REPOLARIZATION OF TUMOR-INFILTRATING MYELOID CELLS TO AUGMENT CAR T CELL THERAPIES

by

Weichuan Luo

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STATEMENT OF COMMITTEE APPROVAL

Dr. Philip S. Low, Chair

Department of Chemistry

Dr. Timothy L. Ratliff

Department of Comparative Pathobiology

Dr. Qing Deng

Department of Biological Science

Dr. Tony R. Hazbun

Department of Medicinal Chemistry and Molecular Pharmacology

Approved by:

Dr. Christine A. Hrycyna

To my family, for the unconditional love and support.

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LIST OF ABBREVIATIONS

4T1-mCD19 cells	Mouse CD19 expressing 4T1 cells
7-AAD	7-Aminoactinomycin D
CAFs	Cancer-Associated Fibroblasts
CAR	Chimeric Antigen Receptor
FA-TLR7a	Folate-conjugated TLR7 agonist
FR	Folate Receptor
MDSCs	Myeloid-Derived Suppressor Cells
MFI	Mean Fluorescence Intensity
NSG	NOD Scid Gamma
scFv	Single-chain variable fragment
TAMs	Tumor-Associated Macrophages
TBI	Total Body Irradiation
TGF-β	Transforming Growth Factor-β
TLR7	Toll-Like Receptor 7
TME	Tumor Microenvironment.

ABSTRACT

Although CAR T therapies have demonstrated efficacy in treating hematopoietic cancers, related CAR T cell therapies have been ineffective in treating solid tumors, in part due to the tumor's immunosuppressive microenvironment. While this immunosuppressive tumor microenvironment (TME) is created by multiple cell types, critical to the suppressive properties of the TME are tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) that express checkpoint co-receptors (e.g. PD-L1), release immunosuppressive cytokines (e.g. TGFβ and IL-10), secrete growth factors (e.g. FGF, VEGF, and PDGF), and inactivate T cells by nitrosylating T cell receptors. Motivated by the fact that TAMs and MDSCs can change when exposed to an opposing immune stimulus, we investigated strategies to reprogram TAMs and MDSCs from their protumorigenic to an anti-tumorigenic state. Here, we demonstrate that targeting of a strongly proinflammatory TLR7 agonist specifically to TAMs and MDSCs can reverse an immunosuppressive TME by converting anti-inflammatory (M2-like) TAMs to pro-inflammtory (M1-like) macrophages and by decreasing the intratumoral content of MDSCs.

Our approach to the targeting of TLR7 agonists to TAMs and MDSCs is based on the fact that tumor-infiltrating myeloid cells over-express folate receptor β (FRβ) that is essentially absent from quiescent myeloid cells and all other cell types. By covalently linking a TLR7 agonist to folate, we demonstrate that the resulting conjugate (FA-TLR7a) accumulates specifically in TAMs and MDSCs, while avoiding uptake in other immune cells and healthy tissues. Because FRβ resides in the same endosome as TLR7, internalization of FA-TLR7a by the TAMs and MDSCs leads to activation of TLR7 and the consequent repolarization of the TAMs and MDSCs to their pro-inflammatory states. Moreover, because other immune cells express no FRβ and since the murine tumor models employed (4T1, CT26, EMT6 cells) also express neither FR nor TLR7, we conclude that the effects of FA-TLR7a described below derive primarily from repolarization of TAMs and MDSCs.

To test the ability of FA-TLR7a to improve the efficacy of a classic anti-CD19 CAR T cell therapy, we transduced the above 3 cancer cell lines with CD19 and tested the ability of anti-CD19 CAR T cells to eradicate the tumors in the presence and absence of FA-TLR7a. Infusion of CAR T cells alone exerted only moderate efficacy, while co-administration of FA-TLR7a greatly

enhanced CAR T cell tumoricidal activity. Flow cytometric analysis of cell surface markers on TAMs and MDSCs demonstrated that the targeted TLR7 agonists shifted TAM markers from M2-like to M1-like while significantly decreasing the numbers of tumor infiltrating MDSCs. Similar analyses of other immune cells revealed increased numbers of T cells and anti-CD19 CAR T cells and enhanced expression of activation markers on the T cells. We conclude that the FA-TLR7 agonist greatly augments CAR T cell efficacy in solid tumors via stimulation of FR^+ TAMs and MDSCs.

CHAPTER 1. INTRODUCTION

1.1 Chimeric Antigen Receptor (CAR) T cells

Chimeric antigen receptors (CARs) are recombinant receptors that provide both antigen-binding and T cell activation functions, which have significant potential for treating cancers because of their tumor-specific activation and killing [1]. An exemplary second-generation CAR consists of a single-chain variable fragment (scFv) for targeting, a CD3 zeta chain for activating, a single cytoplasmic domain of a costimulatory receptor, such as CD28 or 4-1BB, plus hinge and transmembrane domains [2]. The hinge and transmembrane domains are the least commented for CAR design, though they are also important, and most investigators use the hinge and transmembrane domains of CD8α or CD28 [3].

1.2 Current issues of CAR T cell therapy

1.2.1 Limited efficacy against solid tumors

CAR T cell therapies have revolutionized the treatment of hematopoietic cancers by focusing the killing power of a patient's T cells towards the patient's cancer cells. CAR T cell therapies have, however, not proven as effective in treating solid tumors due in large part to an immunosuppressive TME that disables the cytotoxic activities of the engineered T cells [4]. Currently, there are five FDA-approved CAR T-cell therapies, and none of them are for the treatment of solid tumors.

1.2.2 Most preclinical studies are performed in immunodeficient mice.

For preclinical studies, most of the CAR T cells studies are based on NOD scid gamma (NSG) mice, which allows the study of treating human cancer cells with human CAR T cells. However, it is not the best model to predict human clinical outcomes related to TME because NSG mice lack mature T cells, B cells, and functional natural killer (NK) cells and are deficient in multiple cytokine signaling pathways [5]. In the xenograft model, the dynamic interactions between cancer cells and immune cells can not be reflected [6, 7]. Also, the immunomodulatory effects of the anti-tumor drug can not be studied in this model [7, 8]. Thus, a syngeneic system, which is more suited

to probe the cell biology of mice with an intact immune cell environment, is required to provide complementary insight [9]. With a syngeneic mouse model, which has an intact immune system, the activities of CAR T cells and the symptoms of mice patients can be studied in the tumor microenvironment like those of human patients. Therefore, a syngeneic system is significant to study the impact of changing TME on the activities of CAR T cells.

1.3 TAMs and MDSCs play major roles in controlling the TME.

Although this CAR T cell inactivation derives from multiple processes initiated by cancer cells, cancer-associated fibroblasts, and a variety of immune cells [10], among the most immunosuppressive cells are the tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) that secrete IL-10 and transforming growth factor-β (TGF-β) [11-14], nitric oxide and thereby nitrosylate and inactivate T cell receptors [15, 16], upregulate immune checkpoint receptors [17], promote the production of a dense extracellular matrix that can impede penetration of immune cells and drugs [18, 19], and produce immunosuppressive enzymes such as arginase 1, CD39 (NTPDases) and CD73 (ecto-5'-nucleotidase) [20-23]. Taken together, these immune-inhibiting activities probably render TAMs and MDSCs the most effective suppressors of CAR T cell activity in the TME [24-26].

1.4 Repolarization of TAMs and MDSCs with FA-TLR7a.

1.4.1 TLR7 agonist can reprogram TAMs from M2-like to M1-like phenotype.

Not surprisingly, strategies to repolarize these TAMs and MDSCs to a less immunosuppressive phenotype have been the focus of many recent studies [27]. Among these strategies, TLR7 agonist has been reported to repolarize TAM/MDSCs from a tumorigenic M2-like phenotype to a tumoricidal M1-like phenotype [28, 29].

1.4.2 Expression of FRβ on TAMs and MDSCs and suppression function.

Although such TLR7 agonists have proven to be too toxic to administer systemically [30-33], their adverse side effects can be avoided by targeting them selectively to TAMs and MDSCs, thereby avoiding their activation of well-behaving immune cells in healthy tissues. In fact, by exploiting the significant upregulation of FRβ on tumor-infiltrating myeloid cells and the lack of

FRβ expression on almost all healthy cells [34-36], a folic acid conjugate of a TLR7 agonist (FA-TLR7a) has been shown to concentrate the attached TLR7 agonists selectively in TAMs and MDSCs, i.e., avoiding their uptake by myeloid cells in normal tissues [34]. As anticipated, endocytosis of FA-TLR7a by tumor-infiltrating TAMs and MDSCs has been observed to initiate a panoply of signaling events that reprogram the TAM/MDSCs into a more immunostimulating phenotype.

1.4.3 Using FA-TLR7a to repolarize TAMs and MDSCs to M1-like phenotype.

Recognizing the prominent role believed to be played by TAMs and MDSCs in suppressing CAR T cell function, the question naturally arose whether selective targeting of a TLR7 agonist to TAMs and MDSCs might prevent inactivation of CAR T cells in solid tumors. To examine this question, we prepared classical anti-CD19 CAR T cells and evaluated their abilities to suppress solid tumor growth in immune-competent mice in the presence and absence of FA-TLR7a. We report here that a syngeneic murine 4T1 breast cancer cell line transfected with murine CD19 forms aggressive subcutaneous solid tumors in balb/c mice whose growth can only partially be inhibited by administration of anti-CD19 CAR T cells. We further show that co-administration of folate-TLR7a, which cannot enter either 4T1 cancer cells, CAR T cells, or endogenous T cells because they express no folate receptors, significantly augments CAR T cell activity by directly reprogramming the TAMs and MDSCs, leading to complete solid tumor eradication in many instances. Based on additional data showing that folate-TLR7a treatment induces decreases in expression of immunosuppressive markers and concomitant increases in immune cell activation markers on CAR T cells, endogenous T cells, TAMs, and MDSCs alike, we conclude that reprogramming of the TAMs and MDSCs in solid 4T1 tumors can significantly augment the potency of a classical CAR T cell therapies without activating the same immune cells in healthy tissues.

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CHAPTER 2. GENERATION OF A SYNGENEIC MOUSE MODEL TO STUDY THE EFFECTS OF REPOLARIZING TAMS AND MDSCS ON CAR T CELL EFFICACY AGAINST SOLID TUMORS.

2.1 Abstract

To address the major limitations of current CAR T cell technology discussed in Chapter 1, we explored the effects of changing tumor microenvironment on CAR T cells in immune competent mice. For this purpose, we selected and modified appropriate murine cancer cells and murine CAR T cells. In this chapter, we discuss the generation of a syngeneic mouse model to study the impacts of repolarization of TAMs and MDSCs on CAR T cells' efficacy against solid tumors.

To generate the syngeneic mouse model, we first selected the commonly used mouse cancer cell lines that express no FR or TLR7. We then transduced and selected the cell lines with high expression of mouse CD19 and confirmed their stable expression of mouse CD19 by flow cytometry. Next, we made anti-CD19 mouse CAR T cells and verified the in vitro cytotoxicity against the CD19 expressing 4T1 cells. Finally, we optimized the condition of lymphodepletion before CAR T cell infusion to enable the success of CAR T cell infusion with minimized toxicity.

2.2 Introduction

It is essential in the study of TME to distinguish the effects of the drug directly affect cancer cells and the effects result from changing other cells in the tumor. In this study, the drug, FA-TLR7a, is designed to be specifically delivered to TAMs and MDSCs instead of cancer cells [1,2]. In order to determine whether selective repolarization of TAMs and MDSCs in tumor tissues can enhance the anti-tumor efficacy of a classical anti-CD19 CAR T cell therapy without causing systemic toxicity, we needed a cancer cell line that would grow in immune competent mice that lacked both folate and TLR7 receptors but expressed CD19.

First, to identify a cancer cell line that expressed no folate receptors, we screened several commonly employed syngeneic tumor cell lines for FR by evaluating their ability to bind a folate-fluorescein conjugate. 4T1, CT26, and EMT6 cells are all commonly used mouse cancer cell lines and were tested to be FR^-. In addition, the tumors formed by these three cell lines were reported to

have high percentages of FRβ$^+$ TAMs and MDSCs [1]. Next, to determine whether any of these cell lines might also lack a toll-like receptor 7, we examined the same three cell lines by flow cytometry using an antibody to murine TLR7.

Having identified several cancer cell lines that express neither FR nor TLR7, we next undertook to induce CD19 expression in these cells so that they could be treated with a classical anti-CD19 CAR T cell therapy. For this purpose, CT26, 4T1, and EMT6 cells were first transduced with CD19 using a lentiviral vector and then sorted by flow cytometry to select for clones that were found to express higher levels of murine CD19. Following single cell cloning of a cell from each cell line that expressed a high level of CD19, we examined the ability of each CD19 positive cell line to bind the anti-CD19 antibody.

