that were transduced with the CAR constructs were coculture with H460 CD19 in order to activate the CAR expressing THP-1 clones to analyze their polarization upon exposure to tumor cells. After coculture, the THP-1 clones were harvested and RNA was extracted to perform gene expression assay through RT-PCR. Upon activation of the CAR construct, various pro-inflammatory cytokines that all have a role in initiating the immune response, which includes IL-6, IL-1β, IL-12β, and TNFα were all

upregulated. CXCL10, a chemokine that recruits T cells was also upregulated and various surface markers like HLA-DR, which has a role in antigen presentation, and CD86, which has a role in activating T cells, were also upregulated (Figure 5A). On the other hand, upon activation, various anti-inflammatory cytokines that suppressive T cell like IL-10 and TFGβ were downregulated or not effected. Furthermore, M2 related chemokines CCL18 and CCL22, which are associated with recruiting naïve T-cells and T-regulatory cells respectively to the tumor microenvironment have very little change upon CAR activation. The chemokines can recruit an abundance of T-regulatory cells and the suppressive environment due to the secretion of IL-10 in the tumor microenvironment can suppress dendritic cell maturation that can lead to immune tolerance.[40,41] Finally, CD204 and CD206, which are Scavenger Receptor-A and Mannose Receptor respectively and have been reported as TAM associate M2 surface markers that have shown that cancer patients that have high density of these surface markers in tumors sites have worse clinical prognosis.[42,43] M2 associated surface markers have shown to decrease or have little to no change on receptor expression upon CAR activation (Figure 5B). Due to the differing intracellular signals based on the CAR constructs, different levels of cytokines, chemokines, and surface markers expression are observed. As a result, to these differing expression levels, THP-1 CD19 Zeta had a noticeable high expression of various M1 related markers, which could ultimately lead to high toxicity if performed in the clinic. On the other hand, THP-1 CD19 DecFcr has had noticeably lower expression of various M1 related markers, which could mean less toxicity and a safety CAR construct to use in the clinic. Upon activation of the CAR construct, THP-1 that was transduced with the CAR constructs were not observed to polarize to the M2 anti-inflammatory phenotype but rather the M1 pro-inflammatory phenotype.

Chimeric antigen receptor (CAR) T cell immunotherapy has recently been one of the most promising and significantly impactful types of cancer therapy, especially if use to target hematological malignancies. Unfortunately, researchers have not been able to specifically optimize CAR-T immunotherapy to target solid tumors because of the difficulties of bypassing the tumor's immunosuppressive microenvironment. Therefore, rather than optimize T cells to combat solid tumors, in this study we propose the usage of macrophages as a vessel for CAR immunotherapy. Several reports have shown that macrophages have already been associated with tumors, which have been named tumor-associate macrophages (TAMs) and additional tumors secrete various chemokines that recruit myeloid-lineage lymphocytes to the tumor site.[37,38] Furthermore, macrophages have the ability to present antigen, which could ultimately lead to priming of naïve T cells into a secondary immune response that could potentially have an impact of antitumor efficacy.[36] Our study validates the proof of concept that macrophages can be used as a vessel for CAR immunotherapy and potentially be impactful to the field of immunotherapy due to the idea of providing another route of attack for solid tumors.

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