However, because 4T1-mCD19 cells were found to form the more aggressive tumors of the three tumor clones and since their tumors were characterized by greater infiltration of immunosuppressive TAMs and MDSCs [1, 3], we elected to conduct all further studies using the murine CD19-transfected 4T1 cell clone. Moreover, to concurrently maintain an antigenically heterogeneous tumor model, we also retained the CD19-transfected 4T1 cell line that was sorted for high murine CD19 expression but not cloned from a single cell.

Because the 4T1-mCD19 cell clone was derived from balb/c mice, we next needed to produce anti-CD19 mouse CAR T cells from T cells isolated from the same strain of mice. For this purpose, mouse T cells were isolated from the spleens of balb/c mice and transduced with retrovirus to express an anti-CD19 CAR as described elsewhere [4].

Since lymphodepletion is essential for CAR T infusion [5-7], we then optimized the dose of irradiation before CAR T infusion to ensure the success of CAR T infusion while minimizing the toxicity of lymphodepletion.

2.3 Materials and Methods

2.3.1 Confirmation of folate receptor level of 4T1, CT26, and EMT6 cell lines in the folate-deficient environment

Since folate-deficient diet can increase the expression of folate receptors, to confirm that the selected cancer cells are FR$^-$ in the folate-deficient environment, FR$^-$ mouse cancer cell lines 4T1, CT26, and EMT6 cells, and FR$^+$ moues cancer cell line L1210A, were cultured in folic acid-free Roswell Park Memorial Institute (RPMI) 1640 (Gibco, Ireland) containing 10% heat-inactivated

fetal calf serum and 1% penicillin-streptomycin in 5% CO2 at 37°C for a week. Then the cells were detached by 1μM EDTA + 1μM EGTA and stained with folate-FITC (10nM) for 1 hour at room temperature. All the samples were then washed three times with PBS and analyzed by flow cytometry for folate-FITC binding. The stained samples were protected from light during the whole process.

2.3.2 Test the TLR7 expression level in 4T1, CT26, and EMT6 cells

To test the TLR7 expression levels on 4T1, CT26, and EMT6 cells, these cells were detached using 0.25% trypsin and washed once with PBS. The cell lines were fixed and permeabilized with Cyto-Fast™ Fix/Perm Buffer Set (BioLegend, CA). TLR7+ mouse cancer cell line 24JK was treated with the same steps as a positive control. All four cell lines were incubated with anti-mouse CD16/CD32 (BioLegend, CA) in 100 μl volume for 15 minutes at room temperature. The cell lines were then incubated with anti-mouse TLR7-PE antibody (BD Biosciences) for 20 minutes at room temperature in the dark. All the samples were then washed two times with 1 ml PBS and analyzed by flow cytometry. The stained samples were protected from light during the whole process.

2.3.3 Transduction of syngeneic cancer cell lines

CT26, 4T1, and EMT6 cells were first transduced with mouse CD19 linked with GFP using a lentiviral vector. HEK-293T cells and Lipofectamine 3000 were used to produce the lentivirus, and polybrene (8μg/mL) was used during transduction. The transduced cells were then sorted by flow cytometry to select clones that were found to express higher levels of GFP and murine CD19.

2.3.4 Selection of single cell clone tumor model

Flow cytometry sorted CD19 expressing 4T1 cells were diluted to 1 cell/200μl in RPMI 1640 media and seeded into 96 well plates with 100μl/well. Later, the wells with only one colony were detached with 0.25% trypsin and transferred from one well to one well into 96 Well Black/Clear Bottom Plates. Then each well in the plates was screened using Opera Phenix™ High Content Screening System to select the clones with high GFP expression. The selected single cell clones were then expanded to around 1×10^7 cells and stained with anti-mouse CD19-PE antibody to compare the mouse CD19 expression level. The cells from the single cell clone with the highest

mean fluorescence intensity (MFI) were aliquoted and stored in liquid nitrogen for later studies. Single cell clones of CD19 expressing CT26 and EMT6 cells were produced using the same procedure. The expression levels of CD19 on all three selected CD19 expressing murine cancer cell lines were checked by flow cytometry after one month of in vitro culturing.

2.3.5 Production of murine CAR T cells

To target mouse $CD19^+$ cancer cells, mice T cells were transduced to express anti-mouse CD19 CAR (MSGV1-1D3-28Z.1-3 mut). MSGV1-1D3-28Z.1-3 mut was a gift from James Kochenderfer & Steven Rosenberg (Addgene plasmid # 107227; http://n2t.net/addgene:107227; RRID:Addgene_107227) [4]. Mice T cells isolated from mouse spleens through negative selection (EasySep™ Mouse T Cell Isolation Kit, STEMCELL). After isolation, mouse T cells were activated with anti-CD3/CD28-conjugated Dynabeads for 24 hours. The activated T cells were then transferred into RetroNectin coated plates for transduction. Second transduction was performed one day after the first transduction to improve the expression of anti-mouse CD19 CAR on mouse T cells. Since the anti-mouse CD19 scFv is derived from an antibody produced by rats, an anti-rat IgG $F(ab')_2$ Fragment conjugated with Alexa Fluor 594 was used to stain the transduced and non-transduced mice T cells.

2.3.6 In vitro cytotoxicity of anti-mouse CD19 CAR T cells against $CD19^+$ cancer cells

To confirm the in vitro cytotoxicity of anti-mouse CD19 CAR T cells against $CD19^+$ cancer cells, 4T1-mCD19 was co-cultured with anti-CD19 CAR T cells or non-transduced T cells overnight in 96 well plates. The effector cells to target cells ratios include in this study are 1:1, 2:1, and 5:1. The next day, gently wash all the wells once with PBS to remove most of the suspended cells. Then add 50 µl of 0.25% trypsin to each well and incubate the plate at 37 °C for 20 minutes. Then all the cells in each well were pipetted and collected. Each well was washed twice with RPMI 1640 media containing 10% FBS to collect all the cells and neutralize the trypsin. From co-culturing to washing, all the wells had the same total volume. Fifteen minutes before running flow cytometry for cell counting, 1 µl 7-Aminoactinomycin D (7-AAD) was added to each well to distinguish living and dead cells. The T cells and 4T1-mCD19 cells were distinguished by size and

GFP level. The speed and volume of each sample taken by flow cytometer were set to be the same to compare the remaining living 4T1-mCD19 cells in each well.

2.3.7 Optimization of lymphodepletion conditions prior to CAR T cells infusion

The dose of irradiation was optimized to minimize the toxicity of irradiation and keep good lymphodepletion pre-conditioning for CAR T infusion. Balb/c mice were treated with 3, 4, or 5 Gy of irradiation to compare the efficiency of lymphodepletion and the impacts on mice body weight changes by using different doses. Blood from each mouse was collected one day after irradiation, and the red blood cells were removed with Red Blood Cell (RBC) Lysis Buffer. After running the flow cytometer, the percentage of PBMCs decrease for each irradiation dose was calculated. The body weight of each mouse was measured three times per week until the average body weight of each group recovered to the original level.

2.4 Results

2.4.1 Folate receptor level of 4T1, CT26, and EMT6 cells in the folate-deficient environment

As shown in the flow cytometry histograms of Fig. 2.1, panel A, CT26, 4T1, and EMT6 cells were all found to lack any detectable FR, even after culturing the cells in the folate-deficient medium to try to induce upregulation of FR. For the FR^+ cell line L1210A, more than 99% of the cells can bind to folate-FITC.

2.4.2 TLR7 level of selected cancer cell lines

Next, to determine whether any of these cell lines might also lack a toll-like receptor 7, we examined the same three cell lines by flow cytometry using an antibody to murine TLR7. As seen in Fig. 2.1, panel B, none of the same cells were found to express TLR7. Similar results through RT-PCR were previously reported by other groups [8, 9].

2.4.3 Production of single cell clones of CD19 expressing mouse cancer cells.

As seen in Fig 2.2, panel A-B, single cell clones in well C6, C8, C11, D9, and F7 have the highest GFP level. Among these clones, clone in well F7 has the highest mouse CD19 level. As

seen in Fig. 2.2, panel C, the single cell clones of all three murine syngeneic cancer cell lines were observed to express murine CD19 stably.

2.4.4 Production of murine CAR T cells

As shown in Fig. 2.3, panel A, the resulting CAR T cells were routinely generated with ~20 % efficiency, as shown by the fraction of mouse T cells that stained positive for anti-rat IgG Alexa Fluor 594. These CAR T cell preparations were employed in all subsequent studies.

2.4.5 In vitro cytotoxicity of anti-mouse CD19 CAR T cells against $CD19^+$ cancer cells

As shown in Fig. 2.3, anti-CD19 CAR T cells were effective in killing 4T1-mCD19 cell lines with nearly 100% efficiency when incubated at a 5:1 effector to tumor cell ratio. In contrast, nontransduced T cells lacking an anti-CD19 CAR showed only minimal cytotoxicity against the same tumor cell clones. The % cytotoxicity was calculated using the following equation, where TC and TE are the counts of live GFP^+ cancer cells in the absence and presence of CAR T cells, respectively [10]:

$$\% \text{ cytotoxicity} = ((TC-TE)/TC) \times 100$$

2.4.6 Optimization of lymphodepletion conditions prior to CAR T cells infusion

As shown in Fig. 2.4, panel A, all three doses reduced the percentages of PBMCs in one day after irradiation. However, 3 Gy irradiation caused only ~46% decrease of PBMCs, while 4 Gy and 5 Gy depleted ~67 and 79% PBMCs, respectively. On the other hand, 5 Gy irradiation induced significant decreases in body weights (Fig. 2.4, B). 5 Gy irradiation resulted in the most significant body weight loss and the slowest recovery. In comparison, 3 Gy or 4 Gy irradiation treatment did not cause significant body weight loss, and the mice recovered faster. Although the mice treated with 5 Gy irradiation recovered to their original body weights after nine days, since the infusion of CAR T cells could also cause body weight loss, the impact of irradiation should be minimized to avoid substantial toxicity. Finally, 4 Gy irradiation was selected since it can induce better lymphodepletion effects than 3 Gy but have more negligible impacts on the bodyweight than 5 Gy.

2.5 Discussion

To overcome the limitations of CAR T cells in treating solid tumors, several researchers modified the design of CAR T cells. For example, inducible expression of cytokines, including IL-12, IL-18, IL-7, IL-15, and IL-21, has been included in CAR T cells design to augment CAR T cells against tumors [11-16]. In addition, other studies have shown that co-expression of chemokine receptors such as CXCR2 or CXCR1 helps CAR T cell trafficking and infiltration [17-19]. In addition, there are studies using CAR T cells to kill cancer-associated fibroblasts (CAFs) [20-26], TAMs [27] and MDSCs [28] to change the TME to achieve better anti-tumor efficacy.

All these studies demonstrate the significance of changing the tumor microenvironment for CAR T therapy against solid tumors. In this study, we used a syngeneic solid tumor model to study the impact of FA-TLR7a on CAR T cell therapy through repolarizing TAMs and MDSCs.

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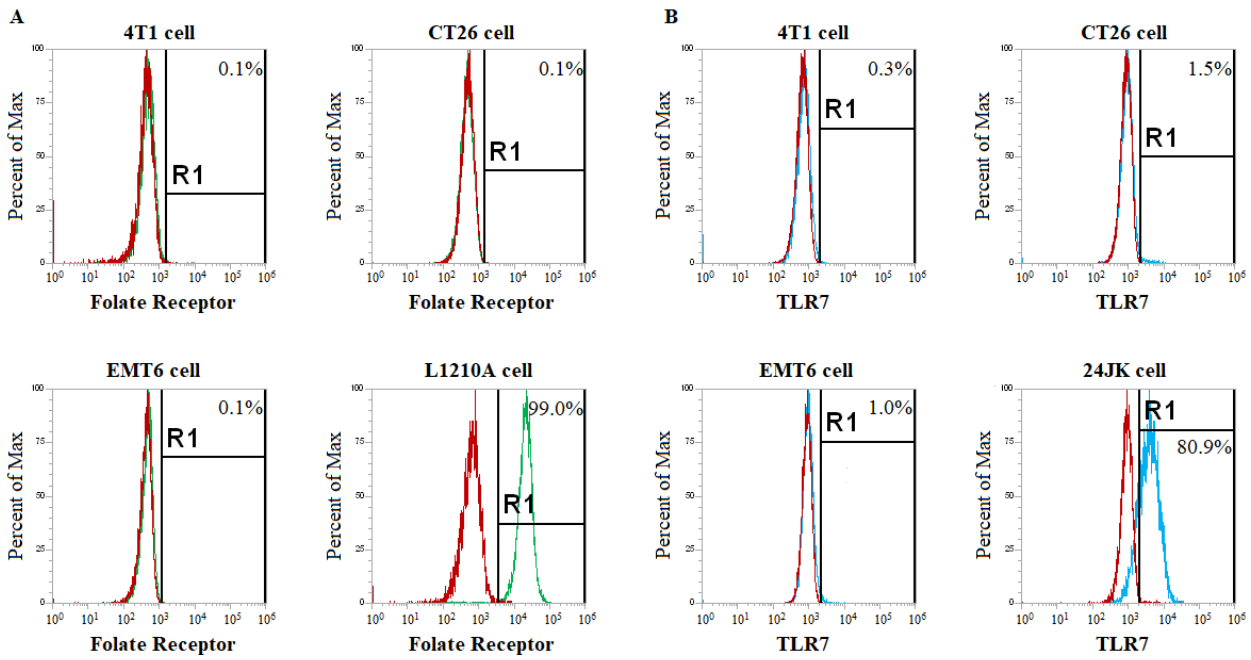

Figure 2.1 (A) Folate receptor level of 4T1, CT26, EMT6, and L1210A in folate-deficient media for one week. The histogram is the overlay of stained and non-stained samples of each cancer cell line. (B) TLR7 level in 4T1, CT26, EMT6, and 24JK cells. The histogram is the overlay of stained and non-stained samples of each cancer cell line.

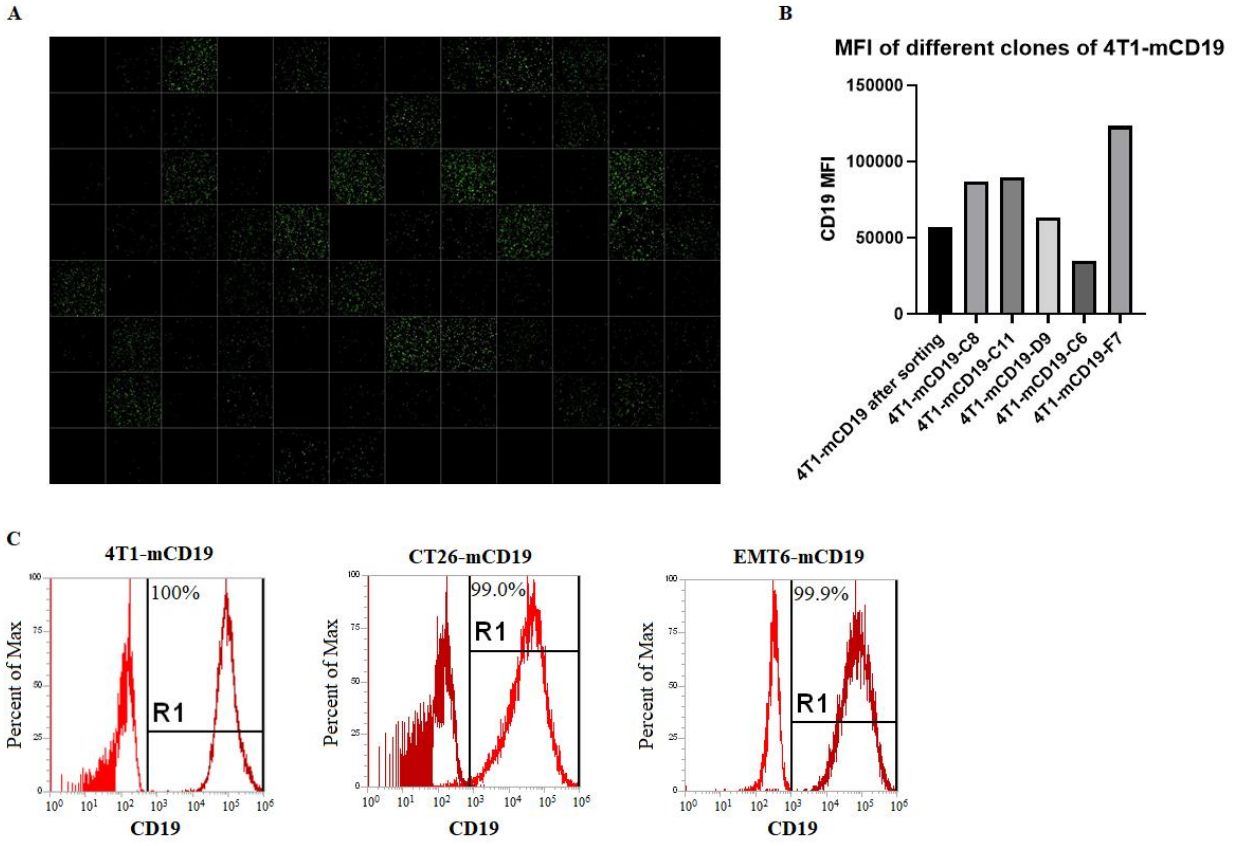

Figure 2.2 (A) GFP levels of different single cell cloning of CD19 expressing 4T1 cells in 96-well plate. (B) MFI of CD19-PE staining for selected clones of CD19 expressing 4T1 cells. (C) Mouse CD19 level of 4T1-mCD19 cells, CT26-mCD19 cells, and EMT6-mCD19 cells after one-month in vitro culturing.

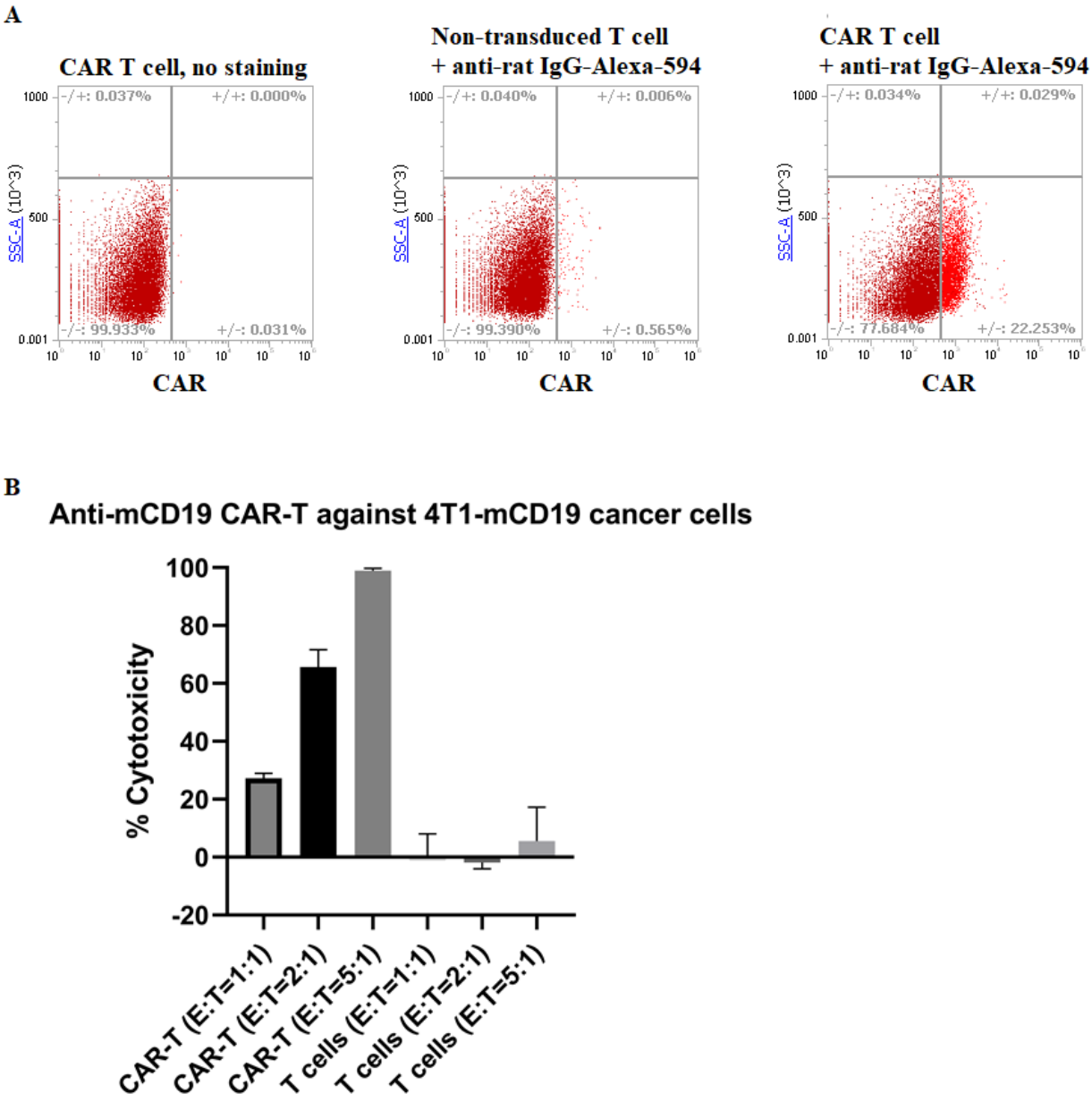

Figure 2.3 (A)Transduction efficiency of anti-mouse CD19 CAR T cells. (B) In vitro cytotoxicity of anti-mouse CD19 CAR T cells against 4T1-mCD19 cancer cells.

Lysis of 4T1-mCD19 cells in culture by anti-mouse CD19 CAR T cells or non-transduced mouse T cells (Effector:target cell ratio = 5:1). Bar graphs represent mean ± S.D. n=3.

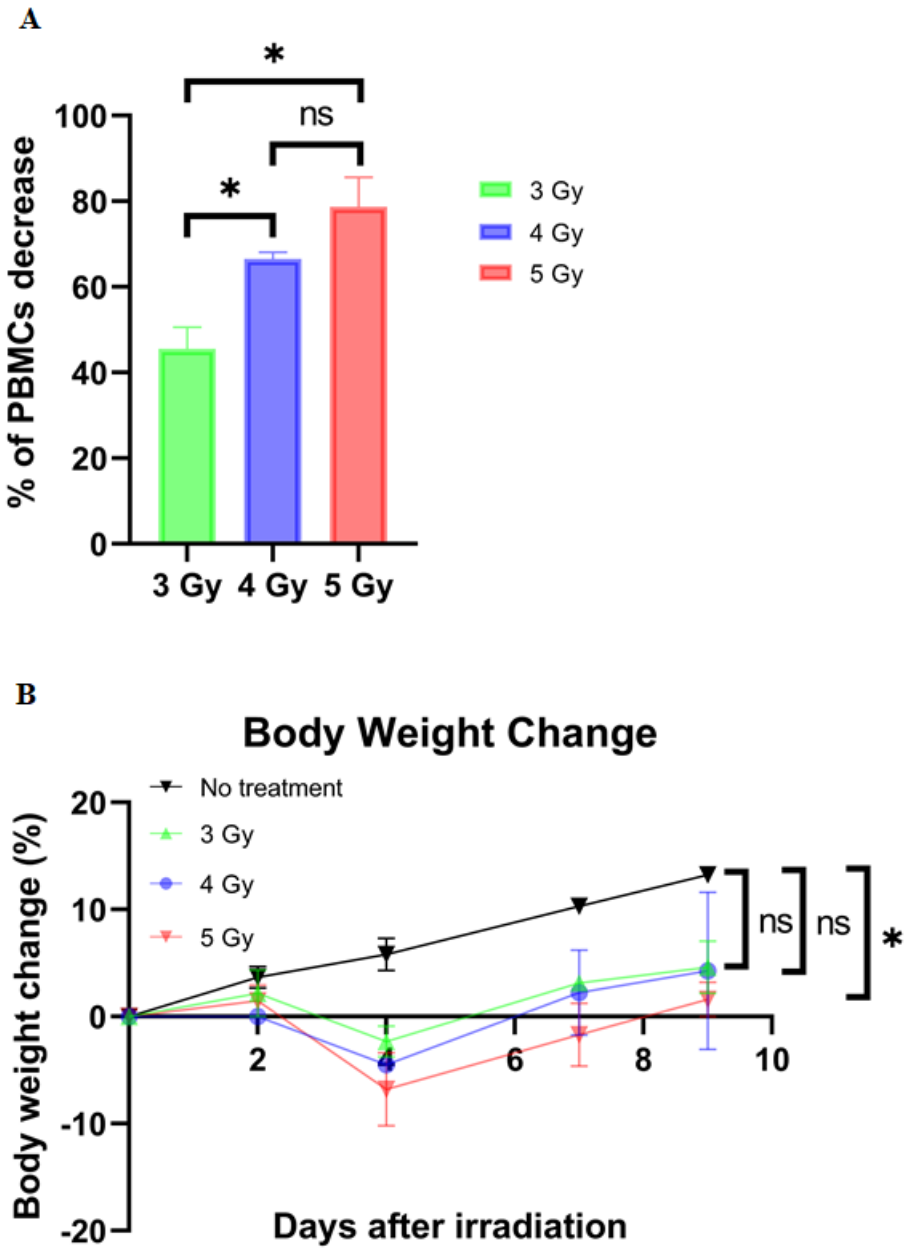

Figure 2.4 (A) Percentage of PBMCs decrease one day after different doses of irradiation. (B) Body weight changes after different doses of irradiations.

CHAPTER 3. ANALYSIS OF ANTI-MOUSE CD19 CAR T CELL EFFICACY IN VIVO

3.1 Abstract

To study the efficacy of anti-CD19 CAR T cells in vivo, we infused CAR T cells into the mice with 4T1-mCD19 tumors. CAR T treatment alone exhibited only a moderate ability to suppress tumor growth, while concurrent administration of FA-TLR7a significantly improved CAR T cell potency, with 4 of the 9 treated mice exhibiting a complete response that was maintained for more than a month without further therapy. In addition, there is no significant body weight loss observed, indicating that the specific delivery of TLR7 does not cause systemic toxicity. Further, the cured mice are resistant to rechallenge of 4T1-mCD19 cells one month after the complete tumor regression. Finally, when the same treatment was applied to larger tumors or heterogeneous tumors, the significant suppression of tumor growth was also observed.

3.2 Introduction

To study the efficacy of anti-CD19 CAR T cells in vivo, we injected the mouse subcutaneously with CD19 expressing 4T1 cells and then infused anti-CD19 CAR T cells to treat the mice with 4T1 tumors. To determine whether repolarization of the TAMs and MDSCs in the TME from a protumorigenic to antitumorigenic phenotype might improve CAR T cell potency, we simultaneously treated an identical cohort of CAR T cell-infused mice with the TAM/MDSC-targeted TLR7 agonist, FA-TLR7a. Since a new form of FA-TLR7a was used in this study, its function has been tested before using it for in vivo studies. The new version of FA-TLR7a is around three times more potent but has similar pharmacokinetic properties compared with the old version (data not shown). In our previous studies, the optimized dosing concentration and frequency of the old version of FA-TLR7a was 10 nmol/mouse, five times/week [1, 2]. Therefore, in the first study, we used 3 nmol/mouse, five times/week to treat the tumor-bearing mice.

Next, to determine whether the 5 times/week FA-TLR7a dosing frequency was required for optimal enhancement of CAR T cell therapy, we tested the impact of a variety of dosing frequencies on CAR T cell efficacy. Next, we optimized the dosing frequency of the FA-TLR7a to augment the CAR T cells.

Although using single cell clone of 4T1-mCD19 is an excellent way to maintain the stability and consistency of the tumor model for the pre-clinical studies, it may not reflect all the challenges in a heterogeneous tumor. Since different subpopulations of cancer cells co-exist inside the same tumor (intratumour heterogeneity) [3-5], we also created an antigenically heterogeneous tumor model and performed similar studies.

Studies by other laboratories have revealed that the immunosuppressive properties of solid tumors can change with tumor size, where the properties of infiltrating TAMs and MDSCs in late-stage cancers are different from the ones in early-stage cancers. It has been reported that the TAMs in late-stage breast cancers are more protumoural, while the TAMs in early-stage breast cancers have anti-tumor effects [6]. Moreover, the percentage of infiltrated MDSCs is elevated in the late-stage tumors compared with the early-stage tumors [7, 8]. Therefore, we studied the efficacy of the treatment of FA-TLR7 and CAR T cells on different sized 4T1 tumors.

3.3 Materials and Methods

3.3.1 Evaluation of the impact of co-administration of FA-TLR7a on anti-CD19 CAR T cell therapy on the growth of CD19-expressing syngeneic murine tumors.

5×10^4 of 4T1-mCD19 cells (single cell clone) were injected subcutaneously into each balb/c mouse. To test the efficacy of anti-CD19 CAR T cells against $CD19^+$ cancer cells in vivo, we infused ~1 million CAR T cells in a mixture also containing approximately 4 million non-transduced murine T cells and compared each 4T1 tumor's growth in the presence and absence of the infused CAR T cell mixture. One day before CAR T infusion, when the average tumor size reached 50 mm^3, 4 Gy total body irradiation (TBI) was performed on each mouse for lymphodepletion. The treatment of FA-TLR7a or PBS was started when the average tumor sized reached 50 mm^3 and continued 5 times/week until the end of the study. The mice treated with both CAR T cells and FA-TLR7a, with CAR T cells only, with FA-TLR7a only, or with PBS were compared in this study.

To determine whether either the CAR T cell therapy or the addition of FA-TLR7a might have caused significant systemic toxicity, the weights of all mice were recorded over the entire time course of treatment.

3.3.2 Evaluation of the effect of FA-TLR7a dosing frequency on CAR T cell efficacy.

To optimize the dosing frequency of FA-TLR7a to augment CAR T cells in solid tumors, different dosing frequencies of FA-TLR7a were applied to CAR T treated balb/c mice with 4T1-mCD19 (single cell clone) tumors. The treatment started when the average tumor sizes reached 50 mm^3, and the dosing frequencies range from 2 times/week to 5 times/week (Fig 3.2, panel A). The dosing frequency was also optimized in an antigenically heterogeneous tumor model by using flow cytometry sorted different clones of CD19 expressing 4T1 cells.

3.3.3 Rechallenge the cured mice with $CD19^+$ cancer cells.

As noted above, only mice treated with both CAR T cell immunotherapy and FA-TLR7 experienced complete tumor cell eradication. To determine whether the combination therapy was able to induce a sustained anti-tumor immunity, we rechallenged the cured mice by injecting 5×10^4 4T1-mCD19 cells into the left (contralateral) flank of cured mice. A cohort of tumor naïve balb/C mice treated with the same number of 4T1-mCD19 cells was used as a control.

3.3.4 Evaluation of the effect of co-administration of FA-TLR7a on the anti-CD19 CAR T cell therapy for 4T1-mCD19 tumors with different sizes.

5×10^4 4T1-mCD19 cells (single cell clone) were injected subcutaneously into each Balb/c mouse. One day before CAR T cell infusion, 4 Gy total body irradiation (TBI) was performed on each mouse for lymphodepletion. To analyze the suppression effects of anti-CD19 CAR T cells for different tumor sizes, the initial dose of FA-TLR7a or PBS was started when the average tumor sizes reached 50, 90, or 130 mm^3 and continued 5 times/week until the end of the study. The mice treated with both CAR T cells and FA-TLR7a, with CAR T cells only, with FA-TLR7a only, or with PBS were compared in this study.

3.4 Results

3.4.1 Evaluation of the impact of co-administration of FA-TLR7a on anti-CD19 CAR T cell therapy on the growth of CD19-expressing syngeneic murine tumors.

As shown in Fig. 3.1, panel A, treatment of 4T1-mCD19 tumor-bearing mice with the murine CAR T cell mixture or FA-TLR7a only was found to suppress tumor growth significantly

but incompletely. However, concurrent administration of FA-TLR7a and with CAR T cells invariably improved CAR T cell potency. The largest number of complete responses was observed, with 4 of 9 mice showing no evidence of residual cancer when therapy was started when tumors were ~50mm^3. Moreover, as shown in panel B, there was no significant weight loss among the groups, suggesting that neither component of the CAR T cell + FA-TLR7a therapy was significantly toxic.

3.4.2 Evaluation of the effect of FA-TLR7a dosing frequency on CAR T cell efficacy.

Next, to determine whether the 5x/week FA-TLR7a dosing frequency was required for optimal enhancement of CAR T cell therapy, we tested the impact of a variety of dosing frequencies on CAR T cell efficacy (Fig. 3.2A). As seen in Fig 3.2, panel B, treatment with 3 nmol FA-TLR7a 5 times/week augment CAR T therapy the most, while treatment with 3 nmol FA-TLR7a 2 times/week had no significant impact on the efficacy of CAR T therapy in the 4T1-mCD19 tumor. The injection of FA-TLR7a using dosing frequency 2 could significantly suppress the development of 4T1-mCD19 tumors, with 2 of 7 mice showing no evidence of residual cancer. However, the dosing frequency 1, which is five injections per week, was still the best dosing frequency because 4 of 9 mice with this treatment had complete responses. Similar results by using lower dosing frequency (two times/week) or lower dose (1 nmol/injection) were observed when using heterogeneous CD19 expressing 4T1 tumor model (Fig 3.3). However, in the heterogeneous tumor model, the impact of each treatment on the tumor size change was diminished, and there was no complete tumor regression observed. Based on these data, all further studies were performed on mice treated 3nmol/dose, 5 doses/week with FA-TLR7a.

3.4.3 Rechallenge the cured mice with CD19$^+$ cancer cells.

As shown in figure 3.4, panel A. all (6/6) of the cured mice exhibiting a complete response that was maintained for more than a month in the absence of any further therapy. Further, they were resistant to rechallenge of 4T1-mCD19 cancer cells. Only one cured mouse had a small tumor developed (smaller than 50 mm3) during the study, and it disappeared in less than a week. All the cured mice are tumor-free at the end of the study when the average tumor size for the control group reached 1000 mm^3.

3.4.4 Evaluation of the effect of co-administration of FA-TLR7a on the anti-CD19 CAR T cell therapy for 4T1-mCD19 tumors with different sizes.

As shown in fig 3.5, significantly reduced tumor sizes were observed for all treatment groups. However, though the co-administration of FA-TLR7a significantly augments the CAR T cells efficacy for all tumor sizes, complete tumor eradication was not observed for the 90 or 130 mm^3 sized groups. Moreover, the ability of CAR T cells to retard tumor growth decreased as the size of the tumor prior to initiation of therapy increased. Intriguingly, this trend was not observed for FA-TLR7a treatment. As shown in fig 3.1A and fig 3.5, the FA-TLR7a treatment had a similar impact on suppressing tumor development among the 50 or 130 mm^3 sized tumors, which indicates that the ability of FA-TLR7a to suppress tumor development is consistent for different sized tumors.

3.5 Discussion

Currently, CAR T therapy is not efficient in fighting solid tumors, and the immunosuppressive TME created by TAMs and MDSCs is one of the major reasons. Although TLR7 agonists can repolarize TAMs and MDSCs [9, 10], systemic administration of TLR7 agonists is too toxic to be used in clinics [11-14]. Moreover, the treatment with TLR agonist alone has only moderate anti-tumor efficacy [1]. Here we demonstrated that by using FA-TLR7a, the TLR7 agonist could be delivered to the $FR\beta^+$ TAMs and MDSCs inside the tumor through intravenous injection without causing any obvious systemic toxicities. Also, it can still change the properties of other infiltrating immune cells, thereby greatly augmenting the potencies of CAR T cell therapies. FA-TLR7a works the best when injected five times per week, while 2 injections per week are not sufficient to induce additional anti-tumor efficacy on CAR T cells. Further, the cured mice became resistant to the rechallenge of the same cancer cells. However, even with the optimized treatment, complete tumor eradication was not observed in all the mice treated with CAR T cells and FA-TLR7a. In addition, although the efficacy of FA-TLR7a is consistent for different sized tumors, the ability of CAR T cells to retard tumor growth decreased as the size of the tumor prior to initiation of therapy increased. The possible reasons include suppression of CAR T cell activity, blocking of CAR T infiltration, and loss of antigen on 4T1 cells. To find the real reason for the incomplete response, it is necessary to analyze the changes in immune cells and cancer cells inside the tumors.

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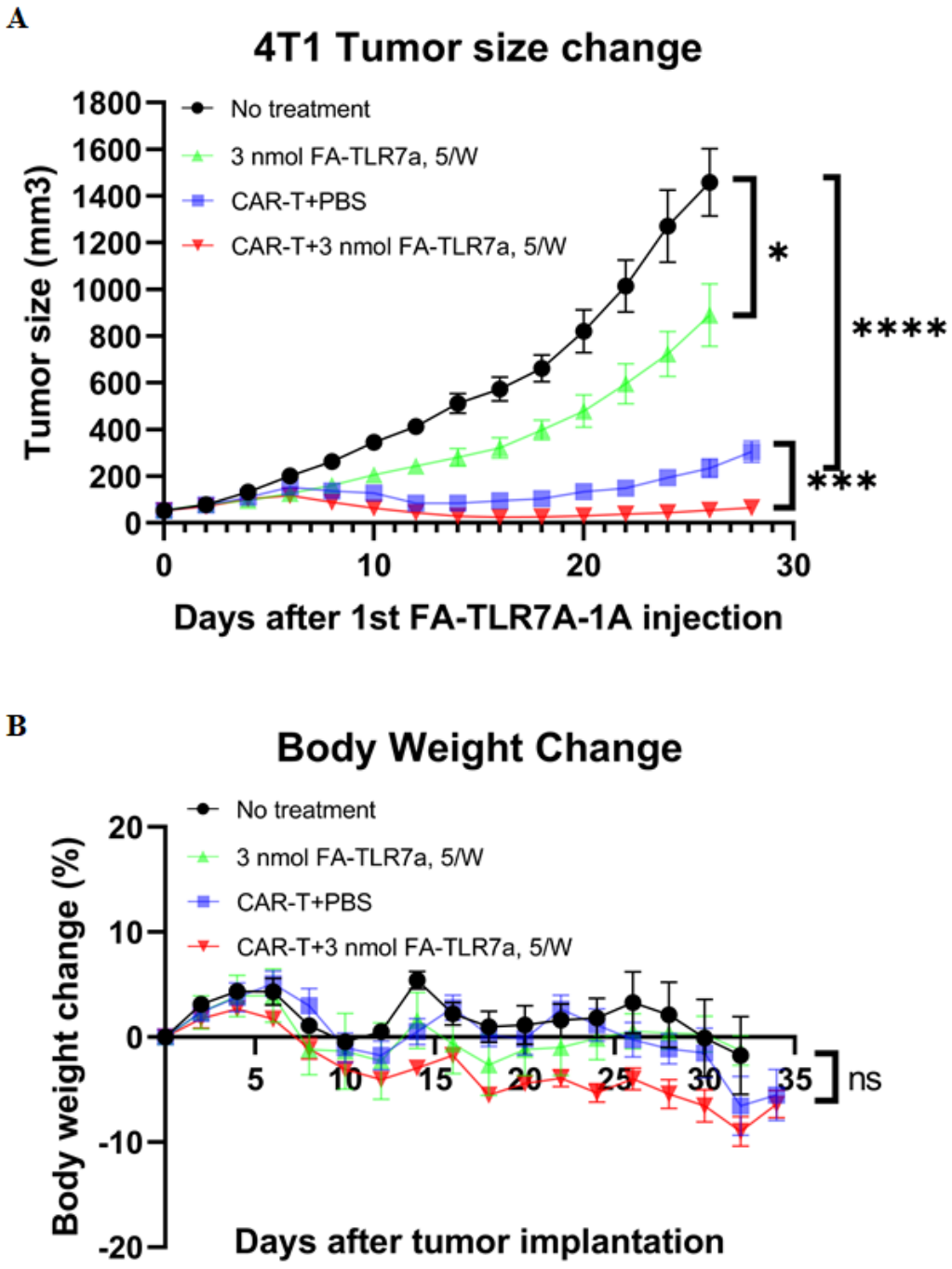

Figure 3.1 (A) The tumor size changes of mice treated with or without CAR T cells and FA-TLR7a. (B) The body change of the mice treated with or without CAR T cells and FA-TLR7a.

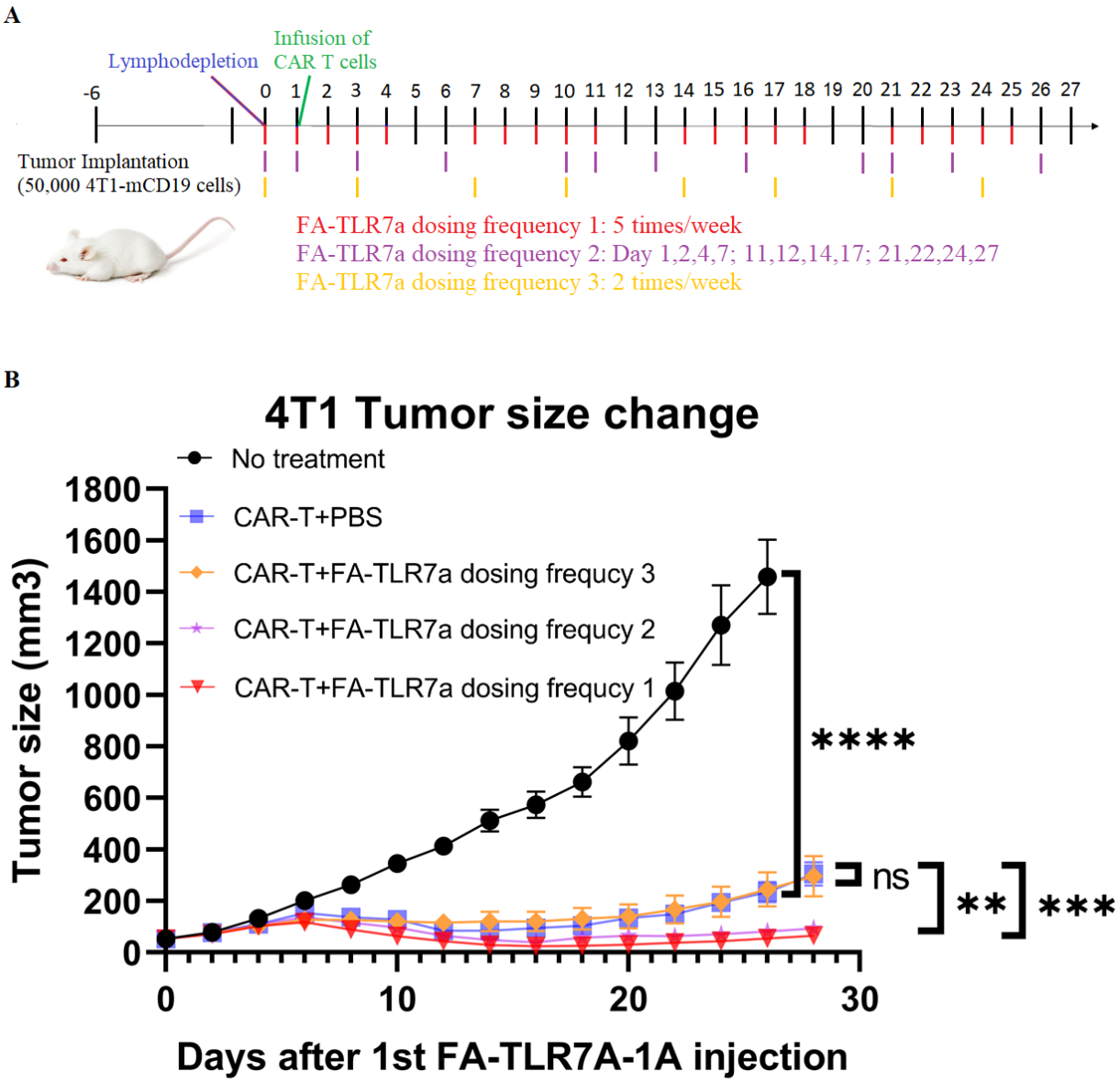

Figure 3.2 (A) The timeline of different dosing frequencies of FA-TLR7a. (B) The tumor size changes of mice treated with CAR T cells and different dosing frequencies of FA-TLR7a.

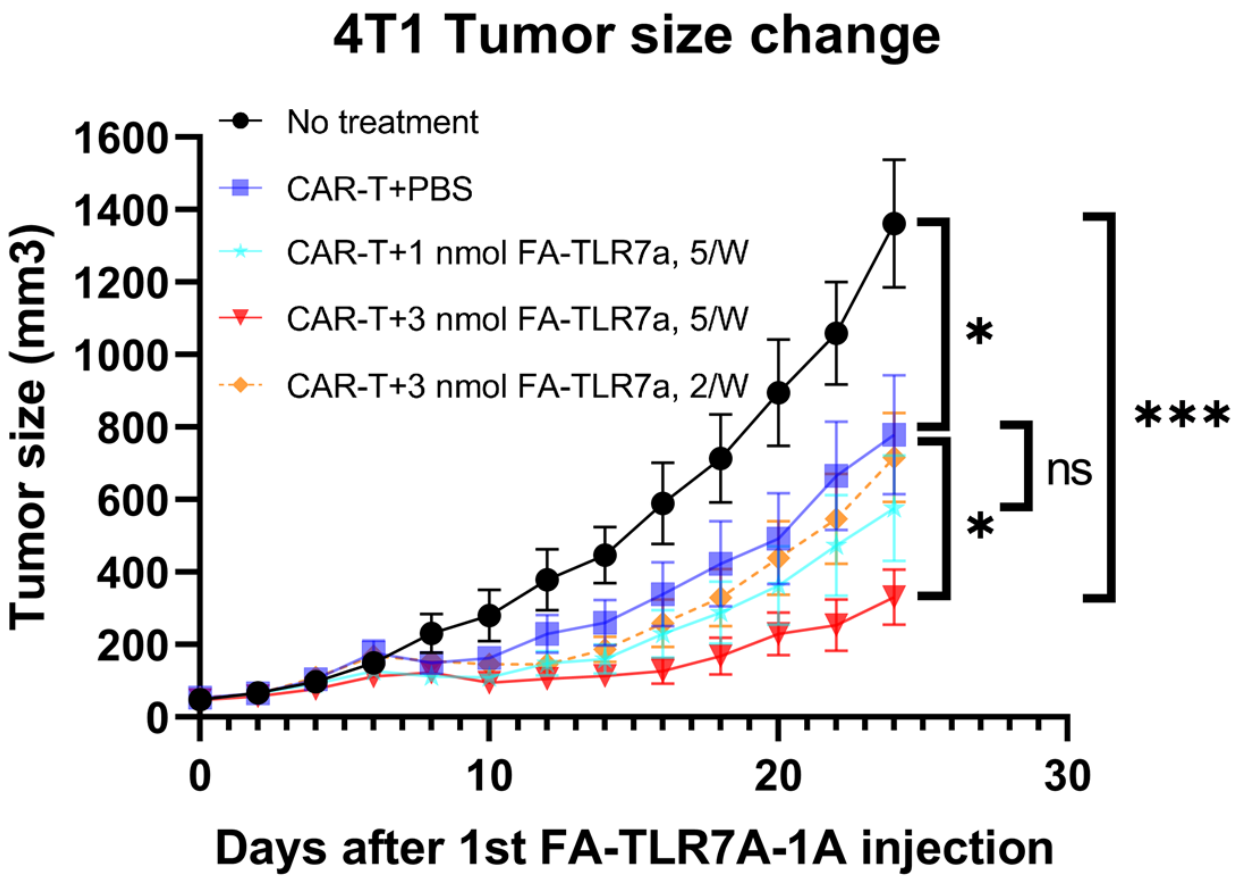

Figure 3.3 The tumor size changes of mice treated with CAR T cells and different dosing frequencies of FA-TLR7a in antigenically heterogeneous tumor model.

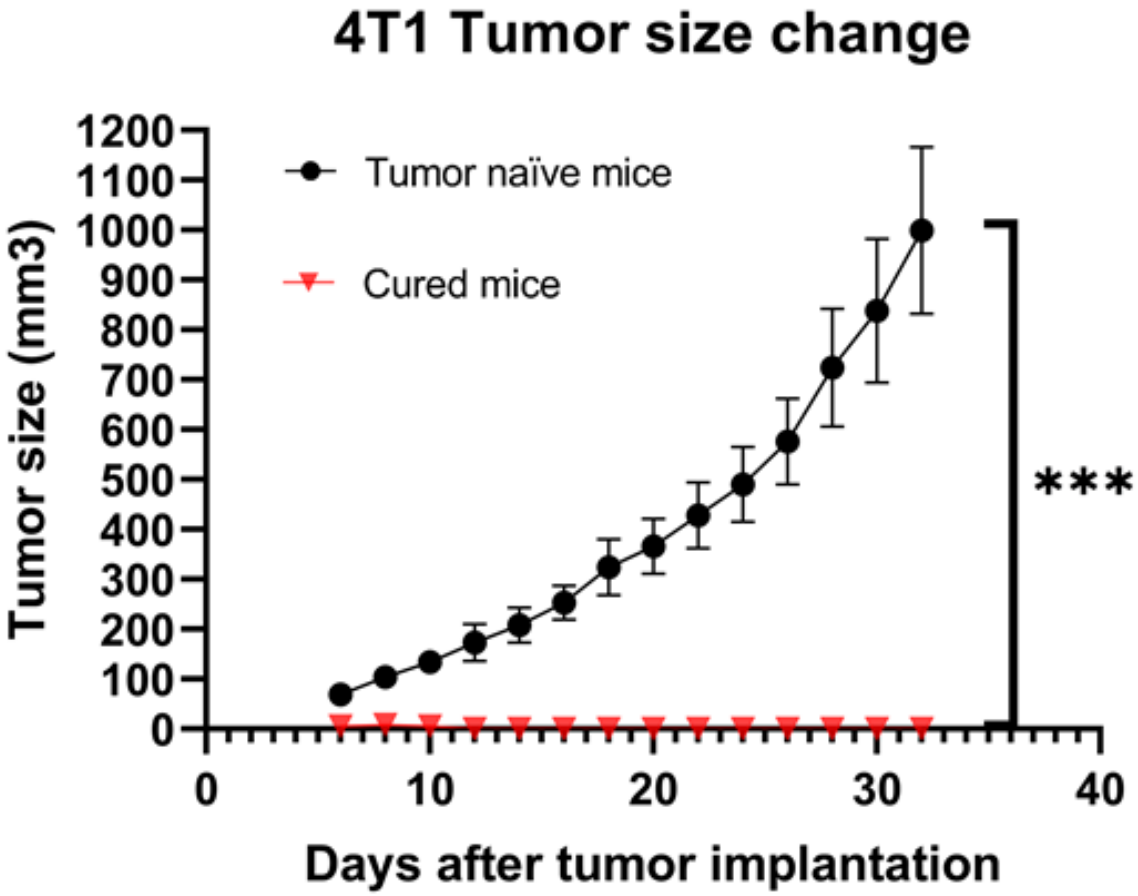

Figure 3.3.4 Tumor size change after rechallenging the cured mice

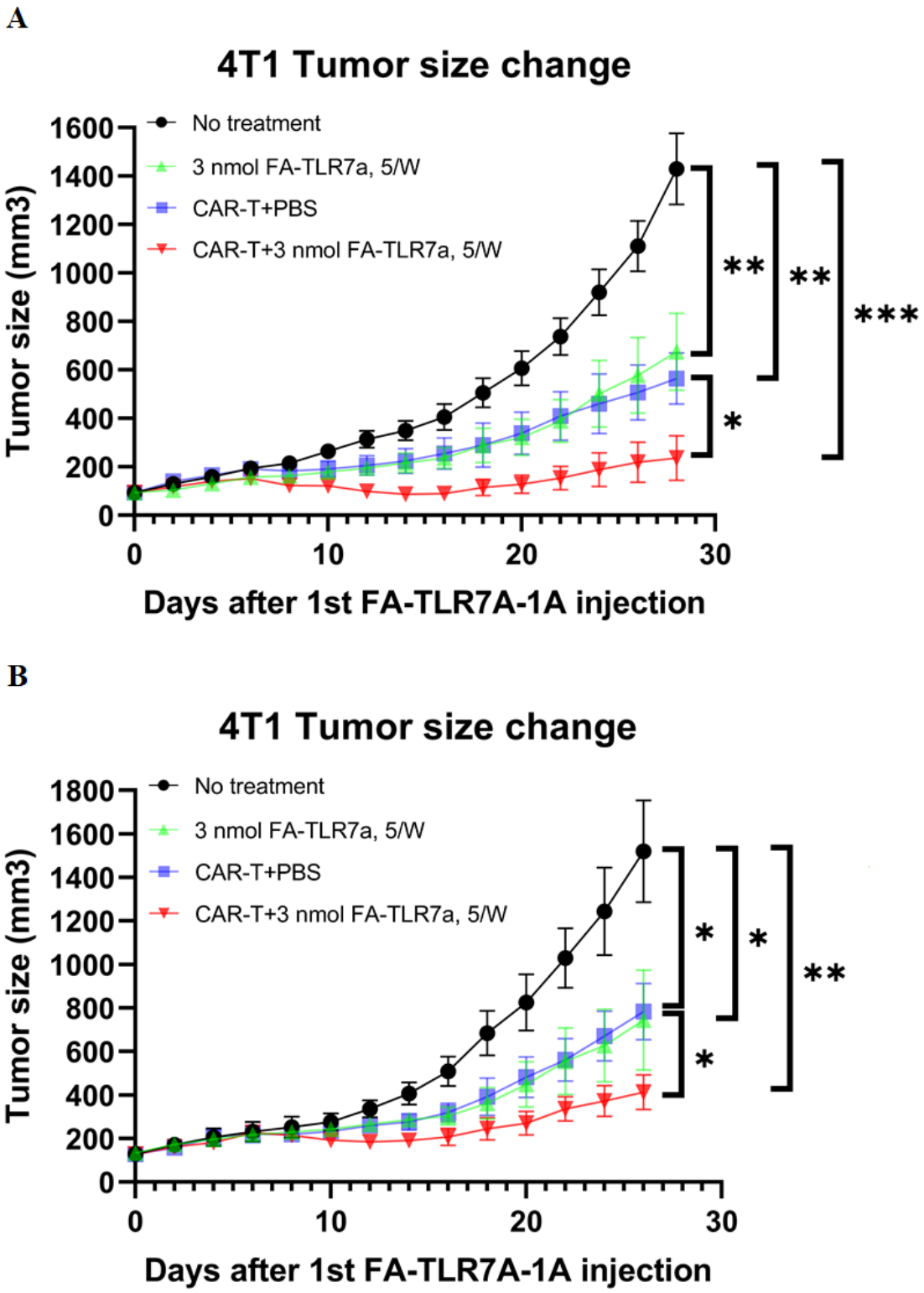

Figure 3.5 (A) The tumor size changes of mice with treatment started when the average tumor size reached 90 mm^3. (B) The tumor size changes of mice with treatment started when the average tumor size reached 130 mm^3.

CHAPTER 4. EVALUATION OF THE MECHANISM UNDERPINNING THE EFFECTS OF MACROPHAGE TARGETED TLR7 AGONISTS ON CAR T CELL THERAPY

4.1 Abstract

Since the hypothesis underpinning this study was that the targeted stimulation of TAMs and MDSCs with a TLR7 agonist might enhance CAR T cell efficacy by shifting the TME to a more inflammatory state, we next examined whether the immune cells in the solid tumor microenvironment (TME) might have changed to a more tumoricidal phenotype. For this purpose, all tumor tissue remaining after termination of the above therapies was resected, subjected to gentle digestion to facilitate isolation of cancer and stromal cells, and then analyzed by flow cytometry to characterize the polarization states of the TAMs and MDSCs. Treatment of FA-TLR7a significantly increased the M1/M2 ratio, while decreased the percentages of MDSCs inside the tumor. Also, the number of total and activated CAR T cells was increased after FA-TLR7a treatment. After checking the CD19 expression level on 4T1 cells isolated from 4T1 cells, we conclude that loss of antigen could be the major reason for incomplete tumor regression after CAR T cell treatment, especially in heterogeneous tumors. In addition, we rechallenge the cured mice with the same cancer cells, and all the cured mice were resistant to the same cancer cells.

4.2 Introduction

Since the mice treated with both anti-CD19 CAR T cells and FA-TLR7a had complete responses or significantly smaller tumor volume, we next wished to determine whether the improved potency of CAR T cells in the presence of the FA-TLR7a might have derived from a change in the properties of TAMs and MDSCs. We euthanized all the tumor-bearing mice and digested the tumors for flow cytometry analysis.

Next, to test the specificity of the delivery of FA-TLR7a, we checked the impact of the injection of FA-TLR7a on macrophages and T cells in spleens. The splenic macrophages were previously reported to be FR^- [1], so the injection of FA-TLR7a should not have a significant impact on them. Similarly, since the splenic T cells and their surrounding cells are all FR^-, they should not be directly or indirectly affected by the injection of FA-TLR7a.

In clinical trials, 70-90% of the patients treated with anti-CD19 CAR T cells had completed remission [2-7]. However, for the patients with incomplete remission or relapse after anti-CD19 CAR T cells, loss of CD19 expression, including antigen escape, antigen downregulation, or lineage switch, is one of the major reasons [8, 9]. Previous studies have shown that a small group of CD19 negative population could become the major population under the selection pressure of anti-CD19 CAR T cells [10, 11]. This could be the potential reason that our anti-CD19 CAR T therapy was less efficient in the heterogeneous tumor model, even with the co-administration of FA-TLR7a. However, since the incomplete tumor regression was also observed in the single cell clone-derived tumor, there must be another reason. Sotillo et al. [12] and Orlando et al. [13] have observed that the mutation of the CD19 gene also caused antigen escape. To analysis the potential antigen loss on 4T1 cells in homogeneous and heterogeneous tumors after CAR T cell treatment, we compared the percentages of $CD19^+$ 4T1 cells inside the tumor. Further, the 4T1 cells isolated from 4T1-mCD19 tumors treated with or without CAR T cells were cultured in vitro to check the potential changes in CD19 expression level and other phenotypes.

4.3 Materials and Methods

4.3.1 Comparison of the effects of FA-TLR71a on TAMs and MDSCs in the presence and absence of anti-CD19 CAR T cells

All the tumor-bearing mice were euthanized, and the tumors were isolated and digested with Tumor Dissociation Kit (Miltenyi Biotec). The digested tumor cells were resuspended and stained with Zombie Violet (Biolegend, CA) and antibodies for flow cytometry analysis. To check the M1/M2 ratios of macrophages inside the tumors, digested cells were first stained with anti-mouse F4/80-APC and washed with PBS two times. The stained cells were then fixed and permeabilized with Cyto-Fast™ Fix/Perm Buffer Set (BioLegend, CA). The cells were then incubated with the anti-mouse arginase-1-PE antibody and the anti-mouse iNOS-APC-eFluor 780 (eBioscience) for 20 minutes at room temperature in the dark. All the samples were then washed two times with 1 ml PBS and analyzed by flow cytometry. To check the percentages of MDSCs inside the tumor, digested cells were stained with anti-mouse CD11b-APC-eFluor 780 (eBioscience) and anti-Gr-1-Alexa-595 (Biolegend, CA) and then washed two times with PBS. The splenocytes were isolated by gently pressing the spleens through the 70 μm cell strainer using 10 ml syringe plungers. The isolated cells were then stained with Zombie Violet and antibodies for flow cytometry analysis.

All the samples were incubated with anti-mouse CD16/CD32 (BioLegend, CA) for 20 minutes on ice before stained with antibodies.

4.3.2 Comparison of the effects of FA-TLR7-1a on T cells and CAR T cells in solid $CD19^+$ 4T1 tumors

The digested tumor cells were resuspended and stained with Zombie Violet (Biolegend, CA) and antibodies for flow cytometry analysis. To check the number of infiltrated T cells and activated T cells inside the tumors, digested cells stained with anti-mouse CD3-APC (Invitrogen) with or without anti-mouse CD25-PE (Biolegend, CA) or anti-mouse CD69-PE (Biolegend, CA) on ice for 20 minutes and washed with PBS for two times. All the samples were incubated with anti-mouse CD16/CD32 for 20 minutes on ice before stained with antibodies. To check the number of infiltrated CAR T cells and activated CAR T cells inside the tumors, digested cells were stained with an anti-rat IgG $F(ab')_2$ Fragment conjugated with Alexa Fluor 594 for 30 minutes on ice and washed twice with PBS before adding anti-mouse CD16/CD32 and other antibodies.

4.3.3 Loss of CD19 expression on 4T1-mCD19 cancer cells after CAR T treatment.

To check the expression of mouse CD19 on the 4T1 cells at the end of the study, the digested cells were stained with Zombie Violet (Biolegend, CA) and antibodies for flow cytometry analysis. After Zombie Violet staining, the digested cells were stained with anti-mouse EpCAM-APC (Biolegend, CA) and anti-mouse CD19-PE (Biolegend, CA) on ice for 20 minutes and washed with PBS two times. All the samples were incubated with anti-mouse CD16/CD32 for 20 minutes on ice before stained with antibodies. Further, the cells isolated from homogeneous 4T1-mCD19 tumors treated with or without CAR T cells were cultured in vitro to check the potential change of the CD19 expression level on isolated 4T1-mCD19 cells.

4.4 Results

4.4.1 Comparison of the effects of FA-TLR71a on TAMs and MDSCs in the presence and absence of anti-CD19 CAR T cells

As shown in Fig. 4.1, panel A, treatment with FA-TLR7a doubled the ratio of M1:M2 macrophages as measured by iNOS:Arginase 1 ratio. For the groups without CAR T cell treatment,

the administration of FA-TLR7a increased the M1/M2 ratio from 0.36 to 0.72. Intriguingly, the treatment of CAR T cells increased the M1/M2 ratio tremendously, with a 2.5 to 4.3 ratio increase for mice cotreated with FA-TLR7a. However, there is no significant difference in the percentages of macrophages inside the tumors for the groups treated with or without FA-TLR7a (Fig 4.1, panel B). This indicates that although the FA-TLR7a repolarized the TAMs and increased the M1/M2 ratio, it did not increase the infiltration of macrophages. Interestingly, there are significant differences in the percentages of macrophages inside the tumors between the groups with or without CAR T treatment. The observation that CAR T cells treatment can increase the M1/M2 ratio and macrophage infiltration indicates that CAR T cells also have an impact on tumor infiltrated macrophages. Moreover, for CAR T cells treated mice, there is a significant decrease in the percentage of MDSCs inside the tumors between the groups with or without concurrent administration FA-TLR7a (Fig 4.1, panel C). However, for the mice without CAR T cell treatment, the difference is not significant.

The impact of FA-TLR7a on TAMs was not observed in splenic macrophages. As shown in Fig 4.1, panel D and E, the systemic treatment of FA-TLR7 agonist does not have a significant impact on the M1/M2 ratio or the percentages of splenic macrophages. These results demonstrated that the impact of FA-TLR7a is specific to FR^+ tumor-associated myeloid cells, and the systemic administration of FA-TLR7a is accurate and safe.

4.4.2 Comparison of the effects of FA-TLR7-1a on T cells and CAR T cells in solid $CD19^+$ 4T1 tumors

As shown in Fig 4.2, panel A-C, no significant impact on the number of infiltrated total T cells or activated T cells inside the tumors was observed with FA-TLR7a treatment alone. However, treatment of FA-TLR7a significantly increased the number of infiltrated total and activated T cells and CAR T cells inside the tumors (Fig 4.2, panel D-E). For the mice without CAR T cell treatment, the tumor sizes were too large at the end of the study. Therefore, treatment with FA-TLR7a alone, though increased the M1/M2 ratio compared to control treatment (no treatment), may not be enough to reprogram the immunosuppressive TME and further increase the activated T cells. For the groups treated with anti-CD19 CAR T cells, there are more activated CAR T cells because the immunosuppressive TME was changed by both killing of 4T1-mCD19 cancer cells with CAR T cells and the repolarization of the TAMs and MDSCs using FA-TLR7a.

4.4.3 Loss of CD19 expression on 4T1-mCD19 cancer cells after CAR T treatment.

Even though all tumors in the homogeneous tumor model were generated from a single CD19 positive 4T1 cancer cell clone, the residual lesions in those mice with refractory tumors were found to lose CD19 positive population, suggesting that the resistance mechanism stemmed from the loss of tumor antigen and not exhaustion of the CAR T cells. As shown in Fig 4.3, for the mice without CAR T cells treatment, 70-80% of $EpCAM^+$ cells are $CD19^+$, while for the cohort of mice treated with CAR T cells, only 10-20% $EpCAM^+$ cells are $CD19^+$. Similar results were observed in the heterogeneous tumor model.

Surprisingly, the selection pressure of CAR T cell therapy even caused morphological alternations of 4T1-mCD19 cells. As shown in Fig 4.4, panel A, the 4T1-mCD19 cells isolated from CAR T cells treated tumors became less adhesive, while no morphology alternation was observed for the 4T1-mCD19 cells isolated from the tumors without CAR T cell treatment. Further, the expression level of CD19 on mutated 4T1-mCD19 cells gradually recovered during in vitro culturing. As shown in Fig 4.4, panel B, the percentage of $CD19^+$ 4T1 cells and CD19 expression level gradually increased in the first three weeks of in vitro culture. However, the increase in CD19 expression stopped after three weeks, and the CD19 expression level became stable but lower than the cells without mutation. As expected, for the 4T1-mCD19 cells isolated from tumors without CAR T cell treatment, 100% of 4T1 cells keep the high-level expression of mouse CD19, which demonstrated that the single cell clone of 4T1-mCD19 cell is stable in vivo without selective pressure from CAR T cells.

4.5 Discussion

We have shown here that FA-TLR7a can reprogram TAMs and MDSCs in the TME from a tumor-promoting to tumor-suppressing phenotype without changing macrophages in healthy tissues. By reprogramming the TAMs & MDSCs, FA-TLR7a also changes the properties of other infiltrating immune cells, including CAR T cells and normal T cells, thereby greatly augmenting the potencies of CAR T cell therapies. Since the infiltration and activation of CAR T cells were improved with the co-administration of FA-TLR7a, the incomplete tumor eradiation was not caused by the disfunction of CAR T cells.

Intriguingly, examination of the 4T1 cells from residual tumors with heterogeneous or homogeneous origins revealed that the resistant cancer cells all have a lower level of CD19 expression, suggesting that their failure to respond completely to the therapy derived from their ability to suppress the expression of CD19. Further, we conclude that the loss of CD19 expression was caused by at least three different mechanisms, including CD19 gene mutation, selection by immune pressure, and antigen downregulation. First, the difference in CD19 expression levels on 4T1 cells isolated from different cohorts of mice indicates that there was a mutation related to CD19 expression. Following the mutation, the selective pressure of anti-CD19 CAR T cells resulted in a decrease in the percentage of $CD19^+$ 4T1 cells inside the tumors. Also, since the upregulation of CD19 expression on isolated 4T1 cells was observed during in vitro culturing, the posttranscriptional downregulation of CD19 expression was probably involved during the tumor development to avoid the anti-CD19 CAR T cells.

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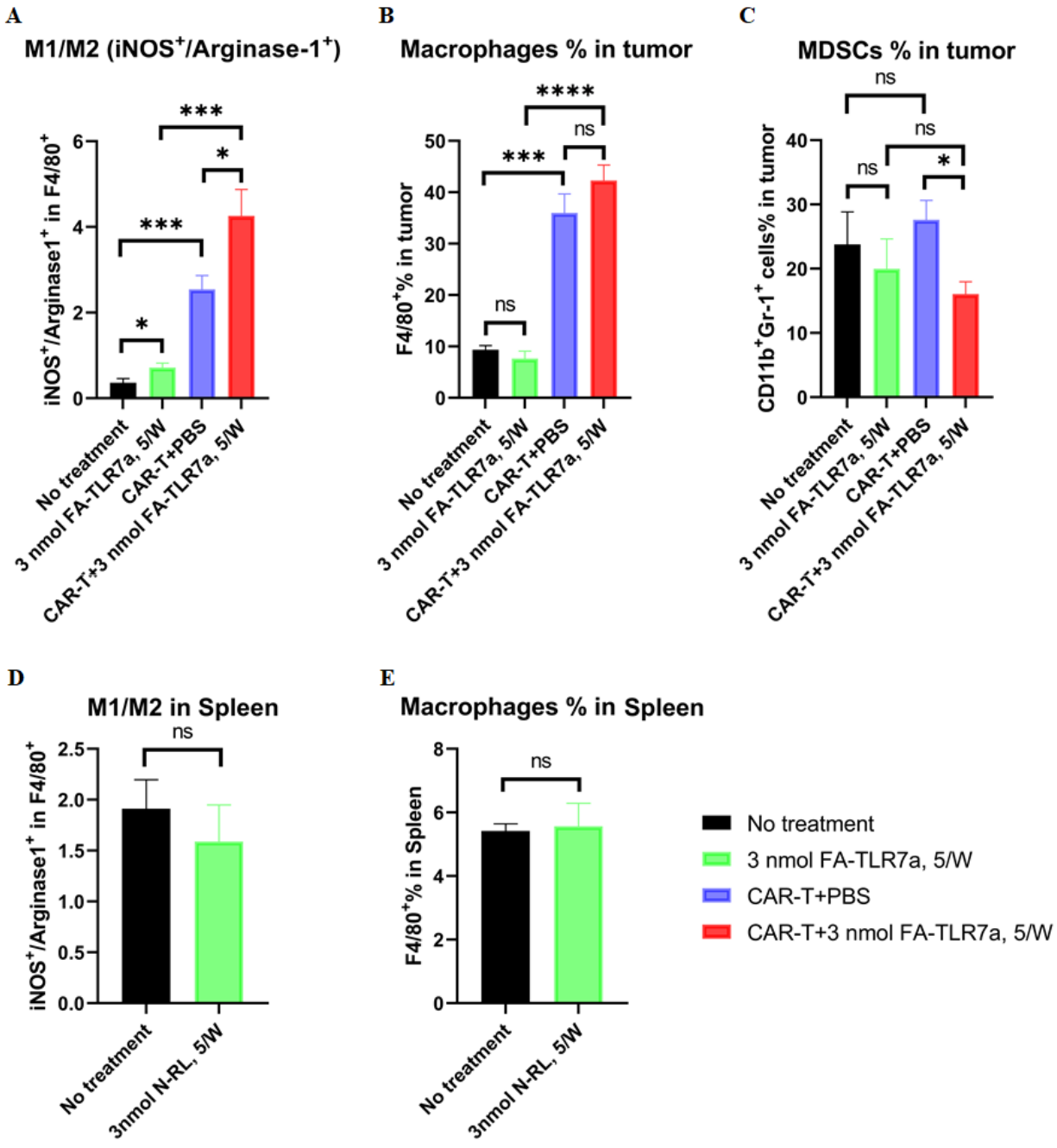

Figure 4.1 Impact of FA-TLR7a-1A on TAMs and MDSCs. (A) M1/M2 ratios of macrophages inside the tumor. (B) Percentages of macrophages inside the tumors. (C) Percentages of MDSCs inside the tumors. (D) M1/M2 ratios of macrophages inside the spleens. (E) Percentages of macrophages inside the spleens.

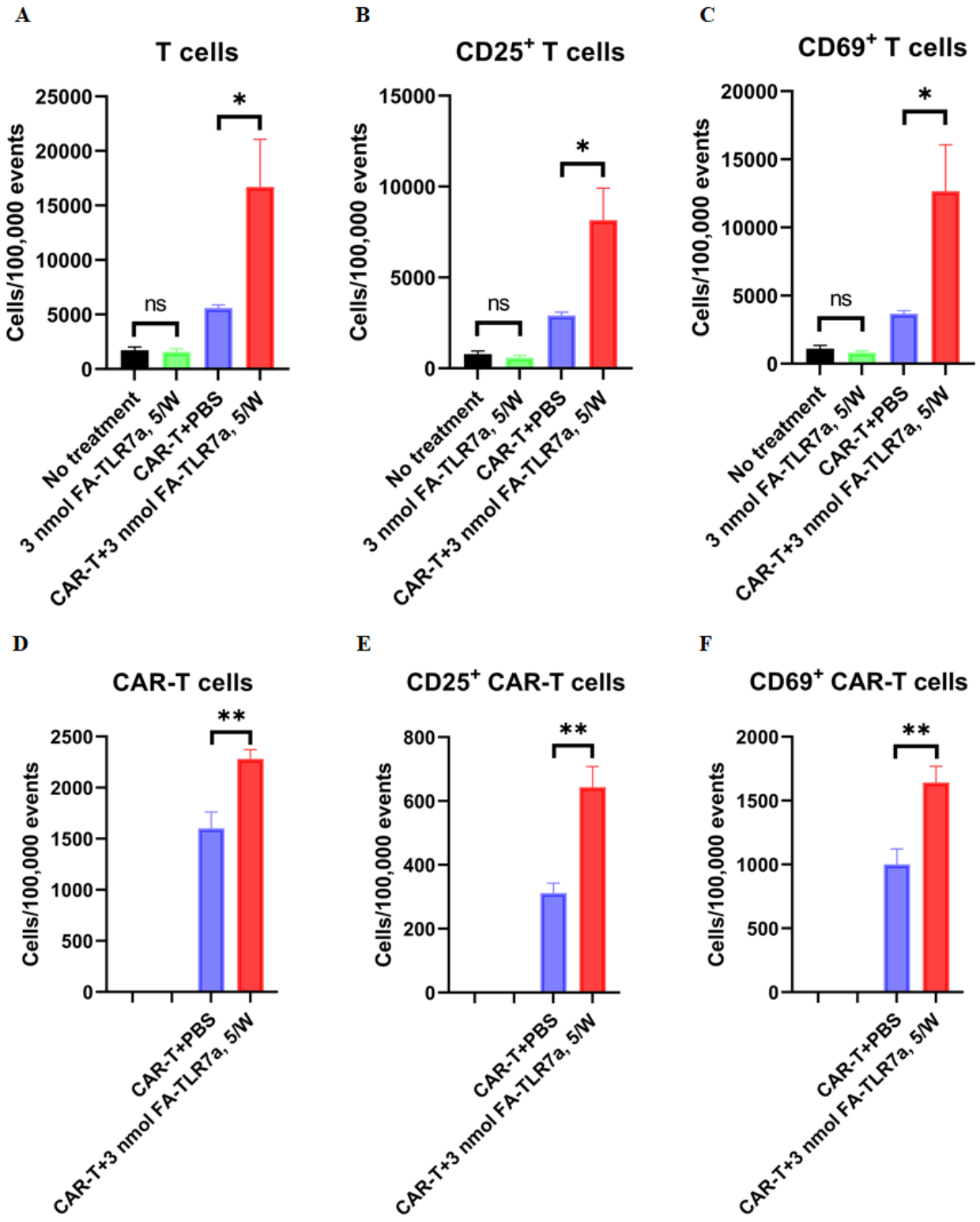

Figure 4.2 Impact of FA-TLR7a-1A on T cells and CAR T cells. (A-C) The number of total T cells, CD25$^+$ T cells, or CD69$^+$ T cells per 100,000 cells inside the tumor. (D-F) The number of total anti-CD19 CAR T cells, CD25$^+$ anti-CD19 CAR T cells, or CD69$^+$ anti-CD19 CAR T cells per 100,000 cells inside the tumor.

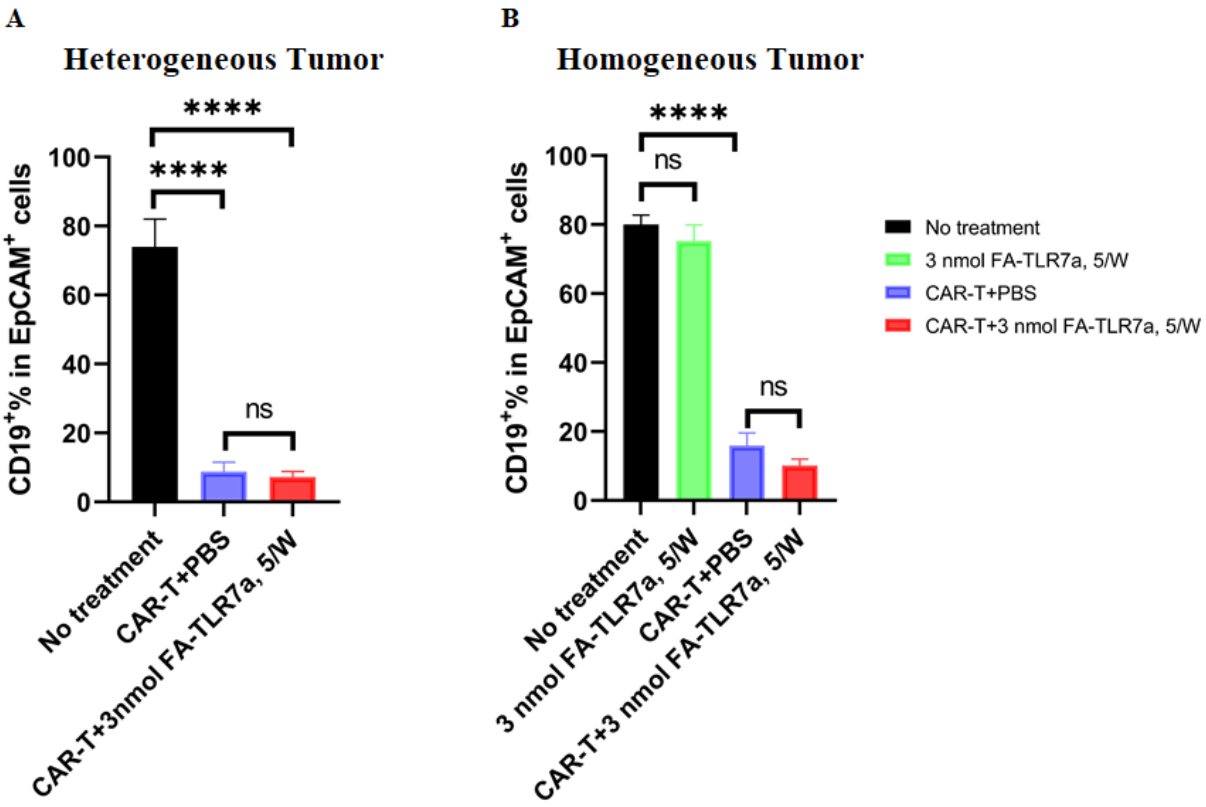

Figure 4.3 Loss of CD19 expression on CAR T treated cancer cells.

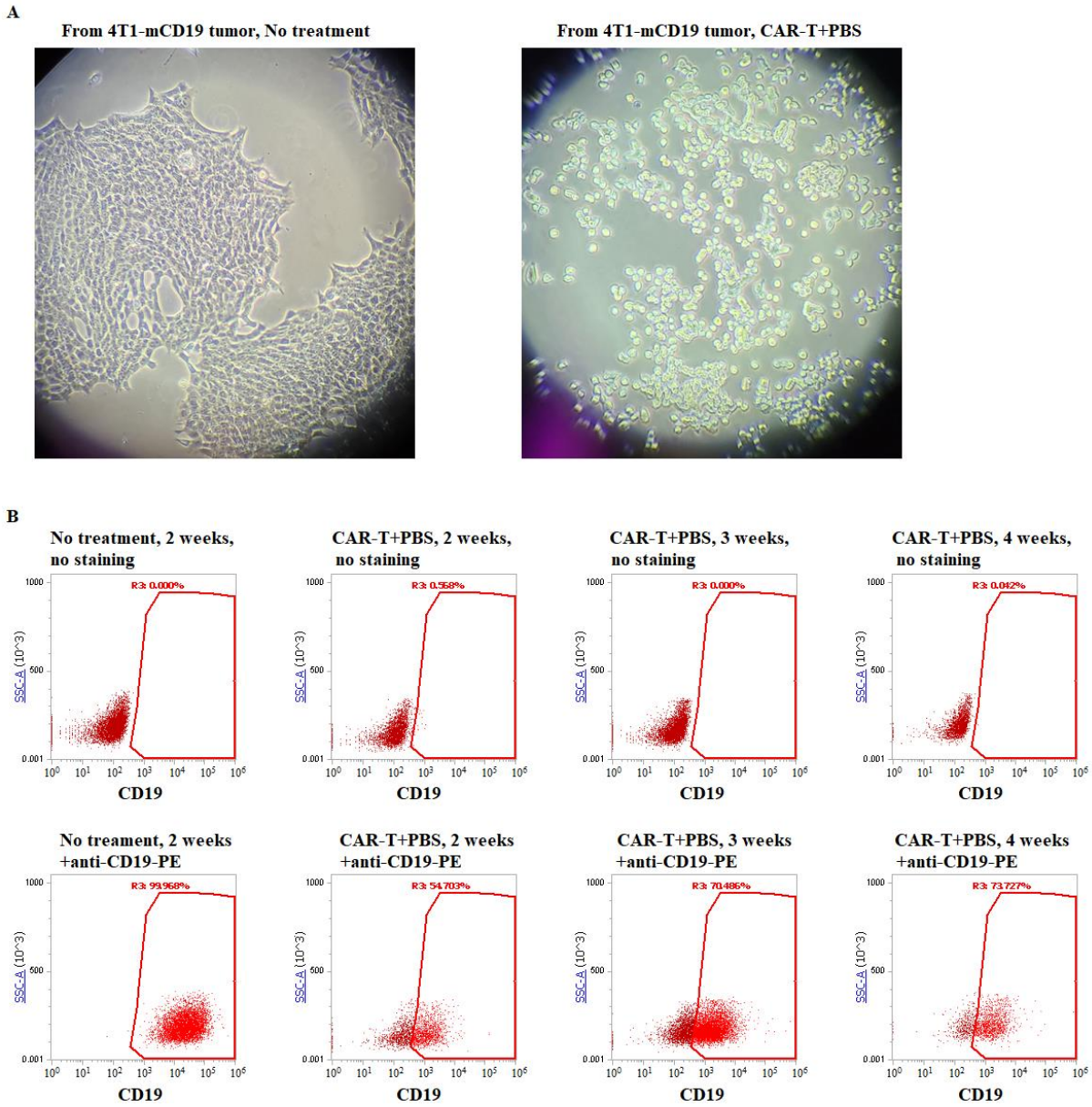

Figure 4.4 Mutation and downregulation of CD19 of 4T1-mCD19 cancer cells. (A) Cell morphology comparison of 4T1 cells isolated from 4T1-mCD19 tumors treated with or without CAR T cells. (B) Mouse CD19 expression of isolated 4T1-mCD19 cells.

CHAPTER 5. CONCLUSIONS

Since elevated TAMs and MDSCs correlated with poor survival for most solid tumors [1-4], repolarizing TAMs and MDSCs can be a universal therapy for various solid tumors. With FA-TLR7a, TAMs and MDSCs can be specifically repolarized without affecting cells in other tissues. Although the repolarization of TAMs and MDSCs with FA-TLR7a is not sufficient to completely cure the tumors, it further reprograms the TME to be less immunosuppressive and creates room for other anti-tumor therapies, including CAR T cell therapies.

Here we demonstrate that with both FA-TLR7a and CAR T cells treatment, the solid tumors in immunocompetent mice can be completely cured. Co-administration of FA-TLR7a can augment CAR T cells efficacy by specifically repolarizing tumor-infiltrating myeloid cells without causing significant systemic toxicity. Although CAR T therapy alone has a significant impact on TAMs and MDSCs, and other immune cells in the solid tumor, the role of FA-TLR7a treatment is essential. Complete tumor eradication was not observed in the cohorts of mice treated with only CAR T cells, and the anti-tumor efficacy of the CAR T cells is FA-TLR7a dose-dependent.

However, the complete response rate is less than 50% and was only observed when the treatment started early. After checking all the residual tumors, we found that loss of antigen under the selection pressure of CAR T cells is the major reason for incomplete tumor killing. To solve this problem, CAR T cells that can target more than one antigen are needed. The anti-CD19/CD20 and anti-CD19/CD22 dual CARs have been used in clinical trials to treat hematological malignancies [5, 6]. In addition, CAR T cells target both HER2 and IL13Rα2 have been reported to have better efficacy than single-target CAR T cells in a xenograft glioma model [7]. Further, we have designed the anti-FITC CAR T cells that can target multiple antigens when using different FITC-conjugated targeting ligands [8]. For the MDA-MB-231 tumors that express both PSMA and CA IX, when the combination of two different tumor tags was used to target both antigens, the anti-tumor efficacy was significantly better than using either one of the FITC conjugated targeting ligand.

In addition, it is essential to speed up the preparation of CAR T cells to minimize the waiting time before the treatment. As shown in this study, complete remission could be achieved if the treatment started early. For the early-stage tumors, CAR T cells are more likely to kill all the cancer cells before their mutation to avoid antigen escape. However, the current manufacturing

process of producing CAR T cells is personalized and complex, which makes it slow and expensive. To minimize the waiting time, “off-the-shelf” allogeneic CAR T cells [9] or in vivo generated CAR T cells [10-12] are needed for immediate treatment after diagnosis.

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VITA

Weichuan Luo was born and grew up in Jiangsu, China. He began his journey in the field of biological science when he attended the College of Science at Purdue University, which is located in West Lafayette in Indiana. After completing a Bachelor's degree in Biological Science, to further pursue his research interest and explore the various field of life science, he continued his study in the same university and joined the Interdisciplinary Life Science Program (PULSe) in 2015. Here he had opportunities to work in the laboratory in different research fields. In 2016, he joined Dr. Philip S. Low's group and started his research on immunotherapy and infectious diseases. In Low's group, he worked on a number of projects, including reprogramming tumor-associated macrophages to enable CAR T cell eradication of solid tumors, CAR T cell therapies against virus-infected cells, and the development of canine CAR T cells. After several years of research, he completed his Ph.D. degree in May 2021.

PUBLICATIONS

[1] Weichuan Luo and Philip S. Low. Combination of small molecule drug conjugate and CAR-expressing cytotoxic lymphocytes. (Patent, submitted)

[2] Xin Liu, Weichuan Luo, Boning Zhang, Yong Gu Lee, Madduri Srinivasarao, and Philip S. Low. Design of ligand-targeted imaging and therapeutic agents for the diagnosis and treatment of influenza virus infections. (Submitted)

[3] Weichuan Luo, John V. Napoleon, and Philip S. Low. Reprogramming tumor-associated macrophages to enable CAR T cell eradication of solid tumors. (AACR Abstract, submitted)

[4] Weichuan Luo, John V. Napoleon, and Philip S. Low. Repolarization of tumor-infiltrating myeloid cells to augment CAR T cell therapies. (Manuscript in preparation)